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Original Article

HOTTIP is upregulated in acute myeloid leukemia that indicates a poor prognosis

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Abstract: LncRNA HOTTIP (HOXA transcript at the distal tip) gene is located in physical contiguity (chr 7p15.2) with HOXA13 and directly controls the HOXA locus gene expression by interaction with the WDR5/MLL complex. HOX genes encode transcription factors regulating embryonic development and cell fate. Recent evidence highlighted HOTTIP plays a crucial role in some solid tumors. However, little is known about the role of HOTTIP in acute myeloid leukemia (AML). In this study, We evaluated HOTTIP expression in bone marrow of de novo AML patients, AML-CR (complete remission) patients and normal controls by real-time quantitative reverse transcription-PCR (qRT-PCR), and then we analyzed the relationship between the HOTTIP expression level and the clinicopathological parameters of AML. The results showed that HOTTIP is markedly upregulated in de novo AML patients compared with those of AML-CR patients and normal controls; the higher expression level of HOTTIP in AML patients was significantly correlated with NCCN high risk group and higher blast cells. In conclusion, our study indicated that HOTTIP is highly expressed in AML patients, and the levels of HOTTIP are associated with AML patients' clinical progression.

Keywords: Acute myeloid leukemia, long non-coding RNA, HOTTIP, prognosis

Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults with an incidence of 3-4 per 100,000 persons per year. AML is a genetically heterogeneous disorder characterized by the somatically acquired genetic changes in hematopoietic progenitor cells altering normal mechanisms of self-renewal, proliferation, and differentiation. Greater insight into the genetic background of the disease fostered the extension of disease classification and pretreatment risk-categorization by gene mutations. In order to improve outcome in AML, multiple studies aimed at genetic characterization of AML have been performed inthe hopes of furthering our understanding of AML pathogenesis and identifying new therapeutic approaches [1, 2].

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 remodeling, gene transcription, and protein transport and trafficking [3]. Recently, more and more studies have shown that lncRNA are dysregulated in a wide variety of cancers [4, 5].

HOTTIP activates genes of HOXA cluster during differentiation in primary human fibroblasts [6]. HOTTIP is transcribed from the 5' end of the HOXA locus and binds to WD repeat-containing protein 5 (WDR5), a component of mixed-lineage leukemia (MLL) complexes, recruiting the MLL complex to form H3K4 methylation markers [7, 8]. Normal expression of HOTTIP is necessary to maintain active chromatin and the correct positioning of the MLL complex, which leads to the activation of the 5' genes of the HOXA cluster [7], abnormal activities of MLL complexes through dysregulation of the lncRNAs that recruit this chromatin remodeling complex to the HOX gene cluster results in abnormal hematopoiesis. The HOXA gene cluster encodes transcriptional regulators critical for human hematopoiesis, so its functions in

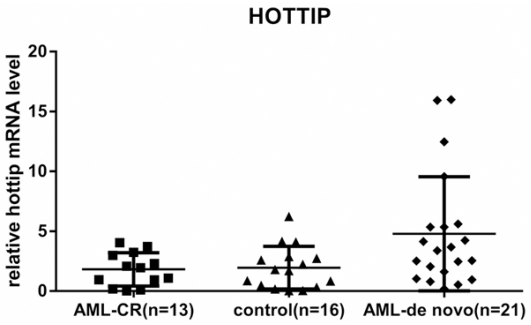

Figure 1. HOTIP expression was significantly higher in AML-de novo patients (n=21) than in AML-CR patients (n=13) and controls (n=16). Relative HOTTIP expression was determined using qRT-PCR with GAPDH as an internal control.

the homeobox pathways have been implicated in leukemogenesis [9].

Recently, HOTTIP has been determined to be a negative prognostic indicator in some solid-tumor patients [5, 10, 11]. Nevertheless, little is known about the impact of HOTTIP on AML. To understand the role of HOTTIP in AML, we investigated the expression level of HOTTIP in AML and analyzed its relationship to clinical pathological features.

Materials and methods

Patients and samples

A total of 34 AML cases were enrolled in our study including 21 de novo AML patients and 13 cases who had achieved complete remission (CR). 21 de novo AML patients were diagnosed in the Hematology Department of Tianjin Medical University General Hospital between February 2014 and November 2014 according to the "2008 WHO adult acute myeloid leukemia (non acute pro-myelocytic leukemia) diagnosis guidelines" and "2008 WHO adult acute pro-myelocytic leukemia diagnosis guidelines". 13 cases achieved complement remission (CR) were enrolled as AML-CR group. 16 iron-deficiency anemia (IDA) patients diagnosed according to the international criteria of IDA in our department were enrolled as control group. We collected 2 ml EDTA anticoagulant fresh bone marrow samples from each patient and BM sample of de novo AML patients were collected before intervention. We also collected the clinical and pathological characteristics for each patient, including age, sex, and blood routine test. This study was approved by the Ethics Committee of Tianjin Medical University. Informed written consent was obtained from all patients in accordance with the Declaration of Helsinki.

FCM analysis

Fresh heparinized BM samples (100 uL) were stained with CD34-FITC (BD Pharmingen, San Diego, CA, USA) and CD45-PerCP (BD Pharmingen, San Diego, CA, USA). After incubation in the dark for 30 min at 4°C, cells were incubated with 1 ml erythrocyte lytic solution (BD PharMingen) for 10 min at room temperature and washed three times with PBS. Finally, at least 100000 cells were acquired and analyzed on a FACSC alibur flow cytometer (BD Biosciences). We gated on CD45/SSC to characterize leukemic blasts. The blast population occupies the position with SSC in the low to moderate range, and CD45 of weak to moderate intensity.

RNA extraction and qRT-PCR analyses

Total RNA from human bone marrow was extracted using the TRIzol reagent (Life Technologies, Scotland, UK) according to the manufacturer's protocol. 1 μg total RNA was reverse transcribed in a final volume of 20 ul using random primers under standard conditions using the PrimeScript RT Master Mix (Takara, Dalian, China). After the RT reaction, qRT-PCR was performed using the BIO-RAD iQ5 Real-Time System (BIO-RAD, Hercules, CA, USA), and SYBR Green (Tiangen, Beijing, China) was used as a double strand DNA-specific dye. The sequences of primers specific for HOTTIP (forward, 5'-CCTAAAGCCACGCTTCTTTG-3'; reverse, 5'-TGCAGGCTGGAGATCCTACT-3') were synthesized by GENEWIZ (Suzhou, China). GAPDH was used as a housekeeping gene for standardizing theexpression of targeted mRNA. The sequences of primers specific for GAPDH (forward, 5'-CCGGGAAACTGTGGCGTGATGG-3'; reverse, 5'-AGGTGGAGGAGTGGGTGTCGCTGTT-3') were also synthesized by GENEWIZ (Suzhou, China). qRT-PCR cycling program: 95°C for 15 min, followed by 40 cycles at 95°C for 10 s and 64.4°C for 30 s. After normalization of the data according to the expressionof GAPDH mRNA, the levels of HOTTIP were calculated using the 2-ΔΔCt method [(Ct, hot tip-Ct, GAPDH) sample-(Ct, hot tip-Ct, GAPDH) control].

Table 1. Relationship between HOTTIP expression levels and clinicopathological parameters of de novo AML samples

Clinicopathologic features	Number	Relative expression of HOTTIP (Mean ± SD)	*P*-value
Gender			0.3589
Male	11	6.102±6.152	
Female	10	4.358±6.101	
NCCN groups			0.0466*
Low and medium risk	15	3.078±2.511	
High risk	6	10.75±8.816	

*P<0.05.

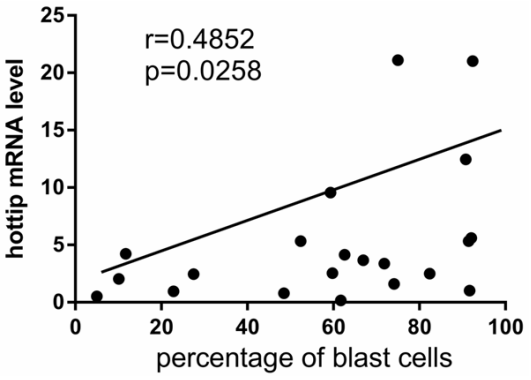

Figure 2. Relationship between the frequency of the BM blast cells and the hottip mRNA level in de novo AML group.

Statistical analysis

Data were presented as mean ± standard deviation in the text and figures and were analyzed with GraphPad Prism 6 Software. Pearson's correlation coefficient was used to determine the correlation between HOTTIP-mRNA level and the frequency of BM blast cells. Statistical significance was evaluated with the Student's t-test for HOTTIP-mRNA level. A *P* value of <0.05 was considered statistically significant.

Results

Clinical data of patients

There were 21 cases of de novo AML patients, male 11 cases, female 10 cases, mean age 50.61±16.04 years old. Among them, 2 cases of acute myeloid leukemia differentiation type (M2), 7 cases of acute promyelocytic leukemia (M3), 7 cases of acute myelomonocytic leukemia (M4), 3 cases of acute monocytic leukemia (M5), and 2 cases of acute red leukemia (M6). According to the 2014 American National Comprehensive Cancer Network (NCCN) guidelines based on cytogenetic and molecular characteristics, all the patients were divided into low risk group, medial risk group and high risk group, including 15 cases of patients with low or medial risk group, 6 cases with high risk group. There were 13 cases of AML-CR patients, male 5 cases, female 8 cases, mean age 43.6±16.5 years old. They included 3 cases of AML-M2, 8 cases of AML-M3 and 2 cases of AML-M4. The data about the percentage of bone marrow blast cells and the percentage of CD34+ cells assayed with FCM were not shown.

HOTTIP was upregulated in de novo AML patients

The level of HOTTIP was detected in 21 de novo AMLsamples, 13 AML-CR samples. And 16 control samples by qRT-PCR, and normalized to GAPDH. HOTTIP expression was significantly up-regulated in de novo AML compared with AML-CR patients and controls. However, there were no significant differences between AML-CR patients and controls (**Figure 1**).

Correlations between elevated HOTTIP and clinicopathological characteristics

In order to examine the clinical importance of the HOTTIP overexpression, the correlation between clinicopathological parameters of de novo AML samples and level of HOTTIP expression was investigated. Analyses showed that the elevated HOTTIP level had significantly correlation with NCCN high risk group. However, there were not any relationships between HOTTIP and patients' gender or leukemia subtype (**Table 1**). Moreover, in de novo AML group, there were positive correlations between the frequency of BM blast cells and the expression of HOTTIP Mrna (r=0.4852, P<0.05) (**Figure 2**); there were no correlations between the frequencies of BM CD34+ cells, blood HB level, PLT counts and WBC counts with the expression of HOTTIP mRNA (data were not shown).

Discussion

So far more than 10,000 lncRNAs have been identified in the human genome [3] and several

lncRNAs have been associated with hematopoietic cancers [12]. HOTAIRM1 (HOX antisense intergenic RNA myeloid 1) plays a role in the myelopoiesis through modulation of gene expression in the HOXA cluster, knockdown of HOTAIRM1 quantitatively blunted RA-induced expression of HOXA1 and HOXA4 during the myeloid differentiation of NB4 cells, and selectively attenuated induction of transcripts for the myeloid differentiation genes CD11b and CD18 [13]. In addition, the level of ANRIL was increased in many AML and ALL patients [14], and the abundance of MEG3 was decreased in myeloid leukemia [15] but BIC was increased in B cell lymphoma [16].

Up to date, 231 lncRNAs have been annotated within the 4 HOX loci [17].

Among these newly described lncRNAs, a lncRNA named HOTTIP located in physical contiguity (chr 7p15.2) with the HOXA13 gene has been recently functionally characterized [7]. HOTTIP is expressed from development to adulthood in lumbo-sacral anatomic regions and it can directly coordinate and control the activation of several 5' HOXA genes by interacting with the WDR5/MLL complex [7]. Until now, many studies have showed that increased expression of HOTTIP is associated with malignant progression and poor survival in various solid cancer types. Hence, HOTTIP may be considered as a potential target for diagnosis and treatment of various cancer types.

A study in hepatocellular carcinoma (HCC) indicates that HOTTIP was the most significantly up-regulated lncRNA in human HCCs, even in early stage of HCC formation. Functionally, knock-down of HOTTIP attenuated HCC cell proliferation in vitro and markedly abrogated tumourigenicity in vivo. In addition, knock-down of HOTTIP also inhibited migratory ability of HCC cells and significantly abrogated lung metastasis in orthotropic implantation model in nude mice. HOTTIP is an antisense lncRNA mapped to the distal end of the HOXA gene cluster. Knock-down of HOTTIP significantly suppressed the expression of a number of HOXA genes [10]. Quagliata et al also confirmed higher HOTTIP expression in non-neoplastic liver compared to non-

tumoral area tissue, the study also showed that high expression of HOTTIP is always coupled with increased HOXA13 levels and conversely low HOTTIP levels correlate with low HOXA13 expression: the combination of clinico-pathological and expression data indicates that high levels of HOTTIP/HOXA13 expression are associated with metastasis formation and predict HCC patients' clinical outcome [5]. Another study profiled 90 well-annotated mouse lncRNAs from cultured mouse keratinocytes after deleting the vitamin D receptor (VDR) and found that HOTTIP and several well-known oncogenes are significantly increased in VDR deleted keratinocytes, so this finding is a novel mechanism that predisposes the VDR deficient mice to skin cancer formation [11].

The results of our study indicated that the expression of HOTTIP was upregulated in de novo AML patients compared with normal controls and AML-CR patients. Moreover, HOTTIP expression was found to be significantly higher in NCCN high risk group. These findings indicate that HOTTIP may play a direct role in the modulation of AML progression and may be useful as a novel prognostic marker for AML. These results were also consistent with the findings about hot tip in other cancers before. In addition, we also found that BM blast cells and hot tip mRNA level were positively correlated.

However, it should be noted that the sample size of this study is not very big, thus each leukemia subtype has few cases, so we cannot thoroughly compare whether there is any difference in HOTTIP level between them. Moreover, although several lncRNAs have been identified the association with hematopoietic cancers, the mechanism of the effect of these lncRNA on hematopoietic malignances is not very clear. Our study also failed to answer this question and we will precede thorough research on it in the future.

In summary the expression of HOTTIP was upregulated in de novo AML patients, and highly expressed HOTTIP is associated with a poor clinicopathological prognostic stratification, but its exact function and mechanism remains to be further studied.

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Disclosure of conflict of interest

None.

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