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**RESEARCH NOTE**

# Identification of an acute myeloid leukaemia associated noncoding somatic mutation at 3′ end of HOXA cluster

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**Abstract.** Noncoding somatic mutations have been demonstrated to play important role in tumourigenesis. Here we show that there exists an acute myeloid leukaemia associated noncoding somatic mutation at 3′ terminal of conserved *HOXA* cluster. The mutation was identified in the bone marrow blasts but not peripheral blood mononuclear cells or buccal cells of two M3 (acute promyelocytic leukaemia, APL) type patients from 45 acute myeloid leukaemia patients. The mutation also existed in a pair of twins one of them developed acute myeloid leukaemia M4 (acute myelomonocytic leukaemia) type. The mutation resides in about 2-kb downstream of *HOXA1* gene where a functional retinoic acid response element is located and also bound by histone demethylase KDM3B. Reporter assay showed that the mutation results in the upregulation of transcriptional activity and unresponsiveness to retinoic acid receptor. To sum up, we identified a new acute myeloid leukaemia associated noncoding somatic mutation.

**Keywords.** acute myeloid leukaemia; noncoding somatic mutation; HOXA cluster.

## Introduction

Noncoding DNA constitutes ∼98% of human genome. In spite of this, most cancer-associated somatic mutations have been thought to reside only in coding but not in noncoding region of the human genome (Ding *et al.* 2012). This situation, however, has changed tremendously due to the introduction of next-generation sequencing (NGS) technology. Multiple noncoding somatic mutations have been found to be associated with different cancer types (Khurana *et al.* 2016). These cancer-associated somatic mutations can occur in any regions out of coding region, namely enhancer, silencer, insulator, promoter, 5′ UTR, 3′UTR, intron as well as splice sites and may affect gene regulation from transcription, splicing, mRNA stability to translation (Diederichs *et al.* 2016).

The clustered Hox family of homeobox genes are evolutionarily highly conserved genes that comprise four genomic clusters (A–D) in mammals (Alharbi *et al.* 2013). As one of the most ancient gene families, aberration of Hox genes are found to be deeply involved in tumourigenesis (Abate-Shen 2002). Interestingly, HOXA family members are especially associated with haematopoiesis and leukaemogenesis (Alharbi *et al.* 2013). 5′-end HOXA play positive regulatory role in leukaemogenesis. Among 6817 genes, *HOXA9* is the single most highly correlated gene associated with the prognosis of acute myeloid leukaemia (AML) (Golub 1999). *HOXA9* fusion to *NUP98* are found in human AML and drives AML in mouse models (Ghannam *et al.* 2004). In particular, HOXA9 plays a fundamental role in MLL-rearranged AML survival (Faber *et al.* 2009). Besides, HOXA4, HOXA5, HOXA7

and HOXA10 all play pivotal role in both haematopoiesis and leukaemogenesis (Argiropoulos and Humphries 2007; Alharbi *et al.* 2013). On the other hand, 3′-end HOXA may play negative regulatory role in leukaemogenesis.

Consistent with its role as one of the most ancient and highly conserved multigene loci in the animal kingdom, HOXA cluster undergoes complex genomic organization to fulfill delicate cross-regulation (De Kumar and Krumlauf 2016). In addition to coding regions of HOXA cluster, there are multiple noncoding regions including HOTAIRM1 and HOTAIRM2, between HOXA1 and HOXA2, HOXA-AS2 between HOXA3 and HOXA4, HOXA-AS3 between HOXA5 and HOXA7, HOXA10-HOXA9, HOXA10-AS and mir-196b between HOXA9 and HOXA11, HOXA11-AS between HOXA11 and HOXA13, and HOTTIP at 5′ upstream of HOXA13 (De Kumar and Krumlauf 2016). Mouse Hoxa cluster also contains Halr1 at 3′ downstream of HOXA1. In addition to these *trans* transcribed noncoding regions, there are multiple *cis* noncoding regions in HOXA cluster. In particular, retinoic acid response elements (RAREs) play important regulatory role for HOXA, and especially HOXA1 expression (Kolm and Sive 1995; Dupé *et al.* 1997; Thompson *et al.* 1998). There are three RAREs in mouse HOXA cluster, two of which are located at 50-kb 3′ downstream of HOXA cluster and third is located at 2-kb 3′ downstream of HOXA cluster (De Kumar and Krumlauf 2016). Transcriptional cofactors such as KDM3B is reported to bind to RAREs but not promoter of HOXA1 in HOXA cluster (Xu *et al.* 2018).

Here, we report the identification of a potential AML associated noncoding somatic mutation in 3′ end of HOXA cluster where HOXA1 proximal RARE is located at. Functional studies demonstrated that this mutation play a robust regulatory role for transcriptional regulation associated with HOXA1 proximal RARE.

## Materials and methods

### *Bioinformatics*

Genome browser of University of California Santa Cruz (UCSC) was used to search for SNPs in HOXA1 proximal RAREs. dbSNP database and Ensembl was used to determine the ancestral allele and minor allele frequency (MAF) of SNPs. Ensembl was used to determine population genetics of SNPs. Potential DNA binding to regulatory regions spanning SNPs based on ChIP sequencing was predicted according to the information provided by The Encyclopedia of DNA Elements (ENCODE).

### *Patients*

Peripheral blood (PB), bone marrow, and buccal samples were collected from 45 AML patients and PB/buccal samples were collected from 114 normal donors as controls. Genome DNA were obtained from peripheral blood, bone marrow, or buccal samples. The detailed information of patients was described previously (Xu *et al.* 2016). No related individuals were incorporated in the study. The study received ethics approval from the Commission for Scientific Research in Weifang Medical University and was administered in accordance with the ethical standards of the Declaration of Helsinki, second revision. Participants gave written consent after being informed about the study orally and in writing.

### *DNA extraction*

Genome DNA was extracted from fresh peripheral blood, bone marrow and buccal cells of AML patients and normal donors using QIAamp DNA Blood Mini kit (Qiagen, Shanghai, China) according to manufacturer's instruction.

### *DNA sequencing*

The DNA sequences (706 bp) flanking Human HOXA1 proximal RAREs were amplified using PrimeSTAR HS DNA Polymerase (Takara, Dalian, China). Forward primer is 5′-TAGGCACCTGGGATAACT-3′ and reverse primer is 5′-CTTGTGGCATCTGAAATAA-3′. The amplified fragments were ligated into pUCm-T vector (Sangon Biotech, Shanghai, China) and the positive clones were picked for plasmid sequencing.

### *Cell culture and transfection*

293T cells were cultured in DMEM medium containing 10% FBS. All cell culture were incubated in 5% $CO_2$ atmosphere at 37°C. Plasmids transfection was performed using Lipofectamine 3000 following the manufacturer's procedures (Life Technologies, Grand Island, USA).

### *Luciferase assay*

706-bp DNA fragments containing HOXA1 RAREs from individual AML patients were cloned into pGL3-promoter plasmids (Promega, Madison, USA) by *Bam*HI and *Sal*I restriction enzymes (New England Biolabs). Primers were: forward 5′-CGCGGATCCTAGGCACCT GGGATAACT-3′ and reverse 5′-ACGCGTCGACCTTG TGGCATCTGAAATAA-3′. Buccal and bone marrow DNA from an AML M3 (APL) patient were used as templates. PCR products and constructed plasmids were sequenced to confirm genotypes. pRL-TK (Promega) was used as control plasmid. Luciferase and renila activities were detected using Promega Dual-Luciferase Reporter Assay System. The assay was finished in Sirius L Tube

luminometer (Berthold Detection systems, Pforzheim, Germany).

### *Statistical analysis*

Statistical analysis was performed using SPSS 19.0 package. Quantitative results are reported as mean ± standard deviation. Comparisons with a $P$ value $\leq 0.05$ were considered statistically significant.

## Results and discussion

### *Identification of AML associated noncoding somatic mutation at 3′ downstream of HOXA cluster*

We previously found that *HOXA1* proximal enhancer where RAREs reside showed strong H3K9 methylation signal and was bound by KDM3B (Xu *et al.* 2018). Bioinformatics analysis showed that *HOXA1* proximal enhancer contains not only RAREs but also CE2, an important regulatory element for *HOXA1* expression reported previously (Thompson *et al.* 1998). Further analysis identified a SNP (rs62454376) from dbSNP 150. Considering the importance of this conserved region, we sequenced *HOXA1* proximal enhancer in 45 AML patients. Interestingly, we found somatic mutations in the flanking region of *HOXA1* proximal enhancer, with one located between RARE and CE2, from bone marrow but not peripheral blood mononuclear cells (PBMCs) in two patients from nine M3 (APL) types in a total 45 AML patients (figure 1). Mutation A (A/T) occurs at rs62454376 (A/G) whereas mutation B has not been reported in any SNP database as far as we known. Since the sample size is small, we could not conclude that frequency of HOXA1 3′-end somatic mutations is 2/45 in AML or 2/9 in AML M3 (APL). Independent cohort experiment with bigger sample size need to be performed to answer this question. Interestingly, the mutations identified also existed in a pair of twins one of them developed acute myeloid leukaemia M4 (acute myelomonocytic leukaemia) type (data not shown). We also sequenced multiple AML cell lines including HEL, K562, NB4, MUTZ3 and MUTZ8 for somatic mutations at HOXA1 3′ end but failed to detect any mutations (data not shown).

### *Identified noncoding somatic mutation at 3′ end of HOXA cluster shows robust regulatory activities*

To learn if the mutation is a bystander or is involved in tumourigenesis of AML, especially M3 AML (APL), we performed functional study. We constructed 706-bp sequences flanking HOXA1 RARE that contains either identified somatic mutations or not and also RARE and CE2 into reporter vector. We then cotransfected reporter with somatic mutations or not (reference sequences) plus transcription factor RAR, PML/RAR, and cofactor KDM3B into 293T cells. Luciferase assay showed that wild reporter responded to PML/RAR activation whereas KDM3B repressed PML/RAR induced transcription. By contrast, reporter with somatic mutations showed much higher constitutive activities, lower response to PML/RAR, and higher repression by KDM3B compared to wild reporter, as shown in figure 2. Our data obtained from luciferase assay indicated that wild reporter responded to PML/RAR activation and KDM3B repressed PML/RAR induced transcription, suggesting the mutations that resides at HOXA1 3′ end where RARE is located may interfere with the binding of potential transcriptional factors such as PML/RAR and transcriptional cofactors such as KDM3B. The outcome of this complex regulation may lead to the downregulation of HOXA1 that may negatively regulate leukaemogenesis especially in M3

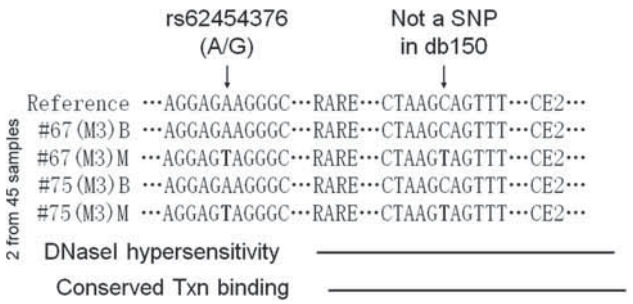


**Figure 1.** Identification of noncoding somatic mutation at 3′ end of HOXA cluster. 3′ end of HOXA cluster was shown. Reference sequence was from UCSC genome browser (Dec. 2013, GRCh38/hg38). The exact somatic mutations were indicated. Mutation A occurs at the position rs62454376. DNase I hypersensitivity and conserved transcriptional binding (RAR etc.) region were predicted by the UCSC genome browser.

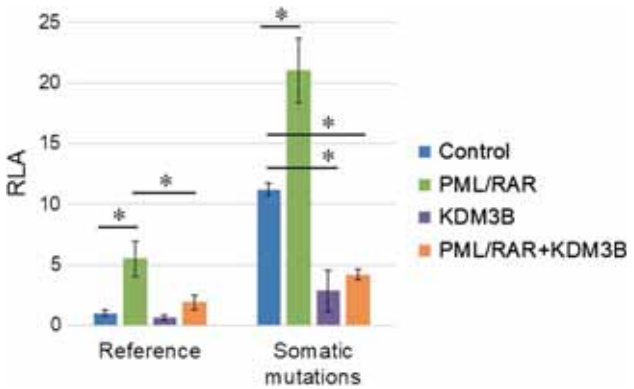


**Figure 2.** Transcriptional activities of the reporters with wild type and mutated HOXA1 enhancer. Wild type and mutated reporters with endogenous control pRL-TK were cotransfected with PML/RAR and KDM3B into 293T cells. After 48 h transfection, cells were collected and lysed to detect fluorescence activities. Three independent experiments were performed. $P$ value smaller than 0.05 was regarded as significant.

AML (APL). PML/RAR fusion may synergize with non-coding mutations of HOXA enhancer to contribute to M3 AML (APL) initiation.

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