Long Noncoding RNAs in Patients With Acute Myocardial Infarction

Mélanie Vausort, Daniel R. Wagner, Yvan Devaux

Rationale: Long noncoding RNAs (lncRNAs) constitute a novel class of noncoding RNAs that regulate gene expression. Although recent data suggest that lncRNAs may be associated with cardiac disease, little is known about lncRNAs in the setting of myocardial ischemia.

Objective: To measure lncRNAs in patients with myocardial infarction (MI).

Methods and Results: We enrolled 414 patients with acute MI treated by primary percutaneous coronary intervention. Blood samples were harvested at the time of reperfusion. Expression levels of 5 lncRNAs were measured in peripheral blood cells by quantitative polymerase chain reaction: hypoxia inducible factor 1A antisense RNA 2, cyclin-dependent kinase inhibitor 2B antisense RNA 1 (ANRIL), potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1 (KCNQ1OT1), myocardial infarction–associated transcript, and metastasis-associated lung adenocarcinoma transcript 1. Levels of hypoxia inducible factor 1A antisense RNA 2, KCNQ1OT1, and metastasis-associated lung adenocarcinoma transcript 1 were higher in patients with MI than in healthy volunteers (P<0.01), and levels of ANRIL were lower in patients with MI (P=0.003). Patients with ST-segment–elevation MI had lower levels of ANRIL (P<0.001), KCNQ1OT1 (P<0.001), myocardial infarction–associated transcript (P<0.001), and metastasis-associated lung adenocarcinoma transcript 1 (P=0.005) when compared with patients with non–ST-segment–elevation MI. Levels of ANRIL were associated with age, diabetes mellitus, and hypertension. Patients presenting within 3 hours of chest pain onset had elevated levels of hypoxia inducible factor 1A antisense RNA 2 when compared with patients presenting later on. ANRIL, KCNQ1OT1, myocardial infarction–associated transcript, and metastasis-associated lung adenocarcinoma transcript 1 were significant univariable predictors of left ventricular dysfunction as assessed by an ejection fraction ≤40% at 4-month follow-up. In multivariable and reclassification analyses, ANRIL and KCNQ1OT1 improved the prediction of left ventricular dysfunction by a model, including demographic features, clinical parameters, and cardiac biomarkers.

Conclusions: Levels of lncRNAs in blood cells are regulated after MI and may help in prediction of outcome. This motivates further investigation of the role of lncRNAs after MI. (Circ Res. 2014;115:668-677.)

Key Words: myocardial infarction ■ prognosis ■ RNA, long noncoding

A large proportion (>80%) of the human genome is transcribed, but only <2% is subsequently translated into proteins.1-3 This implies that the vast majority of DNA sequences are transcribed as non–protein-coding RNAs. Among noncoding RNAs, short noncoding RNAs, such as microRNAs, have been extensively investigated, both in terms of potential disease biomarkers and therapeutic targets. A novel class of noncoding RNAs, longer than 200 nucleotides and termed long noncoding RNAs (lncRNAs), has recently emerged.4-5 In contrast to microRNAs, the mechanisms of gene regulation by lncRNAs are much more complex and involve both activation and inhibition of gene expression, as well as modulation of chromatin architecture.6-8 lncRNAs play significant roles in developmental processes.9 Also, whereas lncRNAs seem to constitute attracting biomarkers and therapeutic targets in the oncology field,10 the knowledge of the role of lncRNAs in cardiovascular disease is limited.

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Two recent studies reported that lncRNAs regulate cardiac development.11,12 A transcriptomic analysis with deep sequencing revealed that lncRNAs are dynamically regulated in the failing heart and after left ventricular (LV) assist device implantation, suggesting that lncRNAs may play important roles in the pathogenesis of heart failure and in reverse remodeling.13 A lncRNA named cardiac hypertrophy–related factor has been shown to regulate cardiac hypertrophy.14 Furthermore, it is thought that lncRNAs play a role in cardiovascular ageing.15 However, although we16 and others17 recently reported that...
expression levels of lncRNAs are regulated in the cardiac tissue after myocardial infarction (MI), it is unknown whether MI affects the levels of lncRNAs in peripheral blood cells. Five lncRNAs suspected to be associated with cardiac pathology were investigated in the present study: hypoxia inducible factor 1A antisense RNA 2 (aHIF), cyclin-dependent kinase inhibitor 2B antisense RNA 1 (ANRIL), potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1 (KCNQ1OT1), miRNA-associate transcript (MIAT), and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1).

We show that expression levels of selected lncRNAs were changed in peripheral blood cells from patients with acute MI and were associated with LV dysfunction.

Methods

Patients

Four hundred fourteen patients with acute MI enrolled in the Luxembourg Acute MI Registry and treated with primary percutaneous coronary intervention were enrolled in this study. A total of 274 patients had acute ST-segment-elevation MI (STEMI), which was defined by the following: (1) clinically significant ST-segment elevation (>1 mm); (2) occluded major coronary artery: thrombolysis in MI, 0 flow in the left anterior descending, circumflex, or right coronary artery; (3) peak creatine kinase activity >600 U/L (3× higher than the upper limit of the reference interval). A total of 140 patients had non–ST-segment–elevation MI (NSTEMI). 

Biochemical Analyses

Cardiac troponin T was measured using a fourth-generation assay from Roche performed on a Cobas e601 equipment, 0.01 μg/L being the lower detection limit and 0.03 μg/L being the cardiac troponin T concentration reproducibly measured with a coefficient of variation <10%. Creatine phosphokinase (CPK) activity was measured with a Roche IFCC recommended method on a Cobas e501 instrument. N-terminal pro–brain natriuretic peptide was measured using an Elecsys E2010 analyzer with N-terminal pro–brain natriuretic peptide reagent pack (Roche Diagnostics). The detection limit of this assay is 1 pg/mL.

Statistical Analyses

Mann–Whitney test was used to compare 2 groups of continuous variables, χ² test was used for qualitative data. One-way ANOVA followed by all pairwise multiple comparison procedures using the Holm–Sidak method was used for multiple groups comparisons. Linear regression and Spearman rank correlations were used to evaluate the association between levels of lncRNAs and continuous variables, and logistic regression was used for dichotomized variables. The SigmaPlot version 12.0 software was used for statistical analyses. All tests were 2 tailed, and a P < 0.05 was considered significant.

To evaluate the prognostic value of lncRNAs, receiver operating characteristic (ROC) curves were constructed, and the area under the ROC curves was determined. Univariable analyses with logistic regression were used to evaluate the ability of lncRNAs to predict LV dysfunction. Multivariable analyses were conducted with logistic regression to determine the predictive value of lncRNAs in conjunction with clinical parameters. Odds ratios with 95% confidence intervals were computed. Analysis of deviation with the Wald χ² test to measure significance was performed to address the value of adding a variable to a model. Reclassification analyses were performed to determine the additive value of lncRNAs to clinical parameters and other biomarkers. The integrated discrimination improvement was computed. Prediction analyses were performed on the R version 2.14.2 statistical platform using the packages hmisc, survival, and splines.

Results

Characteristics of the Study Population

Table 1 provides demographic and clinical features of the study population. A total of 414 patients were enrolled in this study, among which 140 (34%) had NSTEMI and 274 (66%) had STEMI. Median age of the MI population was 62% and 73% and were men. All patients had successful revascularization.

A control group of 86 apparently healthy volunteers was enrolled. Median age of this group was 61% and 81% and were men. This control group was comparable in terms of age and sex with the case group of patients with MI (P = 0.15 and P = 0.12, respectively).
Levels of lncRNAs in Healthy Volunteers and Patients With MI
First of all, considering that each patient with MI enrolled in this study received a bolus of heparin and that heparin can affect DNA amplification by polymerase chain reaction, we verified that heparin did not affect the measurement of lncRNAs by polymerase chain reaction (Online Figure I).

Then, we compared the levels of lncRNAs in whole blood cells obtained in PAXgene tubes from patients with MI and healthy volunteers (Figure 1A). MALAT1 was expressed at a high level; MIAT was moderately expressed; aHIF, ANRIL, and KCNQ1OT1 were expressed at lower levels. Levels of aHIF, KCNQ1OT1, and MALAT1 were higher in patients with MI when compared with healthy volunteers. Levels of ANRIL were lower in patients with MI when compared with healthy volunteers, and levels of MIAT were similar in both groups.

Association Between Levels of lncRNAs and Inflammation Markers in Patients With MI
There were positive associations between aHIF and several markers of inflammation measured at admission, such as white blood cell count, percentage of neutrophils, and c-reactive protein. In contrast, aHIF was inversely associated with the percentage of lymphocytes (Table 2). ANRIL was positively associated with the percentage of lymphocytes and monocytes but was inversely associated with white blood cell count, neutrophils, and platelets. MIAT was positively associated with lymphocytes and negatively associated with neutrophils and...
Levels of lncRNAs According to Diagnosis of STEMI Versus NSTEMI

Considering the association between lncRNAs and inflammatory markers, the levels of lncRNAs were adjusted with the white blood cell count of each patient with MI in subsequent analyses.

Patients with STEMI had lower levels of ANRIL, KCNQ1OT1, MIAT, and MALAT1 when compared with patients with NSTEMI (Figure 1B). Levels of aHIF were comparable between patients with NSTEMI and STEMI.

Levels of lncRNAs According to the Number of Diseased Coronary Arteries

One hundred seventy-one patients with MI had 1-vessel disease; 144 patients had 2-vessel disease; and 94 patients had 3-vessel disease. Information was not documented for 5 patients. Levels of lncRNAs were comparable between patients with 1, 2, or 3 diseased coronary arteries (Figure 2).

Levels of lncRNAs in Subtypes of Leukocytes

Next, we investigated the expression of lncRNAs in different subtypes of leukocytes isolated from the blood of healthy donors (Figure 3). aHIF was predominantly expressed by monocytes, and MIAT was predominantly expressed by lymphocytes. ANRIL and MALAT1 showed a similar expression profile with a predominance in monocytes and lymphocytes. KCNQ1OT1 was preferentially expressed by neutrophils and monocytes. Of note, in these samples from healthy donors, we could confirm the high level of MALAT1 observed in samples from patients with MI (Figure 1). These data suggest that lncRNAs have distinct expression patterns in the different populations of blood leukocytes. None of the 5 lncRNAs investigated could be consistently detected into serum, platelet-enriched, or platelet-deprived plasma of healthy volunteers (Ct value >32; Online Figure II).

Association Between Levels of lncRNAs and Ischemic Time

To gain insights into the kinetic of the changes in expression of lncRNAs after MI, we determined the ischemic time of each patient with MI (ie, the delay between chest pain onset and revascularization). Ischemic time was available for 402 patients and was in 5 hours. There was no linear relationship between ischemic time and levels of lncRNAs (Figure 4A). Then, patients were categorized in 3 groups according to ischemic time: <3 hours (early presenters; n=173), 3 to 6 hours (n=141), and >6 hours (n=88). Patients presenting within 3 hours of chest pain onset had elevated levels of aHIF when compared with patients presenting later on (Figure 4B). Together with the observation that levels of aHIF are higher in patients with MI when compared with healthy volunteers (Figure 1A), these data suggest that levels of aHIF are dynamically regulated after MI, with a rapid elevation in the first 3 hours after MI, and a decline over the next few hours.

Prediction of LV Dysfunction by lncRNAs

Analysis of ROC curves, univariable and multivariable analyses with logistic regression models, analysis of deviance, and reclassification analyses were implemented to determine the value of lncRNAs to predict LV dysfunction after MI, as demonstrated by an ejection fraction ≤40% at 4-month follow-up. Among the 414 patients enrolled in this study, 6 patients died within the first 4 months. A total of 332 patients (80%) had an echocardiography at 4-month follow-up, and 62 patients

Levels of lncRNAs According to the Number of Diseased Coronary Arteries

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Risk Factors, and Peak Levels of Cardiac Biomarkers

414 Patients With MI, Inflammatory Markers, Cardiovascular

Table 2. Association Between Expression Levels of IncRNA in 414 Patients With MI, Inflammatory Markers, Cardiovascular Risk Factors, and Peak Levels of Cardiac Biomarkers

<table>
<thead>
<tr>
<th>Blood biomarkers (admission levels)</th>
<th>aHIF</th>
<th>ANRIL</th>
<th>KCNQ1OT1</th>
<th>MIAT</th>
<th>MALAT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell count</td>
<td>Coeff 0.15</td>
<td>−0.10</td>
<td>−0.02</td>
<td>−0.07</td>
<td>−0.08</td>
</tr>
<tr>
<td></td>
<td>(P) value 0.004*</td>
<td>0.04*</td>
<td>0.74</td>
<td>0.16</td>
<td>0.09</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>Coeff 0.15</td>
<td>−0.14</td>
<td>−0.05</td>
<td>−0.13</td>
<td>−0.08</td>
</tr>
<tr>
<td></td>
<td>(P) value 0.002*</td>
<td>0.005*</td>
<td>0.28</td>
<td>0.01*</td>
<td>0.13</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td>Coeff −0.12</td>
<td>0.11</td>
<td>0.04</td>
<td>0.13</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(P) value 0.02*</td>
<td>0.04*</td>
<td>0.42</td>
<td>0.01*</td>
<td>0.16</td>
</tr>
<tr>
<td>Monocytes, %</td>
<td>Coeff −0.10</td>
<td>0.10</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>(P) value 0.05</td>
<td>0.04*</td>
<td>0.39</td>
<td>0.46</td>
<td>0.43</td>
</tr>
<tr>
<td>Platelet count</td>
<td>Coeff 0.04</td>
<td>−0.15</td>
<td>−0.05</td>
<td>−0.13</td>
<td>−0.19</td>
</tr>
<tr>
<td></td>
<td>(P) value 0.44</td>
<td>0.004*</td>
<td>0.37</td>
<td>0.01*</td>
<td>−0.001*</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>Coeff 0.15</td>
<td>−0.02</td>
<td>−0.07</td>
<td>−0.10</td>
<td>−0.04</td>
</tr>
<tr>
<td></td>
<td>(P) value 0.005*</td>
<td>0.70</td>
<td>0.15</td>
<td>0.06</td>
<td>0.36</td>
</tr>
<tr>
<td>MMP9</td>
<td>Coeff 0.24</td>
<td>−0.12</td>
<td>−0.04</td>
<td>−0.05</td>
<td>−0.01</td>
</tr>
<tr>
<td></td>
<td>(P) value &lt;0.001*</td>
<td>0.02*</td>
<td>0.38</td>
<td>0.29</td>
<td>0.88</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Coeff 0.11</td>
<td>0.07</td>
<td>−0.10</td>
<td>−0.02</td>
<td>−0.03</td>
</tr>
<tr>
<td></td>
<td>(P) value 0.03*</td>
<td>0.18</td>
<td>0.07</td>
<td>0.73</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Cardiovascular risk factors

| Age                                 | Coeff 0.01 | 0.17 | −0.05 | 0.02 | 0.03 |
|                                     | \(P\) value 0.88 | <0.001* | 0.33 | 0.73 | 0.60 |
| Sex                                 | Coeff −0.06 | 0.09 | −0.02 | 0.04 | 0.08 |
|                                     | \(P\) value 0.24 | 0.07 | 0.62 | 0.38 | 0.12 |
| Body mass index                     | Coeff −0.03 | 0.09 | −0.02 | 0.04 | 0.08 |
|                                     | \(P\) value 0.54 | 0.07 | 0.62 | 0.38 | 0.12 |
| Diabetes mellitus                   | Coeff 0.02 | 0.18 | 0.03 | 0.05 | 0.11 |
|                                     | \(P\) value 0.66 | <0.001* | 0.50 | 0.29 | 0.02* |
| Hypertension                        | Coeff 0.13 | 0.17 | 0.07 | 0.11 | 0.13 |
|                                     | \(P\) value 0.01* | <0.001* | 0.15 | 0.03* | 0.01* |
| Hypercholesterolemia                | Coeff −0.01 | 0.01 | −0.07 | −0.001 | −0.001 |
|                                     | \(P\) value 0.79 | 0.76 | 0.16 | 0.98 | 0.79 |
| Smoking                             | Coeff 0.04 | −0.01 | 0.09 | 0.12 | 0.08 |
|                                     | \(P\) value 0.44 | 0.76 | 0.06 | 0.02* | 0.09 |

Cardiac biomarkers (peak levels)

| CPK                                 | Coeff 0.18 | −0.14 | −0.09 | −0.13 | 0.003 |
|                                     | \(P\) value <0.001* | 0.005* | 0.07 | 0.005* | 0.95 |
| cTnT                                | Coeff 0.15 | −0.05 | −0.10 | −0.12 | −0.004 |
|                                     | \(P\) value 0.003* | 0.29 | 0.04* | 0.92 | 0.93 |

Associations were determined using unadjusted levels of IncRNAs. aHIF indicates hypoxia inducible factor 1A antisense RNA 2; ANRIL, cyclin-dependent kinase inhibitor 2B antisense RNA 1; Coeff, coefficient; CPK, creatine phosphokinase; cTnT, cardiac troponin T; KCNQ1OT1, potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1; IncRNA, long noncoding RNAs; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MIAT, myocardial infarction–associated transcript; MMP, matrix metalloproteinase; and TIMP1, tissue inhibitor of metalloproteinase-1.

*Significant \(P\) values.

(19%) had an ejection fraction \(\leq 40\%\). The following clinical variables were included in multivariable models and reclassification analyses: age, body mass index, sex, ischemic time, CPK, cardiac troponin T, N-terminal pro–brain natriuretic peptide, white blood cells count, antecedent of MI, diabetes mellitus, hypertension, hypercholesterolemia, and smoking.

ROC curves analysis revealed that IncRNAs were weak predictors of LV dysfunction at 4 months (Figure 5A). Using logistic regression, ANRIL, KCNQ1OT1, MIAT, and MALAT1 were significant univariate predictors of LV dysfunction (Figure 5B). However, this prediction capacity was lost in multivariate analysis in which only peak levels of CPK (odds ratios [95% confidence interval], 0.9997 [0.9994–1.0000]) and diabetes mellitus (odds ratio [95% confidence interval], 0.33 [0.14–0.82]) remained significant predictors of LV dysfunction (Figure 5C). Analysis of deviance showed that addition of ANRIL, KCNQ1OT1, or MALAT1 to a model including all clinical variables improved the prediction (Table 3). Combinations of markers did not improve the prediction. ANRIL and KCNQ1OT1 correctly reclassified a significant proportion of patients misclassified by the multivariable clinical model (Table 3). ANRIL and KCNQ1OT1 combined did not induce a further improvement of prediction. All together, these data suggest that ANRIL and KCNQ1OT1 may help in prediction of LV dysfunction.

Discussion

In this study, we show that expression levels of selected IncRNAs in peripheral blood cells are regulated after MI, are correlated with markers of cardiac injury, are affected by cardiovascular risk factors, and are associated with prognosis.

Peripheral blood cells were chosen as template to measure levels of IncRNAs. In addition to be noninvasive and convenient, this approach has already been proven valuable to identify transcriptomic signatures useful for the diagnosis of coronary artery disease in high-risk patients.20 In addition, RNA expression profiles of blood cells and cardiomyocytes share common features in response to aldosterone treatment in hypertensive rats and are associated with pathological remodeling.21

The rationale for the selection of the 5 IncRNAs investigated in this study is as follows. aHIF, a natural antisense transcript of HIF1α, is overexpressed in the failing heart.22 By modulating the stability of HIF1α messenger RNA, aHIF has the capacity to regulate angiogenesis, an important component of the response of the heart to ischemia. ANRIL is the best replicated genetic risk factor for coronary artery disease23 and regulates genes involved in glucose and fatty acid metabolism.24 KCNQ1OT1 regulates KCNQ1 expression, which deficiency induces congenital long-QT syndrome that can trigger arrhythmias.25 KCNQ1OT1 acts at the epigenetic level, promoting the formation of repressive chromatin structure, and is involved in cardiac development.26 MALAT1 is a long intergenic noncoding RNA mainly expressed in the nervous system. Genome-wide association studies revealed that genetic variation of MALAT1 confers susceptibility to MI.27 However, a causal link between MIAT and MI has not yet been demonstrated. MALAT1, which has been recently renamed nuclear-enriched noncoding transcript 2 because of its accumulation in the nucleus,28 was selected because it regulates alternative splicing and thereby has a strong potential to regulate gene expression and disease. Also, MALAT1 is one of the most highly abundant IncRNAs in mammalian cells. However, to date, MALAT1 has not been associated with heart disease.
First of all, this study shows that expression levels of selected lncRNAs in blood cells of patients are extremely variable, some lncRNAs being expressed at a low level (e.g., aHIF and ANRIL) and others being highly expressed such as MALAT1. Second, we observed that levels of most of the lncRNAs investigated in this study are regulated in blood cells of patients with acute MI when compared with healthy volunteers. Activation of inflammation most probably contributed to the regulation of lncRNA levels in patients with MI. However, the finding that not all lncRNAs are upregulated in patients with MI suggests that changes in lncRNA levels are not a mere consequence of inflammation. Indeed, levels of ANRIL were lower in patients with MI when compared with healthy volunteers. To correct for a potential bias induced by activation of inflammation, expression levels of lncRNAs were adjusted to the whole blood cell count of each patient with MI. Of note, whole blood cell counts of healthy volunteers were not available.

Levels of aHIF were strongly upregulated in patients with MI. This observation is consistent with the induction of HIF by hypoxia and suggests an interplay between HIF and aHIF in the regulation of angiogenesis after MI. The functional role of aHIF and other lncRNAs in the infarcted heart requires further investigation. Third, the observation that aHIF is correlated with white blood cell count and c-reactive protein suggests that the increased expression of this lncRNA may be a direct consequence of the inflammatory response to MI. Investigation of the expression of lncRNAs in different populations of blood cells from healthy donors revealed that monocytes and neutrophils are the main source of aHIF. Several indicators suggest that the upregulation of aHIF in patients with MI is mostly because of neutrophils. First, blood samples from patients with MI were obtained in the acute phase after MI (<12 hours after symptom onset), a phase where mostly neutrophils become activated. Second, there is a strong positive association between aHIF and the percentage of circulating neutrophils. Third, levels of aHIF are negatively associated with ischemic time, showing a decrease in the first few hours after symptom onset. However, it seems that ANRIL is mostly expressed by lymphocytes after MI because it is expressed both by lymphocytes and by monocytes in healthy donors and it is positively associated with lymphocyte count in patients with MI. This observation is intriguing and, together with the concept of...
that lymphocytes play a critical role after MI, suggests that ANRIL might be involved in the response of the heart to ischemic injury. It would be interesting to determine whether ANRIL modulates cardiac function through its known effects on glucose and fatty acid metabolism.

Levels of ANRIL were found to be associated with age, diabetes mellitus, and hypertension. We previously reported significant associations between circulating levels of cardi-ac-enriched microRNAs and cardiovascular risk factors in patients with MI. As for ANRIL, the strongest association between microRNAs and cardiovascular risk factors was age, suggesting that aging affects blood levels of multiple types of RNAs.

Levels of aHIF were positively correlated with CPK and troponin T. This is consistent with an association between aHIF and infarct severity, albeit there was no association with the number of diseased arteries. On the opposite, levels of MIAT were lower in patients with STEMI and were negatively correlated with CPK and troponin T. This suggests that expression of lncRNAs in blood cells is not a mere representation of infarct severity and thereby may provide additional information instead of traditional biomarkers of cardiac injury.
An added value of IncRNAs was indeed found when we investigated the ability of IncRNAs to predict LV dysfunction at 4-month follow-up as demonstrated by an ejection fraction ≤40%. Although IncRNAs were weak predictors of LV dysfunction in ROC curve analysis, multivariable analyses attested that KCNQ1OT1 and ANRIL enhanced the predictive value of a multiparameter model, including demographic features, clinical data, and cardiac biomarkers. Determination of the deviance of logistic regression models and reclassification analyses supported an additive value of IncRNAs in prediction of LV dysfunction. Of note, in multivariable analyses, only CPK and diabetes mellitus were significant predictors of LV dysfunction. N-terminal pro–brain natriuretic peptide had a borderline predictive value (P=0.06). The additive predictive value of ANRIL and KCNQ1OT1 is consistent with the absence of correlation with CPK and with the markers of extracellular matrix turnover matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1, which have been shown to be potential indicators of LV remodeling after MI.15–17 Additional studies are warranted to address the prognostic value of IncRNAs after MI specifically.

Recent studies reported that cardiac and plasma levels of IncRNAs are regulated after MI and can be useful predictors of LV remodeling.17,38 The 5 IncRNAs investigated in our study were not reliably detected into serum, platelet-enriched, or platelet-depleted plasma of healthy volunteers. This observation does not exclude their expression in serum or plasma of patients with MI.11,12 In addition, in mice subjected to pressure overload, Lee et al39 identified 135 responsive IncRNAs. Yang et al13 characterized the transcriptomic profiles of failing human hearts using deep sequencing and reported the regulation of many different IncRNAs. These studies constitute a reservoir for future investigations of the role of IncRNAs in MI and cardiac failure.

Boeckel et al40 reported that heparin affects the quantification of microRNAs by polymerase chain reaction in human blood samples. Because all patients with MI enrolled in the study received a bolus of 5000 IU of heparin, we tested a potential confounding effect of heparin on levels of IncRNAs. We observed that heparin did not affect the quantification of IncRNAs. This apparent discrepancy with the study by Boeckel et al40 can have several explanations. First, the concentration of heparin in patient blood (1 IU/mL of blood) is lower than in heparinized blood collection tubes (11–32 IU/mL). Second, RNA preparation from PAXgene tubes does not require a phenol–chloroform extraction step, which has been performed to measure microRNAs in the study by Boeckel et al,40 and which copurifies nucleic acids with heparin.41 Third, we have measured IncRNAs, whereas Boeckel et al40 have measured microRNAs, which may be more sensitive to interference by heparin than IncRNAs.

This study is limited by the subjective selection of the IncRNAs. We expect that many other IncRNAs are regulated after MI and possess a prognostic value. Large-scale technologies, such as next-generation sequencing, would provide an unbiased approach. A second limitation relies on the lack of white blood cells count for the control group that prevented an unbiased approach. A second limitation relies on the lack of white blood cells count for the control group that prevented a selection of the subtypes of blood cells expressing IncRNAs. We have performed on enriched population of cells obtained from healthy volunteers. Definite identification of the cell population expressing IncRNAs after MI requires concomitant assessment of IncRNAs and specific blood cell markers in blood samples from patients with MI. Finally, this study is limited by the absence of independent validation. Thus, the results should be considered provisional requiring validation through replication in independent study populations and experimentation.

In conclusion, we have shown that several IncRNAs are dysregulated in peripheral blood cells of patients with acute...
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Disclosures

None.

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34. Spinales FG. Matrix myosin remodeling and the matrix metalloproteinases: influence on cardiac function and function. Physiol Rev. 2007;87:1285-1342.


Novelty and Significance

What Is Known?

- A significant portion of the human genome is transcribed into long non-coding RNAs (lncRNAs).
- lncRNAs are implicated in regulating gene expression, cardiac development, and cardiac function.

What New Information Does This Article Contribute?

- Expression levels of selected lncRNAs are altered in peripheral blood cells of patients with acute myocardial infarction.
- Levels of the lncRNAs cyclin-dependent kinase inhibitor 2B antisense RNA 1 and potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1 are associated with left ventricular remodeling 4 months after myocardial infarction.

Emerging data implicate lncRNAs in the pathogenesis of cardiovascular disease, including heart failure. We determined levels of selected lncRNAs in peripheral blood cells in patients with acute myocardial infarction. Levels of 5 selected lncRNAs were altered in peripheral blood cells of patients with acute myocardial infarction. Levels of cyclin-dependent kinase inhibitor 2B antisense RNA 1 and potassium voltage-gated channel, KQT-like subfamily, member 1 opposite strand/antisense transcript 1 were associated with cardiac and inflammatory biomarkers, as well as left ventricular dysfunction at 4-month follow-up. The findings, which show altered levels of selected lncRNAs in peripheral blood cells in patients with acute myocardial infarction, require replication in independent study populations. Likewise, the biological roles of these lncRNAs in acute myocardial infarction and cardiac remodeling remain unclear.
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Supplemental Material

Methods

Measurement of IncRNAs expression
Total RNA was extracted from PAXgene™ tubes using the PAXgene™ blood RNA kit (Qiagen, Venlo, Netherlands) as described by the manufacturer. A second purification and concentration step was performed with the RNeasy® MinElute™ kit (Qiagen). To extract total RNA from subtypes of leukocytes, cells were lysed in TriReagent® (Sigma, Bornem, Belgium) and RNA was extracted using the RNeasy® Micro kit (Qiagen).

The expression of IncRNAs in PAXgene™ samples of MI patients and in different leukocyte subtypes was determined by quantitative PCR. After extraction, RNA quantity was measured using the ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, USA). RNA quality was assessed using the 2100 Bioanalyzer® apparatus (Agilent Technologies, Massy, France) with the RNA 6000 Nano chips. Total RNA was reverse-transcribed using the Superscript II RT kit (Life technologies, Belgium). The absence of genomic DNA amplification was verified by controls without reverse transcription. PCR was performed using a CFX96 device and the IQ™ SYBR® Green Supermix (BioRad, Nazareth, Belgium). PCR primers were designed using the Beacon Designer software (Premier Biosoft, USA) and were chosen to encompass all variants of each IncRNA. PCR primer sequences and a scheme representing primer location is available from the Online Supplement (Online Table I and Online Figure III). Optimal annealing-extension temperature for PCR cycling was determined for each primer pair. PCR conditions were as follows: 3 min at 95°C, 30 s at 95°C, and 1 min annealing-extension (40-fold). Annealing temperature was 56°C for all 5 IncRNAs and 60°C for SF3a1. The specificity of the PCR reaction was confirmed by a melting curve analysis and the sequencing of PCR products. SF3a1 was selected as housekeeping gene for normalization (see details in Online Figure IV). Expression levels were calculated by the relative quantification method (ΔΔCt) using the CFX Manager 2.1 software (Bio-Rad). Expression values of IncRNAs of each MI patient have been adjusted by whole blood cell count. Reproducibility, sensitivity and specificity of the measurements of IncRNAs by PCR have been characterized (Online Figure V).
Online Table I. PCR primers.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>NCBI Accession Number</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Hybridization Temperature (°c)</th>
<th>PCR efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aHIF</td>
<td>NR_045406</td>
<td>GGTCTGCCATCTATTACTT</td>
<td>TCTCAGCATTATAGTCACAA</td>
<td>56</td>
<td>95.4</td>
</tr>
<tr>
<td>ANRIL</td>
<td>NR_003529 (+12 variants)</td>
<td>TTGATGAGAAGAATAAGCC</td>
<td>CTCCCTTGTGTTGTGT</td>
<td>56</td>
<td>108.9</td>
</tr>
<tr>
<td>KCNQ1OT1</td>
<td>NR_002728</td>
<td>ATAGTAGTTGGAGACTTCA</td>
<td>ACTGTATATTTGAATGTGGT</td>
<td>56</td>
<td>104.4</td>
</tr>
<tr>
<td>MIAT</td>
<td>NR_003491 (+3 variants)</td>
<td>TTTACTTTAGAGCAGCAGAA</td>
<td>CTCCCTTGTGGAATCCC</td>
<td>56</td>
<td>102.2</td>
</tr>
<tr>
<td>MALAT1</td>
<td>NR_002819</td>
<td>TATCCCTTGGAGAGATATT</td>
<td>TAAAGAGTCACATTATGTC</td>
<td>56</td>
<td>92.2</td>
</tr>
<tr>
<td>SF3a1</td>
<td>NM_005877 (+1 variant)</td>
<td>GATTGGCCAGCGAAAGCC</td>
<td>TGCCGGAGACAAGTACGTC</td>
<td>60</td>
<td>96.6</td>
</tr>
</tbody>
</table>

ANRIL has 13 variants, MIAT has 4 variants and SF3a1 has 2 variants. PCR primers were chosen to encompass all transcripts. Accession numbers indicated are those from longest transcripts.
**Online Figure I.** Determination of the effect of heparin on PCR results. Blood samples were withdrawn from a healthy volunteer into PAXgene™ RNA tubes. Increasing amounts of sodium heparin were added to mimic the heparin bolus and its decreasing concentration in blood over time. A control condition without heparin was performed. RNA was extracted, and the 5 lncRNAs and the reference gene SF3a1 were measured by PCR. Four replicates were performed for each experimental condition. Shown are Ct values for the 5 lncRNAs and SF3a1 (mean ± SD). No effect of heparin could be detected.
Online Figure II. PCR results for 5 lncRNAs in diverse biological samples. Amplification (left) and melting curves (right) are shown. 1= PAXgene™ cDNA from a pool of MI patients (orange). 2= RT-PCR control without RT enzyme (pink) and PCR negative control without cDNA (red). 3= serum, platelet-enriched and platelet–deprived plasma samples obtained from the peripheral blood of 3 volunteers collected into citrated tubes (green).
**Online Figure III.** Genomic location of PCR primers. LncRNAs are marked in red.

**aHIF**

**ANRIL**

**KCNQ1OT1**
Online Figure IV. Determination of the housekeeping gene for normalization of PCR data. The human geNorm™ Reference Gene Selection Kit (Primerdesign, Southampton, United Kingdom) was applied to 8 MI patients of the study cohort. A panel of 12 candidate housekeeping genes was measured: ACTB, GAPDH, UBC, B2M, YWHAZ, SF3A1, 18S rRNA, CYC1, EIF4A2, SDHA, TOP1 and ATP5B. The average expression stability of each gene was calculated by the Excel-based algorithm NormFinder from geNorm after stepwise exclusion of the least stable housekeeping genes. The best housekeeping genes for accurate normalisation in our experimental system were SF3A1 and TOP1, with an average expression stability M score of 0.087 (panel A). The stability of the 12 candidate housekeeping genes was then analyzed by ranking genes based on intra-group variations and combining all the reference values into a stability value for each candidate housekeeping gene. SF31A was the third best housekeeping gene (panel B). Considering these results, we chose SF31A as housekeeping gene to normalize PCR data. Panel C shows the PCR results using a normalization procedure with the average of the Ct values of the 5 lncRNAs. The differences between NSTEMI and STEMI patients were identical as with the normalization with SF3a1, with higher expression of aHIF and lower expression of MIAT in STEMI patients compared to NSTEMI patients. Panel D shows the raw PCR Ct values of SF3a1 in the entire cohort, attesting for the stability of this reference gene. Indicated P value of 0.52 is between controls, NSTEMI and STEMI (ANOVA).
Online Figure V. Accuracy of the measurement of lncRNAs by PCR. (A) Reproducibility was tested by measurement of the expression values of 5 lncRNAs and SF3a1 from 4 blood samples from the same healthy donor collected in PAXgene RNA tubes. The results showing Ct values for the 5 lncRNAs and SF3a1 indicated a very high reproducibility with very close Ct values and low SDs. The highest variability was for aHIF with a SD of 0.39 Ct, which represent 1.49% of mean Ct. Show are mean Ct values ± SD. (B) Sensitivity and specificity were evaluated for each lncRNA using serial dilutions of cDNA from a pool of MI patients and 3 RT-PCR controls. Dilutions of cDNA were 1/10 to 1/100,000 for SF3a1, MIAT and MALAT1, and 1/10 to 1/31250 for aHIF, ANRIL and KCNQ1OT1. Three controls were added. The first control was the PCR control including water instead of cDNA template. The second control was the control of reverse transcription without RNA template. The third control was the control of reverse transcription without enzyme. PCR controls and controls of RT without RNA were negative, thus confirming the absence of generation of unspecific PCR products. Controls without RT enzyme had very low Ct signals. These results allowed to determine a sensitivity threshold with a Ct value of 30, above which we can consider that the lncRNA is not readily expressed. 1= PAXgene™ cDNA from a pool of MI patients: serial dilutions (green); 2= RT-PCR control without RT enzyme (blue); 3= RT control without RNA (pink) or negative PCR control without cDNA (red).