Predictive value of using plasma long non-coding RNAs ANRIL and HOXA11-AS for in-stent restenosis

ZHIJIANG JIN[1,2], HONGFENG SHEN[1,2], WEI CHA[1,2], HAIJIANG XIA[1,2] and LONGBIN LIU[1,2]

[1]Department of Cardiology, The Affiliated Hospital of Shaoxing University;
[2]Department of Cardiology, Shaoxing Municipal Hospital, Shaoxing, Zhejiang 312000, P.R. China

Received May 25, 2021; Accepted September 20, 2021

DOI: 10.3892/etm.2021.11038

Abstract. In-stent restenosis (ISR) can pose serious challenges for cardiologists following coronary stent implantation. Early identification of patients at high risk of ISR is considered to be effective for its prevention. However, factors that can reliably predict the risk of ISR remain elusive at present. The present study aimed to investigate the possible association between plasma long non-coding RNA (lncRNA) levels and ISR. A total of 410 patients with single-vessel lesion who received drug-eluting stents (DES) were included in the present study. After 12-36 months of follow-up, coronary angiography was performed and ISR was defined as >50% diameter stenosis at follow-up. RT-qPCR was used to measure lncRNA expression. Expression of the lncRNA RNA antisense non-coding RNA at the INK4 locus (ANRIL) was found to be upregulated whereas the lncRNA homeobox A11 antisense (HOXA11-AS) was downregulated in the plasma of patients with ISR compared with that from patients without ISR (P<0.001). Logistic regression analysis revealed that ANRIL [odds ratio (OR)=2.95; 95% confidence interval (CI)=1.68-8.08] was an independent risk factor for ISR, whilst HOXA11-AS (OR=0.58; 95% CI=0.48-0.71) was found to be an independent protective factor for ISR. Receiver operating characteristic (ROC) analysis demonstrated that high ANRIL expression [area under the ROC (auROC)=0.755; 95% CI=0.702-0.803] and low HOXA11-AS levels (auROC=0.712; 95% CI=0.657-0.763) predicted a high risk for ISR, and the combined score of ANRIL and HOXA11-AS (auROC=0.844; 95% CI=0.798-0.884) was more efficient at predicting ISR than either ANRIL or HOXA11-AS alone (P<0.001). In conclusion, increased ANRIL and decreased HOXA11-AS expressions were associated with ISR. However, combined ANRIL and HOXA11-AS plasma levels proved to be more effective at predicting ISR compared with either ANRIL or HOXA11-AS alone, suggesting that the multiplex detection of lncRNAs could be used to predict ISR in the future.

Correspondence to: Dr Longbin Liu, Department of Cardiology, The Affiliated Hospital of Shaoxing University, 999 Zhongxing South Road, Shaoxing, Zhejiang 312000, P.R. China
E-mail: liulongbin@hotmail.com

Key words: long non-coding RNA antisense non-coding RNA at the INK4 locus, long non-coding RNA homeobox A11 antisense, in-stent restenosis

Introduction

Coronary heart disease (CHD) poses a serious threat to human life and health. The number of patients with CHD in China has reached 11 million and is still increasing (1). Since the first successful percutaneous transluminal coronary angioplasty was performed by Gruentzig in 1977 (2), coronary artery intervention has undergone >40 years of development. However, after coronary stent implantation, in-stent restenosis (ISR) continues to be a serious obstacle faced by cardiologists (3). Early identification of patients at high risk of ISR and strengthening antiplatelet management, coupled with effective risk factor (smoking, diabetes, hypertension and hyperlipidemia) management for these patients, are considered to be effective in preventing ISR (4). However, factors that can effectively predict the risk of ISR have remained elusive.

Long non-coding RNAs (lncRNAs) belong to a family of functional RNA molecules that are >200 nucleotides long and do not encode proteins (5). Despite being non-coding, lncRNAs have been reported to regulate a number of cellular and tissue processes, including growth and development, organ formation, bone marrow hematopoiesis, cell apoptosis and cell proliferation (5,6). In addition, lncRNAs have been reported to serve a key role in a number of pathophysiological mechanisms, such as renal artery stenosis and renal failure (7). In the cardiovascular field, lncRNAs play a key regulatory role in the occurrence and development of CHD, hypertension and cardiac insufficiency (5). Ballantyne *et al* (6) previously found that the expression of lncRNA smooth muscle cell-enriched was increased in plaques and plasma from patients with CHD, which could accelerate the progression of atherosclerotic plaques by promoting the proliferation and migration of vascular smooth muscle cells (VSMCs). In addition, a previous study used gene chip technology to study heart tissues from six patients with congenital heart defects and indicated that the lncRNA forkhead box F1 adjacent non-coding developmental regulatory RNA interacted with trithorax-group proteins/mixed lineage leukemia complexes, leading to H3K27 trimethylation and reduction in the expression of

target genes in the heart and cardiac dysplasia (5). In addition, a previous study by Wang *et al* (8) revealed that plasma levels of the lncRNA antisense non-coding RNA at the INK4 locus (ANRIL) were significantly increased in patients with ISR compared with patients without ISR, suggesting that ANRIL may be a prognostic factor for ISR.

In the present study, plasma samples were collected from three patients with ISR and another three patients without ISR. RNA sequencing was used to identify lncRNAs that were significantly differentially expressed between the two groups. From these, ANRIL and homeobox A11-antisense (HOXA11-AS) were selected for their dysregulation in patients with ISR compared with patients without ISR for further investigation of their potential to predict ISR in patients with single-vessel lesions who received drug-eluting stents (DES).

Materials and methods

Patients. The inclusion criterium in the present study was patients with single coronary artery vessel lesions who received DES treatment. The exclusion criteria were: i) Patients with tumors; ii) patients who died during the follow-up; and iii) patients could not undergo another coronary angiography because of other diseases; or iv) patients who refused to undergo a coronary angiography review. The patients were followed up for 2-5 years.

A total of 410 patients with single-vessel lesions who received DES at The Affiliated Hospital of Shaoxing University and Shaoxing People's Hospital (Shaoxing, China) between September 2015 and September 2017 were included in the present study. The following 114 patients were excluded: i) 20 patients died during the follow-up period; ii) 6 patients were diagnosed with tumors; iii) 24 patients could not undergo another coronary angiography because of other diseases, such as leukemia and thrombocytopenia; and iv) 64 patients refused to undergo a coronary angiography review. Following angiography results, ISR was defined as stenosis >50%. A total of 244 individuals were enrolled into the control group and 52 patients were enrolled in the ISR group. Written informed consent was obtained from all patients and the study protocol was approved on February 1, 2015 by the Ethics Committee of the Affiliated Hospital of Shaoxing University (approval no. 2015002).

Data collection. Health habits and medical history of the patients were recorded by a Dr ZJ. The age, sex, weight (body mass index), smoking history, systolic and diastolic blood pressure (SBP and DBP), in addition to other clinical diseases, such as diabetes and myocardial infarction, were also recorded and presented in Table I. Echocardiography was performed by ZJ and LL. The levels of N-terminal pro B-type natriuretic peptide (cat. no. JN19299; Shanghai Jining Shiye), high-sensitivity C-reactive protein (hs-CRP; cat. no. JN18144; Shanghai Jining Shiye) and creatinine (cat. no. JN17987; Shanghai Jining Shiye) were tested by enzyme immunoassay in the central laboratory of The Affiliated Hospital of Shaoxing University (Shaoxing, China).

Sample collection. A total of 6 h later after percutaneous coronary intervention (PCI), 5 ml whole blood was collected from each participant. The separation procedure was performed immediately after sample collection. Blood samples were centrifuged at 1,000 x g for 10 min at 4˚C to separate the blood cells. The supernatant was then centrifuged at 13,000 x g for 10 min at 4˚C to completely remove all cellular contaminants. The plasma was aliquoted into microcentrifuge tubes and stored at -80˚C until further analysis.

RNA sequencing. Equal volumes of serum were collected from three patients with ISR and three patients without ISR in six separate pools. Total RNA was extracted with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and its quality and quantity were assessed using NanoDrop™ 2000c (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The following RNA sequencing protocol was performed by Lianchuan BioTech Co., Ltd. Following DNase treatment (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, cDNA libraries were constructed in a strand-specific manner from 4 μg of DNase-treated RNA using TruSeq Stranded Total RNA Library Prep kit (cat. no. 20020597; Illumina, Inc.) according to the manufacturer's instructions. The samples were denatured to single-stranded DNA with 0.1 M NaOH (final density, 8 pM; concentrations measured by RT-qPCR) and amplified using TruSeqSR Cluster Kit v3-cBot-HS (cat. no. GD-401-3001; Illumina, Inc.) according to the manufacturer's instructions. The libraries were pooled and sequenced on the Illumina NovaSeq 6000 system (Illumina, Inc.) using 150 cycles of paired-end sequencing. High-quality reads were obtained by trimming adapter sequences, invalid and low-quality reads from the raw reads (quality control). The clean reads were then mapped to the human genome by HISAT2 software (v2.1.0; http://ccb.jhu.edu/software/hisat2) using default parameters. Next, transcript assemblies were constructed using StringTie software (v1.3.6; The Center for Computational Biology at Johns Hopkins University) to merge transcripts, and DESeq2 software (v2.11.40.2; Bioconductor, Inc.) was used to compute differential expression.

RT-qPCR. RNA was extracted from the serum of all patients. Total RNA was extracted with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), and its quality and quantity were assessed using NanoDrop™ 2000c (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). RNA was then reverse transcribed into cDNA with a PrimeScript RT Reagent kit according to the manufacturer's protocol (cat. no. RR047A; Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. RT-qPCR was performed with SYBR™ Green PCR Master Mix kit (cat. no. 4368702; Applied Biosystems; Thermo Fisher Scientific, Inc) using a LightCycler® 480 Real-time PCR System [Roche Diagnostics (Shanghai) Co., Ltd.]. GAPDH was used as an internal control. The following primers were used: ANRIL forward, 5'-TGCTCTATCCGCCAATCAGG-3' and reverse, 5'-GGGCCTCAGTGGCACATACC-3'; HOXA11-AS forward, 5'-CGGCTAACAAGGAGATTTGG-3' and reverse, 5'-AGGCTCAGGGATGGTAGTCC-3' and GAPDH forward, 5'-AGCCACATCGCTCAGACAC-3' and reverse, 5'-GCCCAA TACGACCAAATCC-3'. The thermocycling conditions for qPCR were as follows: 95˚C for 10 min; followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec on an LightCycler® 480 Real-time PCR System. Relative lncRNA expression levels

Table I. Baseline characteristics of patients with drug-eluting stent implantation.

Parameter	Total (n=296)	Non-ISR (n=244)	ISR (n=52)	P-values
Male, n (%)	155 (52.4)	123 (50.8)	32 (61.5)	0.169
Age, years	66.1±6.3	65.4±7.2	69.2±5.1	0.231
Body mass index, kg/m^2	21.4±2.3	21.9±2.9	20.8±2.5	0.301
Systolic blood pressure, mmHg	142.5±18.2	132.5±16.5	148.2±17.0	<0.001
Diastolic blood pressure, mmHg	84.8±9.5	82.9±7.3	94.5±9.5	0.042
Acute coronary syndrome, n (%)	82 (27.7)	58 (23.8)	24 (46.2)	0.002
Diabetes, n (%)	124 (41.9)	88 (36.1)	36 (69.2)	<0.001
Smoking, n (%)	92 (31.1)	79 (32.3)	13 (25.0)	0.327
High-sensitivity C-reactive protein (mg/l)	4.5±0.7	5.4±0.5	4.2±0.5	0.311
Low-density lipoprotein (mmol/l)	2.8±0.36	2.4±0.4	3.0±0.3	0.048
Lnc ANRIL relative expression	1.68±0.05	1.55±0.08	2.44±0.1	<0.001
Lnc HOXA11-AS relative expression	9.04±0.36	9.28±0.3	7.53±0.4	<0.001

Values are presented as n (%) or the mean ± SD. Lnc, long non-coding RNA; ANRIL, antisense non-coding RNA at the INK4 locus; HOXA11-AS, homeobox A11-antisense; ISR, in-stent restenosis.

were calculated using the $2^{-\Delta\Delta Cq}$ method normalized to GAPDH (9). Each blood sample was analyzed in duplicate, and all analyses were repeated three times.

Statistical analysis. Continuous variables are expressed as the mean ± SD, whilst categorical variables are presented as counts or percentages. Kolmogorov-Smirnov test was used to ensure that the variables were normally distributed. The differences between continuous variables were tested with unpaired Student's t-test, and the differences between categorical variables were examined by χ^2 test. All statistical analyses were performed using MedCalc (v2016; MedCalc Software Ltd). P<0.05 was considered to indicate a statistically significant difference.

A logistic regression model performed on MedCalc was used to test the association between the levels of ANRIL and/or HOXA11-AS along with other known risk factors for ISR using univariate and multivariate models. The multivariable model was adjusted for SBP, DBP, sex, age, diabetes, acute coronary syndrome (ACS), smoking, hs-CRP, low-density lipoprotein (LDL), ANRIL and HOXA11-AS levels.

To assess differences in prognostic efficiency involving ANRIL and HOXA11-AS, the area under the curve of a receiver operating characteristic analysis (auROC) using MedCalc, which is a measure of discrimination, was calculated. ISR was used to evaluate the diagnostic performance of the scoring systems for patients who underwent PCI. Furthermore, standard indices of validity, such as the Youden index, specificity and sensitivity, were calculated according to the ROC results.

Results

Study population. The smallest sample sizes were calculated using the respective HOXA11-AS and ANRIL values. RT-qPCR quantification indicated that, for HOXA11-AS, the mean and standard deviation for the control group were 9.2850 and 3.020, whereas the mean and standard deviation for the ISR group were 6.18 and 4.4. The α was set as 0.05 and the β as 0.1, therefore the smallest sample size for each group was 30. For ANRIL, the mean and standard deviation for the control group were 1.535 and 0.8717, whereas the mean and standard deviation for the ISR group were 2.69 and 1.0044. The α was set as 0.05 and the β as 0.1, therefore the smallest sample size for each group was 14. In conclusion, the sample size of the present study was sufficient and did not affect the interpretation or generalization of the data.

A total of 410 patients with single-vessel lesions who received a DES at The Affiliated Hospital of Shaoxing University and Shaoxing People's Hospital (China) between September 2015 and September 2017 were included in the present study. After excluding those who did not meet the follow-up criteria, 296 patients remained. Baseline characteristics for each patient were collected from the medical records. As presented in Table I, the mean age of the patients was 66.1±6.3 years, where 52.4% of all patients were male. A total of 17.6% patients (n=52) developed ISR during the follow-up coronary angiography review. SBP, DBP and LDL levels in the ISR group were significantly higher compared with those in the non-ISR group (P<0.05). There were no differences in terms of body mass index, age and hs-CRP between the two groups. In addition, the rate of history of ACS and diabetes was significantly higher in the ISR group (P<0.05).

Expression of ANRIL and HOXA11-AS. To identify potential lncRNAs involved in the development of ISR, RNA sequencing was used to analyze and sequence lncRNAs isolated from the serum of three patients with ISR and three patients without ISR (accession no. GSE182225). As presented in Fig. 1, >30 lncRNAs exhibited differences in expression between the two groups. As ANRIL value for predicting ISR was reported by Wang *et al* (8) and HOXA11-AS was identified as potentially involved in ISR in our previous work (10), ANRIL and HOXA11-AS were selected for further analysis. ANRIL and HOXA11-AS expression levels were subsequently analyzed in all the patients (including 52 patients with ISR and 244 patients without ISR) using RT-qPCR, which revealed that ANRIL expression was

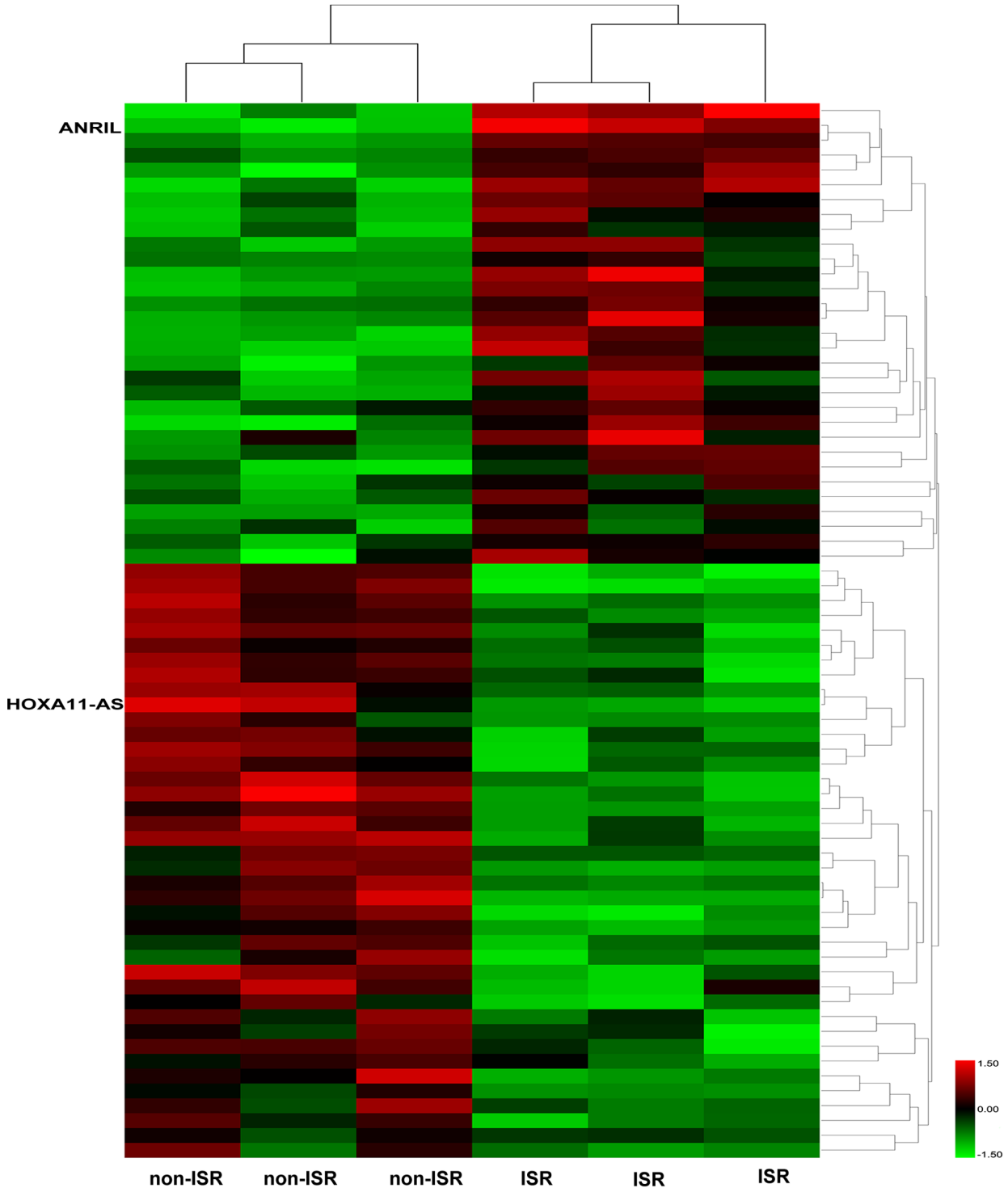

Figure 1. Serum RNA sequencing results of patients with or without ISR. Serum samples were collected from three patients with ISR and three patients without ISR. RNA sequencing results indicated that the levels of several long non-coding RNA expression were different between the two groups. ISR, in-stent restenosis. ANRIL, antisense non-coding RNA at the INK4 locus; HOXA11-AS, homeobox A11-antisense.

significantly increased in the ISR group compared with that in the non-ISR group, whilst HOXA11-AS expression was significantly decreased (P<0.001), as shown in Fig. 2.

Risk factor analysis for ISR. As shown in Table II, univariate logistic regression revealed that SBP (odds ratio (OR)=1.22; 95% confidence interval (CI)=1.03-1.98), DBP (OR=1.09; 95% CI=1.03-1.88), ACS (OR=1.36; 95% CI=1.06-4.89), diabetes (OR=1.92; 95% CI=1.61-4.19), LDL (OR=2.44; 95% CI=1.46-8.04) and ANRIL expression (OR=2.95; 95% CI=1.68-8.08) were significantly associated with ISR (P<0.05). In addition, HOXA11-AS expression was negatively associated with ISR (OR=0.62; 95% CI=0.48-0.71; P<0.05). All variables were then entered into the multivariate logistic regression analysis. As reported by Table II, SBP (OR=1.31; 95% CI=1.06-2.01), DBP (OR=1.05; 95% CI=0.99-1.96),

Table II. Logistic regression analysis for patients with drug-eluting stent implantation, with or without in-stent restenosis.

	Univariate analysis			Multivariate analysis		
Variables	OR	95% CI	P-value	OR	95% CI	P-value
Male	0.99	0.49-1.94	0.722	0.96	0.41-2.07	0.603
Age	0.94	0.68-1.44	0.223	0.98	0.88-1.68	0.245
Body mass index	1.09	0.29-1.99	0.485	1.09	0.30-1.85	0.502
Systolic blood pressure	1.22	1.03-1.98	0.007	1.31	1.06-2.01	0.002
Diastolic blood pressure	1.09	1.03-1.88	0.034	1.05	0.99-1.96	0.046
Acute coronary syndrome	1.36	1.06-4.89	0.041	1.39	1.14-3.27	0.002
Diabetes	1.92	1.61-4.19	0.004	1.88	1.42-6.21	0.009
Smoking	1.05	0.53-1.60	0.692	1.02	0.24-1.38	0.416
High-sensitivity C-reactive protein	1.14	0.78-1.56	0.265	1.09	0.42-1.95	0.301
Low-density lipoprotein	2.44	1.46-8.04	<0.001	2.74	1.78-9.69	0.001
Lnc ANRIL relative expression	2.95	1.68-8.08	0.001	2.68	1.62-7.82	0.001
Lnc HOXA11-AS relative expression	0.62	0.48-0.71	0.001	0.58	0.45-0.72	0.001

OR, odds ratio; 95% CI, 95% confidence interval; Lnc, long non-coding RNA; ANRIL, antisense non-coding RNA at the INK4 locus; HOXA11-AS, homeobox A11-antisense.

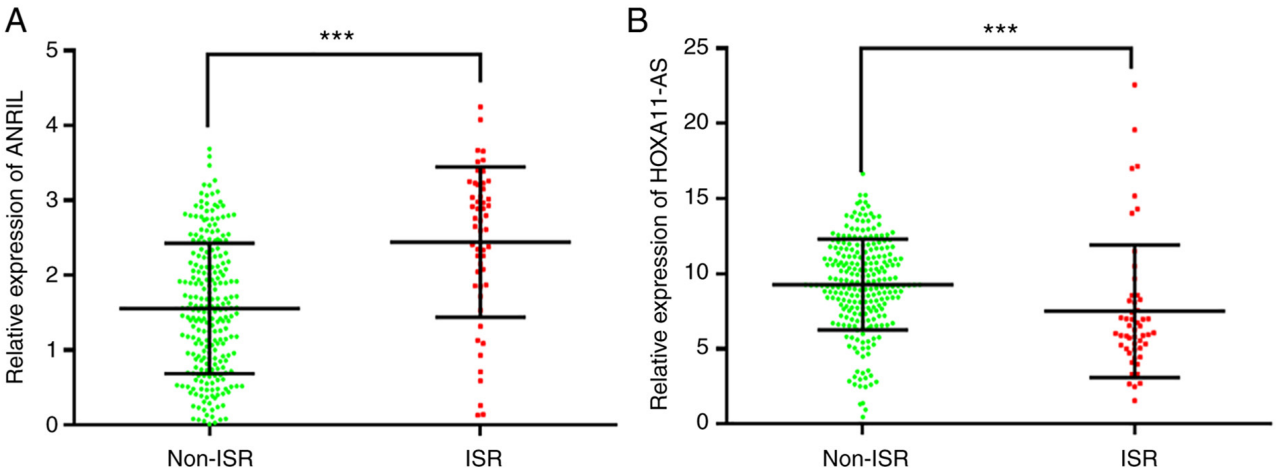

Figure 2. ANRIL and HOXA11-AS expression in the serum of patients with or without ISR. Relative expression of (A) ANRIL and (B) HOXA11-AS in patients with and without ISR, obtained by reverse transcription-quantitative PCR. ***P<0.001. ISR, in-stent restenosis; ANRIL, antisense non-coding RNA at the INK4 locus; HOXA11-AS, homeobox A11-antisense.

ACS (OR=1.39; 95% CI=1.14-3.27), diabetes (OR=1.88; 95% CI=1.42-6.21), LDL (OR=2.74; 95% CI=1.78-9.69) and ANRIL expression (OR=2.68; 95% CI=1.62-7.82) were found to be independent risk factors for ISR (P<0.05), whilst HOXA11-AS expression (OR=0.58; 95% CI=0.45-0.72; P<0.001) was found to be an independent protective factor for ISR.

ROC analysis of ANRIL and HOXA11-AS for ISR. The ability of ANRIL and HOXA11-AS to predict ISR in patients with single-vessel lesions who received a DES is presented in Fig. 3 and Table III. The performance of ANRIL with an auROC of 0.755 (95% CI=0.702-0.803) was higher than that of HOXA11-AS with an auROC of 0.712 (95% CI=0.657-0.763), although the difference was not statistically significant between the two lncRNAs (data not shown). When using the best ANRIL cut-off value of 2.25, the sensitivity was 67.3% and the specificity was 75.8%. By contrast, when using a cut-off value for HOXA11-AS of 7.07, the sensitivity was 67.31% and the specificity was 79.1%. ANRIL and HOXA11-AS were then combined to obtain the ANRIL and HOXA11-AS scores [ANRIL and HOXA11-AS score = -2.3517 + 1.12065 x A NRIL + (-0.17024) x HOXA11-AS], for which coefficients were calculated by multivariate logistic regression, with only ANRIL and HOXA11-AS included. The score revealed an auROC of 0.844 (95% CI=0.798-0.884), which was significantly higher than that of ANRIL or HOXA11-AS (P<0.01). When a cut-off value of -1.67 was used for this score, the sensitivity was 82.7% and the specificity was 73.8%. Therefore, the combined ANRIL and HOXA11-AS score exhibited a better performance than either ANRIL or HOXA11-AS alone in predicting ISR in patients with single-vessel lesions who received a DES.

Table III. ROC analysis of using ANRIL and HOXA11-AS for predicting ISR in patients with drug-eluting stent implantation, with or without in-stent restenosis.

Parameter	auROC	95% CI	P-value[a]	P-value[b]	Youden	Cut-off	Sensitivity (%)	Specificity (%)
ANRIL	0.755	0.702-0.803	<0.001	0.008	0.431	2.25	67.3	75.8
HOXA11-AS	0.712	0.657-0.763	<0.001	0.002	0.464	7.07	67.31	79.10
ANRIL and HOXA11-AS	0.844	0.798-0.884	<0.001	N/A	0.564	-1.67	82.7	73.8

ANRIL and HOXA11-AS score = -2.3517+1.12065 x ANRIL + (-0.17024) x HOXA11-AS. [a]P<0.05 compared with 0.5 (auROC critical value); [b]P compared with auROC of ANRIL and HOXA11-AS. ROC, receiver operating characteristic; auROC, area under the curve of a ROC analysis; 95% CI, 95% confidence interval; ANRIL, antisense non-coding RNA at the INK4 locus; HOXA11-AS, homeobox A11-antisense.

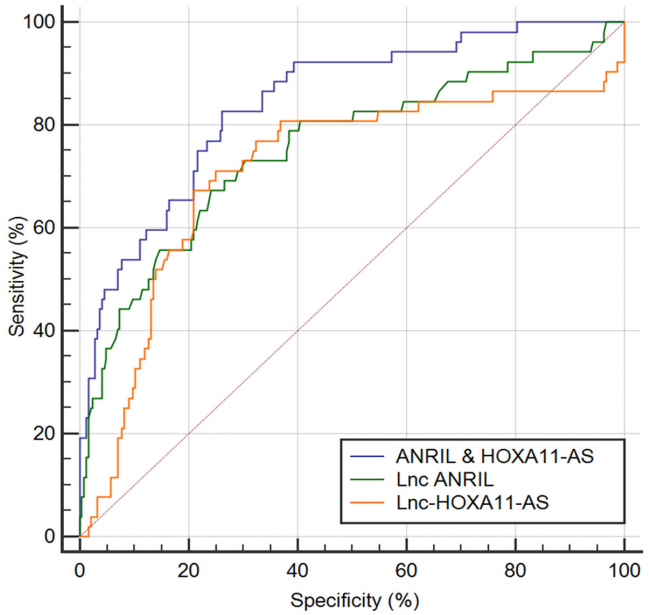

Figure 3. ROC analysis of the potential of ANRIL and HOXA11-AS to predict ISR. ROC analysis was used to test the values of ANRIL and HOXA11-AS for the prediction of ISR. High ANRIL (auROC=0.755; 95% CI=0.702-0.803) expression (green line) and low HOXA11-AS (auROC=0.712; 95% CI=0.657-0.763) expression (yellow line) predicted high risk for ISR (P<0.05). Combined scores of ANRIL and HOXA11-AS (auROC=0.844; 95% CI=0.798-0.884) expression (blue line) had a better performance for the prediction of ISR than ANRIL or HOXA11-AS alone (P<0.05). auROC, area under the curve of a receiver operating characteristic analysis; ISR, in-stent restenosis; Lnc, long non-coding RNA; ANRIL, antisense non-coding RNA at the INK4 locus; HOXA11-AS, homeobox A11-antisense.

Discussion

ISR occurrence is the result of a number of factors. Several studies have shown that the type of coronary vascular lesions, length of the lesions, diameter and length of the implanted stent, types of stent, tandem stent and technical proficiency of doctors can all affect the occurrence of ISR (11,12). In addition, previous studies have revealed that smoking, diabetes and hyperlipidemia are independent risk factors for ISR (13). Sex and age can also affect ISR occurrence (14). Early assessment of the risks of ISR in patients after PCI and controlling the risk factors have been considered to be one of the most effective means to prevent and treat ISR (8,15).

lncRNAs are highly conserved molecular sequences that are also relatively stable in the circulatory system, rendering them potentially reliable molecular markers for disease identification (16). Previous studies have confirmed that some lncRNAs have diagnostic value for CHD (15,17). For example, expression of the lncRNA long intergenic noncoding RNA predicting cardiac remodeling was found to be downregulated during the early phases after a cardiac event, but remained elevated during latter stages after an acute myocardial infarction (AMI) (18). In addition, myosin heavy-chain-associated RNA transcript, a recently described lncRNA that was found to protect the heart from hypertrophic remodeling, demonstrated a positive correlation with the cardiac injury marker cTnT in patients with AMI (5). However, to the best of our knowledge, only one study has addressed the association between plasma lncRNAs and ISR (8). In the present study, plasma samples were collected from patients with and without ISR for analysis. RNA sequencing results indicated that ANRIL expression was upregulated, whilst HOXA11-AS was downregulated in plasma samples from patients with ISR compared with patients without ISR. In addition, the present results identified ANRIL to be an independent risk factor for ISR, whereas HOXA11-AS was an independent protective factor for ISR. Therefore, high ANRIL expression and low HOXA11-AS expression are proposed to be predictors of high ISR risk. Furthermore, the present data identified that the combined score of ANRIL and HOXA11-AS was a superior predictor for ISR compared with either ANRIL or HOXA11-AS alone.

Consistent with the present findings, the value of ANRIL for predicting ISR was previously reported by Wang *et al* (8). Accumulating evidence has demonstrated that ANRIL is expressed in vascular endothelial cells, VSMCs, mononuclear phagocytes and atherosclerotic plaques (19-21). ANRIL can not only regulate protein expression, but also participate in post-translational modifications (20). Abnormal ANRIL expression has been revealed to result in vascular endothelium injury, proliferation, migration, senescence and apoptosis of VSMCs, in addition to mononuclear cell adhesion and proliferation, glycolipid metabolism disorders and DNA damage (21). ANRIL accelerates atherosclerosis development and is a known risk factor for CHD and ISR (8).

HOXA11-AS is a newly discovered lncRNA that has been studied mainly in the context of tumor biology (22). Wang *et al* (23) reported that HOXA11-AS was associated with cell cycle progression in glioma, associated with tumor grade and lead to a poorer prognoses. In another study, Chen *et al* (24) proposed that HOXA11-AS may participate in the occurrence of cervical cancer by regulating HOXA11. Furthermore, two previous studies revealed that HOXA11-AS

expression was associated with metastasis, invasion, staging and prognosis in human ovarian cancer and lung adenocarcinoma (25,26). These studies also proposed that suppression of ovarian cancer by HOXA11-AS did not occur by regulating the HOXA11 gene (25,26). In 2017, a number of studies reported that HOXA11-AS could promote the proliferation of tumor cells by regulating the expression of microRNA (miR)-140-5p, large tumor suppressor kinase 1, miR-124 and peptidyl arginine deiminase 2 (27-31). However, to date, the role of HOXA11-AS in the cardiovascular field has not been previously reported. The present study reported that HOXA11-AS expression was decreased in patients with ISR and can therefore function as a protective factor for ISR.

Multiplex detection demonstrated better predictive performance than individual factors alone, which has also been demonstrated in a number of previous studies (32,33). Tesche *et al* (33) demonstrated that a combination of coronary CT angiography-derived non-calcified plaque volumes, lesion length and remodeling index had a higher predictive value for ISR than each aforementioned alone, which was clinically valuable for the prediction of ISR. Consistent with this, the present results indicated that the combination of ANRIL and HOXA11-AS significantly improved the predictive accuracy for ISR compared with each lncRNA alone. With the rapid development in technology and equipment, it is now possible to simultaneously detect several different lncRNAs from a single sample using multiplex microbead-based assays (34). Therefore, multiplex detection of lncRNAs could represent a promising assay for predicting ISR.

In conclusion, the present results demonstrated that HOXA11-AS expression was downregulated in the plasma of patients with ISR, whereas ANRIL expression was upregulated. ANRIL was found to be an independent risk factor for ISR, whilst HOXA11-AS was an independent protective factor for ISR. In addition, high ANRIL expression and low HOXA11-AS expression predicted a higher risks of ISR. The present study subsequently revealed that the combined score for ANRIL and HOXA11-AS proved to be superior at predicting ISR compared with that for either ANRIL or HOXA11-AS alone. Therefore, despite its limitations (lack of *in vivo* investigations to confirm the role ANRIL or HOXA11-AS in ISR), the present results support the use of the multiplex detection of lncRNAs as a test for predicting ISR in the future.

Acknowledgements

Not applicable.

Funding

The present study is supported by the Basic Public Welfare Research Project of Zhejiang Province (grant no. LGF19H020002) and Science Project of Shaoxing City (grant no. 2018C30021).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The RNA sequencing data was uploaded to GEO database (accession no. GSE182225; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE182225).

Authors' contributions

All authors have read and approved the final manuscript. ZJ and LL designed and performed the research. HS, WC and HX helped collect and analyze the data. ZJ and LL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Committee of The Affiliated Hospital of Shaoxing University (Shaoxing, China; approval no. 2015002). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Hu SS, Gao RL, Liu LS, Zhu ML, Wang W and Wang YJ: The abstract of 2018 report of Chinese cardiovascular disease. Chin Circ Mag 34: 209-220, 2019.
2. Meier B: The first patient to undergo coronary angioplasty - 23-year follow-up. N Engl J Med 344: 144-145, 2001.
3. Lee MS and Banka G: In-stent restenosis. Interv Cardiol Clin 5: 211-220, 2016.
4. Buccheri D, Piraino D, Andolina G and Cortese B: Understanding and managing in-stent restenosis: A review of clinical data, from pathogenesis to treatment. J Thorac Dis 8: E1150-E1162, 2016.
5. Uchida S and Dimmeler S: Long noncoding RNAs in cardiovascular diseases. Circ Res 116: 737-750, 2015.
6. Ballantyne MD, Pinel K, Dakin R, Vesey AT, Diver L, Mackenzie R, Garcia R, Welsh P, Sattar N, Hamilton G, *et al*: Smooth muscle enriched long noncoding RNA (SMILR) regulates cell proliferation. Circulation 133: 2050-2065, 2016.
7. Lai CF, Chen YT, Gu J, Nerbonne JM, Lin CH and Yang KC: Circulating long noncoding RNA DKFZP434I0714 predicts adverse cardiovascular outcomes in patients with end-stage renal disease. Int J Cardiol 277: 212-219, 2019.
8. Wang F, Su X, Liu C, Wu M and Li B: Prognostic value of plasma long noncoding RNA ANRIL for in-stent restenosis. Med Sci Monit 23: 4733-4739, 2017.
9. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
10. Wang P, Meng L, Liu L and Peng F: The value of circulating exosomes-mediated lncRNA HOXA11-AS in the proliferation of vascular endothelial cell by up-regulating SOX4. Journal of Wenzhou Medical Unversity 51: 639-645, 2021 (In Chinese).
11. Shi KQ, Wu FL, Liu WY, Zhao CC, Chen CX, Xie YY, Wu SJ, Lin XF, Chen YP, Wong DK, *et al*: Non-alcoholic fatty liver disease and risk of in-stent restenosis after bare metal stenting in native coronary arteries. Mol Biol Rep 41: 4713-4720, 2014.
12. Bambagioni G, Di Mario C, Torguson R, Demola P, Ali Z, Singh V, Skinner W, Artis A, Cate TT, Zhang C, *et al*: Lipid-rich plaques detected by near-infrared spectroscopy predict coronary events irrespective of age: A Lipid Rich Plaque sub-study. Atherosclerosis 334: 17-22, 2021.
13. Pleva L, Kukla P and Hlinomaz O: Treatment of coronary in-stent restenosis: A systematic review. J Geriatr Cardiol 15: 173-184, 2018.
14. Li MK, Tsang AC, Tsang FC, Ho WS, Lee R, Leung GK and Lui WM: Long-term risk of in-stent restenosis and stent fracture for extracranial vertebral artery stenting. Clin Neuroradiol 29: 701-706, 2019.

15. Kumric M, Borovac JA, Martinovic D, Ticinovic Kurir T and Bozic J: Circulating biomarkers reflecting destabilization mechanisms of coronary artery plaques: are we looking for the impossible? Biomolecules 11: 881, 2021.
16. Schmitt AM and Chang HY: Long noncoding RNAs in cancer pathways. Cancer Cell 29: 452-463, 2016.
17. Thum T and Condorelli G: Long noncoding RNAs and microRNAs in cardiovascular pathophysiology. Circ Res 116: 751-762, 2015.
18. Li M, Wang YF, Yang XC, Xu L, Li WM, Xia K, Zhang DP, Wu RN and Gan T: Circulating long noncoding RNA LIPCAR acts as a novel biomarker in patients with ST-segment elevation myocardial infarction. Med Sci Monit 24: 5064-5070, 2018.
19. Holdt LM, Stahringer A, Sass K, Pichler G, Kulak NA, Wilfert W, Kohlmaier A, Herbst A, Northoff BH, Nicolaou A, *et al*: Circular non-coding RNA ANRIL modulates ribosomal RNA maturation and atherosclerosis in humans. Nat Commun 7: 12429, 2016.
20. Chi JS, Li JZ, Jia JJ, Zhang T, Liu XM and Yi L: Long non-coding RNA ANRIL in gene regulation and its duality in atherosclerosis. J Huazhong Univ Sci Technolog Med Sci 37: 816-822, 2017.
21. Chen L, Qu H, Guo M, Zhang Y, Cui Y, Yang Q, Bai R and Shi D: ANRIL and atherosclerosis. J Clin Pharm Ther 45: 240-248, 2020.
22. Wei C, Zhao L, Liang H, Zhen Y and Han L: Recent advances in unraveling the molecular mechanisms and functions of HOXA11 AS in human cancers and other diseases (Review). Oncol Rep 43: 1737-1754, 2020.
23. Wang Q, Zhang J, Liu Y, Zhang W, Zhou J, Duan R, Pu P, Kang C and Han L: A novel cell cycle-associated lncRNA, HOXA11-AS, is transcribed from the 5-prime end of the HOXA transcript and is a biomarker of progression in glioma. Cancer Lett 373: 251-259, 2016.
24. Chen J, Fu Z, Ji C, Gu P, Xu P, Yu N, Kan Y, Wu X, Shen R and Shen Y: Systematic gene microarray analysis of the lncRNA expression profiles in human uterine cervix carcinoma. Biomed Pharmacother 72: 83-90, 2015.
25. Richards EJ, Permuth-Wey J, Li Y, Chen YA, Coppola D, Reid BM, Lin HY, Teer JK, Berchuck A, Birrer MJ, *et al*: A functional variant in HOXA11-AS, a novel long non-coding RNA, inhibits the oncogenic phenotype of epithelial ovarian cancer. Oncotarget 6: 34745-34757, 2015.
26. Kim HJ, Eoh KJ, Kim LK, Nam EJ, Yoon SO, Kim KH, Lee JK, Kim SW and Kim YT: The long noncoding RNA HOXA11 antisense induces tumor progression and stemness maintenance in cervical cancer. Oncotarget 7: 83001-83016, 2016.
27. Yu W, Peng W, Jiang H, Sha H and Li J: LncRNA HOXA11-AS promotes proliferation and invasion by targeting miR-124 in human non-small cell lung cancer cells. Tumour Biol: Oct 15, 2017 (Epub ahead of print). doi: 10.1177/1010428317721440.
28. Yu J, Hong JF, Kang J, Liao LH and Li CD: Promotion of lncRNA HOXA11-AS on the proliferation of hepatocellular carcinoma by regulating the expression of LATS1. Eur Rev Med Pharmacol Sci 21: 3402-3411, 2017.
29. Xu C, He T, Li Z, Liu H and Ding B: Regulation of HOXA11-AS/miR-214-3p/EZH2 axis on the growth, migration and invasion of glioma cells. Biomed Pharmacother 95: 1504-1513, 2017.
30. Cui Y, Yi L, Zhao JZ and Jiang YG: Long noncoding RNA HOXA11-AS functions as miRNA sponge to promote the glioma tumorigenesis through targeting miR-140-5p. DNA Cell Biol 36: 822-828, 2017.
31. Chen D, Sun Q, Zhang L, Zhou X, Cheng X, Zhou D, Ye F, Lin J and Wang W: The lncRNA HOXA11-AS functions as a competing endogenous RNA to regulate PADI2 expression by sponging miR-125a-5p in liver metastasis of colorectal cancer. Oncotarget 8: 70642-70652, 2017.
32. Japp NC, Souchek JJ, Sasson AR, Hollingsworth MA, Batra SK and Junker WM: Tumor biomarker in-solution quantification, standard production, and multiplex detection. J Immunol Res 2021: 9942605, 2021.
33. Tesche C, De Cecco CN, Vliegenthart R, Duguay TM, Stubenrauch AC, Rosenberg RD, Varga-Szemes A, Bayer RR II, Yang J, Ebersberger U, *et al*: Coronary CT angiography-derived quantitative markers for predicting in-stent restenosis. J Cardiovasc Comput Tomogr 10: 377-383, 2016.
34. Parsa SF, Vafajoo A, Rostami A, Salarian R, Rabiee M, Rabiee N, Rabiee G, Tahriri M, Yadegari A, Vashaee D, *et al*: Early diagnosis of disease using microbead array technology: A review. Anal Chim Acta 1032: 1-17, 2018.