Assessment of The Lnc-CCAT1/miR-155a Regulatory Network in Acute Myeloid Leukemia

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Abstract

Colon cancer-associated transcript_1 (CCAT1) is a long noncoding RNA that maps to chromosome 8q24.21, it was first discovered to be upregulated in colorectal cancer. Recent studies have observed CCAT1 overexpression in primary human solid cancers and cell lines as well as in AML, moreover, it repressed monocytic differentiation and promoted cell growth of HL-60 by sequestering tumor suppressor miR-155. However, the prognostic value of CCAT1/miR-155a pathway in acute myeloid Leukemia (AML) has not been investigated on clinical samples. In this study, the expression levels of CCAT1 and miR_155a were measured in 150 AML patients with standard and high-risk factors; CCAT1 and miR_155a were increased by 2.7 and 5.7 folds; respectively in AML compared to healthy controls. Further, upregulation of both biomarkers was significantly associated with high risk AML. Collectively, these results suggest that CCAT1 and miR_155a can be considered as a diagnostic and prognostic biomarker in AML.

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Introduction

Acute myeloid Leukemia (AML) is a heterogeneous hematopoietic malignancy characterized by the rapid accumulation and malignant proliferation of immature myeloid progenitors in the bone marrow and peripheral blood [1]. Without treatment, AML quickly becomes fatal, and historically, it has always been associated with a poor prognosis [2, 3]. However, AML treatment has markedly improved over the last few decades, with improvements in risk assessment, post-remission chemotherapy and hematopoietic stem-cell transplantation. However, even though complete remission is achieved after chemotherapy by the majority of AML patients, only ~20% of obtained relatively long-term relapse-free survival. Thus, in order to improve the diagnosis, prevention and treatment of this disease, detailed knowledge of the mechanisms that form the basis of AML development and progression must be acquired [4-7].

It has been shown that long non-coding RNAs (lncRNAs) play a crucial role in hematopoietic differentiation and haematological malignancies, including AML [8]. Long non-coding RNAs (lncRNAs) are a heterogeneous class of RNAs that are generally defined as non-protein-coding transcripts longer than 200 nucleotides. lncRNA which was considered as only transcriptional “noise” in the past decades can participate in various critical biological processes, such as chromatin remodelling, gene transcription, and protein transport and trafficking [9]. Recently, more studies have shown that lncRNAs are deregulated in a wide variety of cancers such as pancreatic cancer and hepatocellular carcinoma [8-10]. There is a continually growing list of lncRNAs that are associated with gene expression regulation and diseases [11]. However, very little is known about their precise function. To date, the number of human lncRNA genes is close to 9,000. However, only a few of them have been assigned a role in myelopoiesis.

The colon cancer associated transcripts (CCATs) are a collection of lncRNAs located on different chromosomes that have been both associated with and functionally demonstrated to be involved in the development of human colorectal cancers (CRC). Specifically, three of the best characterized CCAT lncRNAs are CCAT1 (also known as CARLo5), CCAT2 and CCAT6 which was previously known as MYCLo2. CCAT6 gene is located on chromosome 7 [12, 13, 10]. It has

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been identified and characterized as a highly specific marker for human CRC [8]. There is an interaction between MYC (avian myelocytomatosis viral oncogene homolog) and CCAT1 whom are involved in many complex molecular interactions [14, 4]. A studies showed a long-range physical interaction between MYC-335 and the promoter of CCAT1 [8], which has been suggested to regulate MYC post-transcriptionally. MYC has been shown to bind to the promoter of CCAT1 and upregulate its expression and promote proliferation and invasion of colon and gastric cancer cells and has been shown to promote tumor growth and metastasis [15, 16]. Moreover, found that CCAT1 was deregulated in hepatocellular carcinoma (HCC), and CCAT1 expression correlated with the progression of the malignancy and poor prognosis [17, 10, 11]. Adding to the complexity, IncRNAs are involved in multiple cellular events, as well as in tumorigenesis [16].

Colon cancer-associated transcript-1 (CCAT1) gene encodes an IncRNA whose over-activation was observed in AML, however its biological roles in AML has not been reported yet at present (FER). Zhang et al reported that the aberrant upregulation of CCAT1 was detected in French American-British M4 and M5 subtypes of adult AML patients. Referred to these results, a new mechanism of IncRNA CCAT1 in AML was development and suggested that the manipulation of CCAT1 expression could serve as a potential strategy in AML therapy (FER). MicroRNAs (miRNA) are highly conserved non-coding RNAs of approximately 20-24 nucleotides that inhibit gene expression at the posttranscriptional level via binding to imperfect complementary sites within the 30-untranslated regions (3’UTR) of messenger RNAs (mRNAs) [18]. Thus, acting as oncogenes or tumor suppressor genes, miRNAs modulate diverse biological processes including cell cycle progression, proliferation and apoptosis [19].

miRNA-155 as an oncogenic miRNA was first reported in B-cell lymphoma and Chronic lymphocytic leukemia implicating its important oncogenic role in hematological malignancies. However, miR-155 role in solid tumors was more extensively studied and showed to target FOXO3a (Human protein encoded by FOXO3 gene) thus inducing cell survival and chemo-resistance [20]. Many studies have correlated miR-155 prognosis with outcome in several types of cancer. In AML, that high expression of miR-155 independently predicts poor outcome in cytogenetically negative patients and is associated with high-risk FLT3 internal tandem duplication (ITD) mutations [6]. The expression of miR-155 was inversely correlated with disease-free survival and overall survival. It was associated with a gene expression profile enriched for genes involved in cellular mechanisms deregulated in AML (eg. apoptosis and nuclear factor-kB activation) [21]. In previous studies indicated the importance of miR-155 as a therapeutic target, thus the availability of clinically emerging compounds with antagonistic activity to miRNAs will provide the opportunity for therapeutically targeting miR-155 in AML for better clinical outcome [22].

Results

I Demographic and clinical characteristics of AML patients

A total of 150 bone marrow samples for AML patients and 40 non-leukemic samples were enrolled in to study the expression of Lnc_CCAT1 and miR_155a. Of 150 AML cases, a 62% (n=93) of patients were >40 years of age and males more predominate 76% (n=114) than females. In phenotype, M2 patient’s accounts 42% of cases and the majority of cases were of standard risk. At follow up; 62% (n=93) than (n=57) enter in remission (Table 1).

Table 1: Descriptive analysis of the studied subjects.

| Demographic data | AML N=150 | Control N=40 |
|------------------|-----------|-------------|
| Age (years) Mean±SD | 51±15 | 44±14 |
| Range | 27 – 77 | 27 - 27 |
| Gender | | |
| Male | N ( %) | 114 (76) | 32 (63) |
| Females | 36 (24) | 8 (42) |
| Age subgroups | | |
| Favorable ( ≤40) | N (%) | 57 (38) | 93 (62) |
| Unfavorable (>40) | | |
| AML phenotype | | |
| M0 – M1 | 42 (28) | 70 (47) |
| M2 | 38 (25) | 27 |
| M4 – M5 | | |
| Cytogenetic abnormality | | |
| Negative | 69 (46) | 81 (54) |
| Positive | | |
| TLC (x10^9/L) ≤50 | N (%) | 111 (74) | 39 (26) |
| >50 | | |
| Hemoglobin (g/ml) >6 | N (%) | 126 (84) |
Assessment of the Lnc-CCAT1/Mir-155a Regulatory Network in Acute Myeloid Leukemia

II Lnc_CCAT1 is upregulated in AML and higher levels is associated with AML patients at high risk

The expression level of CCAT1 was analyzed in 150 AML patients and expression values was compared to forty healthy controls that were matched for age and sex. The expression of Lnc_CCAT1 was higher by 2.7 folds in AML more than healthy control, p=0.008 (Table 2, Figure 1a). In order to evaluate the prognostic value of Lnc_CCAT1 in AML; CCAT1 overexpression was correlated with clinic pathological features. In comparative analysis, higher expression levels of CCAT1 were statistically significant with high risk factors (p<0.01), higher expression levels were associated with cytogenetic negative AML, TLC>50,000/ul, BM blasts >70%, MRD>0.01 and clinical relapse after induction therapy (Table 2, Figure 1). Controversially; no statistical significance was detected for Lnc_CCAT1 between different AML phenotypes as well as for age, gender and haemoglobin risk subgroups (Table 2).

Table 2: Expression levels of Lnc_CCAT1 and miR-155a between AML and healthy control; between standard and high risk AML groups.

| Variable                      | Lnc_CCAT1 expression (Log10) | miR-155a expression (Log10) |
|-------------------------------|-----------------------------|-----------------------------|
|                               | Median (range)              | Statistics                  | Median (range)              | Statistics                  |
| Studied groups                |                             |                             |                             |                             |
| AML                           | 22 (6.5–133)                | U=295 p=0.008*              | 151 (39 – 340)              | U=8 p=0.001*                |
| Control                       | 8.8 (0.8–18)                |                             | 27 (11 – 46)                |                             |
| Age subgroups                 |                             |                             |                             |                             |
| Favorable (≤40)               | 3.4 (0.9–12)                | U=235 p=0.2                 | 145 (39–340)                | U=276 p=0.7                 |
| Unfavorable (>40)             | 2.9 (0.4–8.6)               |                             | 161 (61–305)                |                             |
| Gender                        |                             |                             |                             |                             |
| Male                          | 3 (0.9–12)                  | U=214 p=0.8                 | 147 (39–295)                | U=171 p=0.2                 |
| Females                       | 3.7 (0.4–6)                 |                             | 174 (41–340)                |                             |
| AML phenotype                 |                             |                             |                             |                             |
| M0 – M1                       | 3.3 (0.4–8.6)               | F=1.4 p=0.3                 | 166 (67–340)                | F=0.7                       |
| M2                             | 2.9 (1.0–12)                |                             | 144 (60–305)                |                             |
| M4 – M5                       | 1.4 (0.9 – 6)               |                             | 131 (39–238)                |                             |
| Cytogenetic abnormality       |                             |                             |                             |                             |
| Negative                      | 4.6 (0.9 – 12)              | U=107 p=0.001*             | 169 (39–340)                | U=210 p=0.05                |
| Positive                      | 1.4 (0.4 – 7.7)             |                             | 129 (41–295)                |                             |
| TLC (x10^9/L)                 |                             |                             |                             |                             |
| ≤50                           | 2.6 (0.4–6.6)               | U=85 p=0.001*              | 144 (39 – 340)              | U=172 p=0.1                 |
| >50                           | 6.2 (1.4–12)                |                             | 173 (61 – 261)              |                             |
| Hemoglobin (grm/dl)           |                             |                             |                             |                             |
| >10                           | 2.9 (0.4–8.7)               | U=115 p=0.2                | 145 (39–340)                | U=132 p=0.3                 |
| ≤10                           | 5.3 (0.9–12)                |                             | 163 (94–261)                |                             |
| Platelet count (x10^12/L)     |                             |                             |                             |                             |
| ≥50                           | 6.2 (1.2–12)                | U=85 p=0.001*              | 183 (39–261)                | U=172 p=0.1                 |
| <30                           | 2.2 (0.4–6.0)               |                             | 144 (41–340)                |                             |
| BM blasts (%)                 |                             |                             |                             |                             |
| ≤70%                          | 1.9 (0.4–6.6)               | U=57 p=0.001*              | 130 (41 – 305)              | U=166 p=0.03*               |
| ≥70%                          |                             |                             |                             |                             |

BM: bone marrow; MRD: Minimal residual disease; TLC: Total leucocyte count; AML: Acute Myeloid Leukemia; M0: Minimally differentiated Acute Myeloblastic leukemia; M1: Myeloblastic leukemia; M2: Myelocytic leukemia; M4: Myelomonocytic leukemia; Monoblastic leukemia.
Assessment of The Lnc-CCAT1/Mir-155a Regulatory Network in Acute Myeloid Leukemia

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III miR_155a is overexpressed in AML and higher levels is associated with AML patients at high risk

To understand the functional significance of miR_155a “an oncogene” reported to be associated with various cancers; miR_155a was analyzed in all AML samples compared with healthy control group. miR_155a expression was significantly increased by 5.7 folds in AML than in control group (p<0.001) (Table2, Figure 1b). In univariate analysis; except for cytogenetic status (p=0.05) and percentage of BM blasts at diagnosis (p=0.03) (Figure1), none of the clinic pathological features tested were statistically significant (Table2), and this may be contributed to the wide variation in the of the clinical AML phenotypes. In addition, a higher level of miR_155a was significantly associated with therapeutic response (p=0.004) and residual BM blasts >0.01 at day 15th after induction therapy (p=0.02) (Table 2, Figure 1).

IV Diagnostic and prognostic performance of Lnc_CCAT1 and miR_155a in AML

The Receiver operating characteristic (ROC) was applied to evaluate whether the Lnc_CCAT1 and miR_155a in AML could be used as diagnostic and prognostic markers. Figure 2 presents the diagnostic potential of Lnc_CCAT1 and miR-155a genes expression in discriminating AML from healthy control (a). Also, the prognostic value of both biomarkers was assessed to measure the ability for prediction of patient’s prognosis with standard and high-risk factors. The AUC was calculated for ROC curve to assess the score performance of each biomarker in AML. AUC: area under the curve.
diagnostic biomarkers in AML. As shown in (Table 3) and figure 2a and b; we found that Lnc_CCAT1 and miR_155a were a potential biomarkers for screening AML patients from healthy controls with AUC of [0.7 (95%CI: 0.6-0.9), 1.0 (95%CI: 0.9-1.0)]; respectively. At the optimum expression cut-off value of 1.2 and 45 for Lnc_CCAT1 and miR_155a, the sensitivities were 74%, 96% and specificities were 70%, 95%; respectively. For standard versus high risk AML factors, significant differences also existed for the two studied biomarkers (Table 3, Figure 2). These findings suggested the Lnc_CCAT1 and miR_155a expression levels might be potential prognostic biomarkers in discriminating high risk from standard risk AML (Table3, Figure 2).

**Table 3:** Diagnostic and prognostic efficiency of Lnc_CCAT1 and miR_155a in discriminating between AML and healthy control; between standard and high risk-AML subgroups.

| Groups                      | Lnc_CCAT1 | miR_155a |
|-----------------------------|-----------|----------|
|                             | AUC       | P value  | 95CI%    | AUC | P value | 95CI%    |
|                             | Lower     | Upper    |         | Lower | Upper    |         |
| Subjects AML/Control        | 0.7       | 0.008i   | 0.6     | 0.9  | 1.0      | 0.001*  |
| MRD (≤0.01/>0.01)            | 0.9       | 0.001*   | 0.9     | 1.0  | 0.7      | 0.02i   |
| Clinical response Remission/relapse | 0.9       | 0.001*   | 0.8     | 1.0  | 0.8      | 0.004*  |
| BM blasts% ≤70%/>70%         | 0.9       | 0.001*   | 0.8     | 1.0  | 0.7      | 0.03i   |
| TLC (x10^3/L) ≤50/>50        | 0.8       | 0.001*   | 0.7     | 0.9  | 0.6      | 0.1     |
| Platelet count (x10^3/L) ≥30/<30 | 0.9       | 0.001*   | 0.8     | 1.0  | 0.7      | 0.08i   |

AUC: area under the curve; CI: confidence interval; BM: bone marrow; MRD: Minimal residual disease; TLC: Total leucocyte count; AML: Acute Myeloid Leukemia; *test is significant at level ≤0.01; t test is significant at level ≤0.05.

**Subjects & methods**

**Study cohort**

The present study enrolled fifty patients with de-novo diagnosed AML, attended at Clinical Haematology Department, Ain Shams Internal Medicine Hospital, Cairo, Egypt between March 2017 and August 2018, twenty healthy controls volunteers present our control cohort. Patients’ clinic pathological characteristics are presented in (Table 1). The samples was collected before the receiving of chemotherapy induction protocol; All sample handling, procedure and storage was the same for leukemic and control subjects. A written informed consent was signed from each patient or his/her parent’s in accordance with the Declaration of Helsinki. Peripheral blood (PB) samples were obtained, with a minimum blast infiltration of 25%. Patients follow up and risk groups stratification of AML was done according to the Berlin-Frankfurt-Munster (BFM) guidelines; it includes; assessment of BM blasts on day 8, BM blasts % on day 15 and detection of residual blast cells to diagnose (MRD). The diagnosis of AML was confirmed according to a morphologic assessment of the Leishman stained smears of the bone marrow aspirates along with special immune-cytochemical stains, immune-phenotyping, and Cytogenetics analysis. Assessment of Minimal residual disease (MRD) was performed using a lineage-specific monoclonal panel; MRD was considered positive when leukemic cells exceeded 0.01% of all marrow nucleated cells on days 15thand 28th.

**I Total and miRNA extraction and purification**

Total mRNA was extracted from mononuclear cells (MNCs) that it is isolated by Ficoll Hypaque density gradient centrifugation from 2 ml PB samples in EDTA bone marrow cells from newly diagnosed patients. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol, RNA concentration and purity were evaluated spectrophotometrically at 260 and 280 nm. RNA integrity was visually confirmed by agarose gel electrophoresis. cDNA was synthesized by reverse transcription reaction using miScript RT Kit (Qiagen, Hilden, Germany).

**II Quantitative Real time PCR (qPCR)**

Long non-coding RNAs “CCAT1, and miR_155a expression analysis: cDNA was amplified using a Hs_CCAT1 RT2 Primer Assay cat no: 330701, assay ID: LPH15969A Primer Assay; amplification was performed using RT2 SYBR Green PCR Kit (Qiagen, Germany) and Hs_ACTB_1_SG QuantiTect Primer assay was used as reference gene. In addition, miR-155a was amplified using Hs_miR-155a Primer Assay (Hs_miR155_2; ID: MS00031486 and Hs_SNORD68_11; ID: MS00033712 as a housekeeper gene (Qiagen, Germany), each sample was amplified using miScript SYBR Green PCR Kit (Qiagen, Germany). The thermal cycling was adjusted according to manufacture instructions. The fluorescence data were collected at extension step. Following amplification, gene expression was calculated using 2^(-∆∆Ct) method.

**III Statistical Analysis**

Statistical analysis was performed using SPSS v.23 (Chicago, IL, USA). The non-parametric Mann–Whitney U test and Wilcoxon Signed Rank test were performed to evaluate the differences of CCAT1and miR-
155a between leukemic vs healthy specimen, and between standard vs high risk AML, respectively. Moreover, to assess the diagnostic and prognostic potential of CCAT1 and miR-155a expression with healthy controls and with patients’ risk factors; Receiving operating Characteristics (ROC) analysis was conducted, then the biomarker sensitivities and specificities were calculated according to the optimum cut-off value. Significance was set at \( p<0.05 \).

Discussion

This study was set out with the aim to evaluate the diagnostic and prognostic value of the Lnc_CCAT1 and miR_155a in Acute Myeloid Leukaemia (AML) and if these biomarkers could predict the clinical response to therapy? In our study, the BM samples of 150 AML patients were analyzed and compared with twenty healthy controls, furthermore, AML patients were classified into standard and high risk subgroups according to favorable and unfavorable risk factors. The expression of Lnc_CCAT1 and miR_155a were analyzed in all samples and their clinical significance in AML was explored.

Per our results, we demonstrate a significant difference in the expression of Lnc_CCAT1 and miR_155a between AML and healthy controls, the expression of Lnc_CCAT1 was increased by 2.7 folds in AML samples than healthy control. Similar finding was obtained for the miR_155a expression which showed 5.7 folds increase. Interestingly, as the expression of CCAT1 and miR_155a are demonstrated to be exponentially increased, the clinic-pathological features were characterized into favorable and unfavorable categories and each was correlated to the expression of Lnc_CCAT1 and miR_155a in order to evaluate their prognostic significance in AML. Meanwhile, the expression of Lnc_CCAT1 and miR_155a was higher in cytogenetic negative-AML and those with BM blast >70%, it was found that; the expression of Lnc_CCAT1 was significantly associated with TLC>50,000/ul. As an attempt to evaluate the significance of Lnc_CCAT1 and miR_155a as predictors for clinical response in AML, the authors correlated their expression levels with patient’s clinical response. Our results revealed a significant association between the expression values of both biomarkers and clinical response (p<0.01); higher expression values of Lnc_CCAT1 and miR_155a was associated with patient’s relapse and residual blasts<0.01 at day 15th of induction chemotherapy. Consistent with these results, it was demonstrated that CCAT1 has an oncogenic behavior in leukemia’s, regulate AML progression, thus; suggesting that CCAT1 could regulate leukemogenesis, therefore, it may serve as a potential target for AML therapies. Lnc_CCAT1 was first found to be upregulated by 235-fold increase in colon cancer (CRC) than normal colonic tissue, 10.8-fold increase in hepatocellular carcinoma, gastric, lung, and breast cancer [8, 23, 24]. Moreover, previous studies have revealed the oncogenic behavior of Lnc_CCAT1 in the development, progression, metastasis, and invasion of many cancer types, therefore; the tumor progression in leukemias, colorectal cancer (CRC), hepatocellular carcinoma, gall bladder cancer, breast cancer, and epithelial ovarian cancer could be contributed to over expression of Lnc_CCAT1 [25].

Moreover; upregulation of miR-155 has been reported by Rao et al who observed overexpression of miR-155 in AML patients by 4-folds, this finding was clinically correlated to cytogenetic negative (CN) AML with FLT3-ITD+ mutations [26]. Similar findings have been reported by other studies; they observed an increase in miR-155 expression levels in the bone marrow blasts of leukemia patients bearing M4 or M5 subtypes of Acute Myeloid Leukemia. On the other hand, downregulation of miR-155a was demonstrated in chronic myeloid and lymphoid neoplasms [27]. In the current study, a transcriptome analysis was performed on CCAT1 in order to identify the upstream gene-gene interaction between CCAT1 and miRNAs regulating genes. Bioinformatics analysis showed high cross-link evidence between miR-155a and the regulation of CCAT1 in leukemia; these data was confirmed from three different data bases. Recent studies have proved Lnc_CCAT1/miR_155a interlink in Leukemias, it was demonstrated that CCAT1 has an oncogenic behavior and it is linked to miR_155a in leukemia’s, it exert his effect through inhibiting myeloid cell differentiation and promoting cell proliferation by operating as a competing endogenous RNA (ceRNA) for the miR-155a, accordingly, they suggested that CCAT1 could regulate leukemogenesis and may serve as a potential target for AML therapies [14].

Based on our results, higher expression of Lnc_CCAT1 and miR_155a was significantly associated with high risk AML and poor prognosis, these findings were in agreement with who reported that higher expression values of CCAT1 correlates with tumor status, lymph node invasion and advanced tumor-nodes-metastasis (TNM) stage, used as a prognostic marker for hepatocellular carcinoma (HCC), correlates with clinical stage and poor overall survival of patients with esophageal squamous cell carcinoma [28, 29]. Demonstrated that higher expression values of miR_155a is considered as an independent prognostic marker for cytogenetically normal (CN) acute myeloid leukemia (AML) and it is significantly correlated shorter disease-free survival (DFS), overall survival (OS) [6, 27, 30].

When the diagnostic performance was tested, the miR_155a was superior to Lnc_CCAT1 as regarding the biomarker sensitivity and specificity. At the optimum expression cut-off value of 1.2 and 45 for Lnc_CCAT1 and miR_155a, the sensitivities were 74%, 96% and specificities were 70%, 95%; respectively. These results were in agreement with various studies that found the over-expression of Lnc_CCAT1 in several solid tumors, such as lung cancer, prostate cancer, colon cancer, colorectal cancer, and leukemia as it is considered to be a marker of poor prognosis [8]. Moreover, previous study has demonstrated that miR_155a is a diagnostic biomarker in mature B-cell lymphomas with 80% sensitivity and 91% diagnostic specificity [5]. We concluded that Lnc_CCAT1 and miR_155a are upregulated in AML and associated with poor prognosis.

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. Therefore, in the current study; we evaluated the role of Lnc_CCAT1/miR-155a/c-Myc pathway “RNA-DNA cross-link” as a potential predictor in stratification of high risk AML. Moreover, to evaluate the impact of these studied biomarkers on disease outcome.

Conflicts of Interest

The authors declare no conflict of interest.
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List of Abbreviations

Acute myeloid leukemia: (AML)
Long non-coding RNAs: (IncRNAs)
The colon cancer associated transcripts: (CCATs)
MicroRNAs: (miRNA)
Colon cancer-associated transcript-1: (CCAT1)

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