Long Non-Coding RNA CCAT1 Acts as a Competing Endogenous RNA to Regulate Cell Growth and Differentiation in Acute Myeloid Leukemia

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Long non-coding RNAs (IncRNAs) are involved in multiple cellular events, as well as in tumorigenesis. Colon cancer-associated transcript-1 (CCAT1) gene encodes an IncRNA whose over-activation was observed in an expanding list of primary human solid tumors and tumor cell lines, however its biological roles in acute myeloid leukemia (AML) has not been reported yet at present. In this study, the aberrant upregulation of CCAT1 was detected in French-American-British M4 and M5 subtypes of adult AML patients. By gain- and loss-of-function analysis, we determined that CCAT1 repressed monocytic differentiation and promoted cell growth of HL-60 by sequestering tumor suppressive miR-155. Accordingly, a significant decrease in miR-155 level was detected in AML patients. Re-introduction of miR-155 into HL-60 cells restored monocytic maturation and repressed cell proliferation. Furthermore, CCAT1 could up-regulated c-Myc via its competing endogenous RNA (ceRNA) activity on miR-155. In conclusion, these results revealed new mechanism of IncRNA CCAT1 in AML development, and suggested that the manipulation of CCAT1 expression could serve as a potential strategy in AML therapy.

INTRODUCTION

Acute myeloid leukemia (AML) is a clonal neoplastic disorder that is characterized by increase in myeloid cell numbers in patients bone marrow and arrest in cell maturation, frequently leading to hematopoietic insufficiency (Saadatpour et al., 2014). It is likely that many different genomic mutations, epigenetic aberrations, or abnormalities in gene expression could contribute to AML progression. Previous studies have shown that the overgrowth of abnormal myeloid blasts and severe blockage in myeloid differentiation are the major hallmarks of AML (Ichikawa et al., 2004; Jeziskova et al., 2015; Sakurai et al., 2014; Su et al., 2015). Therefore, repressing cell growth or overcoming differentiation arrest by manipulating such key regulators becomes the major driving force of this blood cancer.

Recent studies on human transcriptome have demonstrated that a relevant part of the genome is transcribed into the heterogeneous class of long non-coding RNAs (IncRNAs). Similar to protein-coding genes, IncRNAs may have important roles in diverse cellular processes, with their dysregulation already associates with disorders, including leukemia (Khandelwal et al., 2015; Peng et al., 2015; Ye et al., 2014; Zhang et al., 2013). Thus, the investigation of expression and function of IncRNAs could help with our understanding of leukemogenesis and the identification of novel therapeutic targets. Until now, several IncRNAs have been identified in normal or malignant hematopoiesis (Fernando et al., 2015; Hughes et al., 2015; Wang et al., 2015). However, only a few have been mechanically and functionally characterized in the context of AML-associated aberrant gene networks (Hughes et al., 2015).

Colon cancer-associated transcript-1 (CCAT1), a ~2kb IncRNA that maps to chromosome 8q24.21, was first found to be upregulated in colon cancer (Alaiyan et al., 2013; Yang et al., 2013). Recent study has suggested that CCAT1 was also upregulated in gastric and hepatocellular carcinoma tissues (Deng et al., 2015; Mizrahi et al., 2015; Zhu et al., 2015). Although CCAT1 has pivotal roles in different cancers, little is known about its involvement in AML, and the mechanism by which CCAT1 exerts its oncogenic activity remains undefined. In this study, we showed that IncRNA CCAT1 was increased in AML patients, especially the M4 and M5 subtypes (the myelomonocytic subtypes), compared to the normal controls. We further demonstrated that CCAT1 inhibited myeloid cell differentiation and promoted cell proliferation by operating as a competing endogenous RNA (ceRNA) for the miR-155 microRNA (miRNA). C-Myc was subsequently validated as a downstream target of CCAT1 ceRNA activity, and was important for CCAT1 to regulate AML progression, suggesting that CCAT1 regulates miR-155 activity by altering its targeting. Taken together, these results suggest that CCAT1 could regulate leukemogenesis as a ceRNA and may serve as a potential target for AML therapies.

Keywords: AML, CCAT1, IncRNA, miR-155

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MATERIALS AND METHODS

Human samples
The peripheral blood samples from AML patients and normal volunteers were obtained from The Affiliated Hospital of Inner Mongolia Medical University. The informed consent to perform the biological studies was obtained from all of the examined subjects and the related study was approved by the Ethics Committees of the participating hospitals and the Institutional review Board of The Affiliated Hospital of Inner Mongolia Medical University.

Cell lines and cell culture
Human myeloid leukemia cell lines HL-60 was purchased from cell resource center of Shanghai Institutes for Biological Science. HL-60 was cultured in IMDM (Gibco, BRL, UK) supplemented with 10% FCS (Gibco), 50 U/ml penicillin and 50 μg/ml streptomycin (Sigma-Aldrich, USA) at 37°C in 5% CO2. The monocytic differentiation of HL-60 was induced by 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich).

RNA isolation, reverse transcription and quantitative real-time PCR
Total RNA was isolated from the cell harvest using Trizol agents (Invitrogen, USA) according to the manufacturer’s instruction. Quantitative real-time PCR assay was conducted to detect the level of RNA transcripts. Briefly, cDNA was synthesized by M-MLV reverse transcriptase (Invitrogen) from 2 μg of total RNA. Oligo (dT18) RT primer was used for the reverse transcription of miR-155. Quantitative real-time PCR was carried out in the Bio-Rad IQ5 real-time PCR system (Bio-rad, USA) in triplicate with the following cycling conditions: 95°C for 10 min (initial denature); then 40 cycles of 95°C for 15 s, 60°C for 60 s. The miR-155 specific forward primer sequence was designed on the basis of miRNA sequences obtained from the miRBase database. The data were normalized using the endogenous GAPDH or U6 snRNA for mRNA/IncRNA or miRNA, respectively.

Plasmid constructs, Oligonucleotides and cell transfection
For gain-of-function analysis of CCAT1, the full length of CCAT1 was amplified from human cDNA and inserted into pcDNA6.1 (Invitrogen, USA) vectors. For loss-of-function analysis of CCAT1, shRNAs specific to CCAT1 was synthesized and inserted into pSH (System Biosciences, USA) vectors. For miR-155 target analysis, the full length of CCAT1 was amplified and inserted into pMIR-REPORT vectors. The mutant CCAT1 constructs were generated by using the QuikChange site-directed mutagenesis kit (Stratagene, USA). miR-155 mimic or mimic control were purchased from GenePharma (China) and used at a final concentration of 25 nmol/L in transfection by using Lipofectamine 2000 (Invitrogen; USA) and co-transfected with pMIR constructs or empty vectors (final concentration of 2 μg/ml) with a plasmid pRL-TK control vector (final concentration of 1 μg/ml) along with the pRL-TK control vector (final concentration of 1 μg/ml) and miR-155 mimic or mimic control. The plasmid pRL-TK containing Renilla luciferase was used as an internal control. Cells were harvested 48 h post-transfection and assayed with Dual Luciferase Assay (Promega, USA) according to the manufacturer’s instructions. Data were obtained by normalization of renilla luciferase activity to firefly luciferase activity. All transfection assays were performed in triplicate.

RNA pull-down by MS2-MBP
Maltose-binding protein (MBP)-affinity purification was used in this study to identify miRNAs that associated with CCAT1. The MS2-MBP protein was expressed and purified from E. coli following a protocol from the Steitz lab. Three bacteriophage MS2 coat protein-binding sites (5′-catacctacatcgtgctagttgctgggtcatcgtgacgttagtcatctgcatcactacagaccgtacg-3′) were inserted downstream of the wild type or mutant CCAT1 by site-directed mutagenesis using Stratagene’s QuikChange Site-Directed Mutagenesis Kit. To obtain miRNAs associated with the MS2-tagged CCAT1, HL-60 cells were transfected with MS2-tagged CCAT1 constructs, and 10 million cells were used for each immunoprecipitation assay. The cells were harvested 48 h post-transfection and subjected to RNA pull-down analysis as described elsewhere (Peng et al., 2015).

Western blot
Total proteins were extracted from cells or tissues using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin. The protein concentration was determined by BCA Protein Assay Kit (Vigorous, China). 20 μg total protein was loaded to 10% SDS-PAGE gel, probed with mouse or rabbit mAb against c-Myc (Proteintech, 10828-1-AP), and GAPDH (Proteintech, 10494-1-AP) followed by horseradish peroxidase-(HRP) conjugated sheep anti-mouse or rabbit Ig (ZSGB-BIO).

Flow cytometry analysis
The harvested cells were washed with PBS, resuspended in PBS containing 1% FCS and stained with CD11b-FITC and CD14-PE (Biogend, USA) for 30 min at 4ºC. Stained cells were analyzed with a flow cytometer. All the flow cytometry assays were carried out in triplicate.

Cell proliferation assay
HL-60 cells were incubated in 10% CCK-8 (DOJINDO, Japan) diluted in culture medium at 37°C until visual color conversion occurred. Proliferation rates were determined at 0, 24, 48, 72, 96 h after transfection. The absorbance of each well was measured with a microplate reader set at 450 nM and 630 nM. All experiments were performed in quadruplicate.

Statistics
Student’s t-test (two-tailed) was performed to analyze the data. A two-sided P-value of less than 0.05 was considered statistically significant. All statistical computations were performed using SPSS (SPSS Inc., USA).

RESULTS
CCAT1 was significantly increased in AML M4 and M5 subtype samples
To examine whether the CCAT1 was abnormally expressed in
AML, we performed real-time PCR to detect its level in peripheral blood mononuclear cells (PBMNCs) derived from 60 patients with newly diagnosed AML and 60 healthy donors. As shown in Figure 1A, CCAT1 was markedly upregulated in AML patients compared with the normal controls. These AML specimens represent the major French-American-British (FAB) subtypes from M1 to M5: 7 M1, 10 M2, 10 M3, 13 M4 and 20 M5. Furthermore, we compared CCAT1 level in different AML subtypes and found significantly increased CCAT1 expression mainly in the samples of M4 and M5 subtypes (Fig. 1B). The dysregulation of CCAT1 in AML M4 and M5 subtypes might suggest its potential oncogenic role in the myelomonocytic leukemogenesis.

Given that the AML M4 and M5 subtypes might involve monocytic differentiation arrest, we detected the expression changes of CCAT1 during monocytic differentiation of HL-60 leukemia cell lines. Real-time PCR data showed that CCAT1 level gradually decreased (Fig. 1C, the lower panel) during phorbol myristate acetate (PMA)-induced monocytic differentiation of HL-60 cells (Fig. 1C, the upper panel). To further test whether CCAT1 plays an important role in monocytic differentiation, shRNA specific to CCAT1 (si-CCAT1) was transfected into HL-60 cells and the efficiency of CCAT1 knockdown was subsequently confirmed by real-time PCR analysis (Fig. 1D). Flow cytometry data showed that CCAT1 knockdown resulted in higher expression of both CD11b and CD14 in 24 h and 48 h PMA-treated HL-60 cells, when compared with the control (Fig. 1E). On the contrary, the overpresence of CCAT1 (Fig. 1F) reduced the percentage of CD11b+/CD14+ HL-60 cells after PMA treatment (Fig. 1G).

Next, we detected the influence of CCAT1 on leukemia cell proliferation by using CCK-8 assay. As a result, the repression of CCAT1 led to significant decrease in cell proliferation (Fig. 1H), whereas the ectopic expression of CCAT1 increased HL-60 cell growth (Fig. 1I).

**CCAT1 is physically associated with miR-155 in leukemia cells**

The ceRNA hypothesis posits that specific IncRNA can function as sinks for pools of active miRNAs, functionally liberating mRNA transcripts targeted by certain miRNAs (Arancio et al., 2014; Sen et al., 2014; Song et al., 2014). A recent study has indicated that CCAT1 could promote hepatocellular carcinoma progression by functioning as a let-7 ceRNA (Deng et al., 2015).
To determine whether CCAT1 also operates as a ceRNA in leukemiaogenesis, we used the webserver lnciDB (http://gyanxet-beta.com/lnciDB/) to predict potential lncRNA-miRNA interactions. Among the results, we found 3 miR-155 binding sites scattering the CCAT1 transcripts, suggesting its ceRNA potential for another miRNA miR-155, in addition to let-7 (Fig. 2A). Subsequently, we constructed a series of luciferase reporters containing the wild type CCAT1 (pMIR-WT), or a series of mutant CCAT1 with mutations of a single (pMIR-Mut1, 2, 3) or all 3 predicted miR-155 binding sites (pMIR-Mut1–3). We found that transfection of miR-155 mimic into HL-60 cells reduced the luciferase activity of the wild type CCAT1 (pMIR-WT), but not empty vector or all miR-155 site mutant reporter vector (Fig. 2B), suggesting the binding of miR-155 to these sites. Among them, the third miR-155 binding site (#3) seemed to be the strongest interaction site, because the mutation of this sequence almost abolished the effects of miR-155 on luciferase activity (Fig. 2B). We next examined whether the endogenous CCAT1 level was affected by miR-155. As shown in Fig. 2C, CCAT1 expression was reduced by ~3-folds upon miR-155 over expression both before and after PMA induction (Fig. 2C). These results suggested that CCAT1 was negatively regulated by miR-155 in AML cells.

To further validate the direct interaction between miR-155 and CCAT1 at endogenous level, we performed an RNA pull down analysis to examine endogenous miRNAs associated with the MS2 sequence-tagged CCAT1. The precipitated miRNAs were analyzed by real-time PCR and results showed that miR-155 was significantly enriched by the MS2-tagged wild-type CCAT1 (pcDNA-WT-MS2) compared to the empty vector (pcDNA-MS2) or the all miR-155 targeting sites mutant ones (pcDNA-Mut1–3-MS2) in HL-60 cells. These results suggested that endogenous miR-155 was associated with CCAT1 in leukemia cells (Fig. 2D). Accordingly, mutation of the third miR-155 binding site on CCAT1 resulted in a huge decrease in miRNA binding (Fig. 2D). Taken together, these data demonstrated that miR-155 could bind to CCAT1 and CCAT1 could function as a ceRNA in leukemia cells.

**miR-155 is downregulated in AML and functions as a tumor suppressor**

Based on above findings, miR-155 may also play an important role in leukemogenesis. We first assessed the expression changes of miR-155 in the same samples of 60 AML patients and 60 healthy donors. As expected, miR-155 was significantly decreased in AML compared to normal controls (Fig. 3A). Interestingly, similarly to CCAT1, the lower miR-155 was specifically associated with M4 and M5 AML subtypes (Fig. 3B). Accordingly, miR-155 level was upregulated in PMA-induced monocytic differentiation of HL-60 cells (Fig. 3C), which was opposite to its ceRNA CCAT1.  

Next, we still used HL-60 cells to analyze the role of miR-155 in monocytic differentiation. Real-time PCR was first used to measure the level of miR-155 after miR-155 mimic transfection and showed that the miR-155 increased to ~40 to 60-fold (Fig. 3D). Flow cytometry analysis showed that overexpression of miR-155 promoted monocytic maturation as indicated by the increased differentiated CD11b+/CD14+ cells (Fig. 3E). Furthermore, the CCK-8 proliferation assay in the same cells indicated that cell growth was suppressed after transfection with miR-155 mimic (Fig. 3F). Taken together, these results indicated the tumor suppressor role of miR-155 in AML.
Furthermore, to detect whether the functional relevance of miR-155 in AML is regulated by CCAT1's ceRNA activity, we performed a “rescue” assay with co-transfection of pcDNA-CCAT1 and miR-155 mimic into HL-60 cells, followed by PMA treatment. As expected, the introduction of CCAT1 could rescue the miR-155-induced monocytic maturation of HL-60 cells (Fig. 3G), as well as the repression on cell proliferation driven by miR-155 (Fig. 3H). These results suggest that the differentiation-promoting and cell growth-repressing effects of miR-155 is closely connected to CCAT1 regulation in leukemia cells.

**CCAT1 ceRNA activity regulates c-Myc expression in leukemia cells**

Studies have reported that miR-155 could control c-Myc expression in gastric carcinoma cells (Sun et al., 2014). To validate whether miR-155 also targets c-Myc in AML cells, real-time PCR and immunoblotting assay was carried out respectively, and showed that both the mRNA and protein levels of c-Myc were decreased by ~ 2-fold in HL-60 cells transfected with miR-155 mimic compared with the control (Fig. 4A). As a ceRNA, CCAT1 could share the regulatory miR-155 with its target c-Myc, therefore, we wondered whether CCAT1 could influence c-Myc in HL-60 cells. As excepted, overexpression of CCAT1 (pcDNA-CCAT1) increased both the mRNA (Fig. 4B) and protein levels of c-Myc (Fig. 4C). Furthermore, ectopic expression of miR-155 upon pcDNA-CCAT1 transfection abrogated this increase (Figs. 4B and 4C). All these results suggest that the role of CCAT1 in modulating c-Myc is mediated by its competitively binding with miR-155 in HL-60 cells.

Since the oncogenic role of CCAT1 was, at least partly, mediated by its ceRNA activity on miR-155/c-Myc axis, we supposed that the upregulated CCAT1 expression in AML would ultimately lead to c-Myc accumulation. To test this hypothesis, we detected the level of c-Myc mRNA in AML samples. In consistence with CCAT1, c-Myc was markedly increased in AML patients compared to the normal control (Fig. 4D). However, only the AML M5 subtype had significantly higher level of c-Myc mRNA (Fig. 4E). In summary, these data indicated that CCAT1 inhibited myeloid cell differentiation and promoted cell proliferation by operating as a ceRNA for the miR-155, thus altering its target c-Myc.

**DISCUSSION**

The last decade has seen exciting advance in IncRNA research, suggesting that the involvement of IncRNA in human diseases could be far more prevalent than previously appreciated (Zhang et al., 2013). Different studies have shown that changes in IncRNA expression may contribute to the development of different types of cancer, however most of them have not yet been studied in mechanistic details. Recently, it has been demonstrated that endogenous IncRNA could participate in post-transcriptional regulation by interfering with the miRNA pathways, by acting as competing endogenous RNAs (ceR-
Myc were classified according to FAB subtypes. For all quantitative results, the data are presented as the mean ± SEM, and the error bars represent the standard deviation obtained from three independent experiments. *p < 0.05; **p < 0.01.

NAAs (Arancio et al., 2014; Sen et al., 2014). These lncRNAs have miRNA responsive elements (MRE) and act as miRNA sponges to modulate miRNAs available for binding with their own targets (Johnsson et al., 2013). It is also suggested that these ceRNAs are implicated in many biological processes and the disruption of the equilibrium of ceRNAs and miRNAs is critical for tumorigenesis (Johnsson et al., 2013; Yokoyama et al., 2000). For example, IncRNA PTEN-P1 could act as ceRNA of miR-19b and miR-20a to release PTEN tumor suppressor in prostate cancer, glioblastoma, and melanoma, and disruption in the network leads to tumorigenesis in many cases (Poliseno et al., 2010; Yu et al., 2014). HULC IncRNA also blocked miR-372 from binding to its target PRKACB that induces the activation of CREB to modulate self-regulation in hepatocellular carcinoma (Wang et al., 2010). Similar report suggested that H19 acted as a ceRNA to modulate let-7 availability (Kallen et al., 2013). In a recent study, IncRNA-ATB upregulated ZEB1 and ZEB2 by competitively binding to the miR-200 family and then induced EMT and invasion (Yuan et al., 2014). Additionally, Hmga2 promotes lung carcinogenesis both as a protein-coding gene and as a ceRNA dependent upon the presence of let-7 sites (Kumar et al., 2014). Thus, understanding the intricate interplay among diverse RNA species, including protein-coding messenger RNAs and non-coding RNAs such as long non-coding RNAs, pseudogenes and miRNAs will lead to significant insight into gene regulatory networks and have implications in human disease.

CCAT1 is an IncRNA initially characterized in colon cancer. Recently, CCAT1 was found to be crucial for cancer cell invasion and proliferation in both gastric and hepatocellular carcinoma (Mizrahi et al., 2015; Yang et al., 2013; Zhu et al., 2015). However, little is known about the involvement of CCAT1 in leukemia, and the mechanism through which CCAT1 exerts its oncogenic activity remains undefined. Here, we identified CCAT1 as a novel regulator of acute myeloid leukemogenesis.

More importantly, we demonstrated a ceRNA activity of CCAT1 by modulating miR-155 availability in leukemia cells. Furthermore, the oncogenic role of CCAT1 was reinforced by upregulating the expression of c-Myc, a target of miR-155. Interestingly, CCAT1 has been reported to be activated by c-Myc in gastric carcinoma, suggesting the existence of a feedback loop consisting of c-Myc, CCAT1, miR-155, which is likely to be validated in future studies.

Another important finding in this study is to provide novel evidence for a tumor suppressive role of miR-155 in AML by inhibiting cell proliferation and releasing the monocytic differentiation block. miR-155 has been reported to be an oncogenic miRNA due to its up-regulation in diffuse large B cell lymphoma (Eis et al., 2005), Hodgkin lymphoma (Kluiver et al., 2005), CLL (Calin et al., 2005), and AML with FLT3-ITD mutations (Gerloff et al., 2015; Xue et al., 2014). However, miR-155 has also been indicated to be down-regulated in various hematological malignancies: Burkitt’s lymphoma (Kluiver et al., 2006), CML (Rokah et al., 2012) and AML with inv(16) (Jongen-Lavrencic et al., 2008), indicating the complexity of miR-155 regulation in tumorigenesis. In consistent with Palma’s study (Palma et al., 2014), we showed that miR-155 has a pro-differentiation and anti-proliferation role in FLT3-WT AML cells, in contrast to its oncogenic function reported in B cell lymphoma (Eis et al., 2005) and FLT3-ITD mutation AML (Gerloff et al., 2015; Xue et al., 2014). Therefore, these evidences suggested that the gene regulation by miR-155 is complex (Palma et al., 2014), as it targets both tumor suppressor gene or oncogene, and its function in a specific malignancy is likely subject to disease context and cell type.

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