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The role of HOXB2 and HOXB3 in acute myeloid leukemia

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ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous aggressive disease and the most common form of adult leukemia. Mutations in the type III receptor tyrosine kinase FLT3 are found in more than 30% of AML patients. Drugs against FLT3 have been developed for the treatment of AML, but they lack specificity, show poor response and lead to the development of a resistant phenotype upon treatment. Therefore, a deeper understanding of FLT3 signaling will facilitate identification of additional pharmacological targets in FLT3-driven AML. In this report, we identify HOXB2 and HOXB3 as novel regulators of oncogenic FLT3-ITD-driven AML. We show that HOXB2 and HOXB3 expression is upregulated in a group of AML patients carrying FLT3-ITD. Overexpression of HOXB2 or HOXB3 in mouse pro-B cells resulted in decreased FLT3-ITD-dependent cell proliferation as well as colony formation and increased apoptosis. Expression of HOXB2 or HOXB3 resulted in a significant decrease in FLT3-ITD-induced AKT, ERK, p38 and STAT5 phosphorylation. Our data suggest that HOXB2 and HOXB3 act as tumor suppressors in FLT3-ITD driven AML.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous aggressive hematopoietic disorder which is the most common adult acute leukemia accounting for around 80% of cases. Genetic alterations lead to abrogated differentiation of hematopoietic cells. Therefore, the self-renewal ability of those cells is increased, and regulation of normal cell proliferation is disturbed. The major genetic changes include mutation in genes affecting cell proliferation (FLT3, KIT, NRAS/KRAS, JAK/STAT and PTPN11), myeloid differentiation (RUNX1/AML1 and CEBPA), cell cycle regulation or apoptosis (TP53, NPM1), and up-regulation of genes involved in stem-cell maintenance (HOXA, HOXB) [1–4].

The homeobox (HOX) genes are a family of homeodomain-containing transcription factors mainly involved in development. The human HOX genes are clustered in four different chromosomes, 7p15 (HOXA), 17q21 (HOXB), 12q13 (HOXC) and 2q31 (HOXD). HOXA family genes have been thoroughly studied in respect to AML [5–7]. While some studies suggest that HOXB family genes are up-regulated in certain types of AML, their roles in AML have not yet been defined [8–13]. A recent report suggests that HOXB4 expression is elevated in a group of AML patients and higher HOXB4 expression correlated with better prognosis [8]. Another report suggests that over-expression of HOXB6 in NB4 cells or in HL60 cells caused inhibition of the granulocytic and monocytic maturation, respectively [12].

The type III receptor tyrosine kinase FLT3 is expressed in almost all AML, and about 35% of AML patients carry an oncogenic *FLT3* mutation [14]. Among the several mutations that have been found, the internal tandem duplication (ITD) of the sequence that encodes the juxtamembrane domain is the most common mutation in FLT3. The presence of FLT3-ITD mutation portends a poor prognosis in AML. FLT3 mutations also occur in other types of leukemia to a lesser extent, including acute lymphoblastic leukemia [15]. While wild-type FLT3 requires its ligand FL for activation, oncogenic mutants are constitutively active. Activated FLT3 recruits SH2 domain-containing protein through phosphotyrosine residues

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resulting in activation of AKT, ERK, p38, and STAT5 [16–20].

In this report, we show that expression of HOXB2 and HOXB3 genes is upregulated in AML patients carrying the FLT3-ITD mutations. Loss of HOXB2 or HOXB3 expression in patients carrying FLT3-ITD mutations results in enrichment of oncogenic pathways. Overexpression of HOXB2 or HOXB3 significantly inhibits FLT3-ITD-induced cell proliferation and colony formation and further increases apoptosis.

2. Materials and methods

2.1. Plasmids and antibodies

Plasmids expressing human HOXB2 and HOXB3 were generated by ligating the open reading frame (ORF) of the corresponding gene into the retroviral vector pMSCVneo. FLT3-ITD plasmid was described previously [21]. Anti-FLT3 antibody was also described previously [22]. Anti-phosphotyrosine antibody 4G10 was purchased from Millipore (Life Technologies, Carlsbad, CA) and Anti-phospho p38 and anti-p38 antibodies were from BD Biosciences (Franklin Lakes, New Jersey). Anti-phospho-ERK1/2, anti-ERK2, anti-STAT5 and anti-AKT antibodies were from Santa-Cruz Biotechnology (Dallas, Texas) and anti-phospho AKT was from Epitomics (Abcam, Cambridge, UK). Anti-β-actin antibody was from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture and transfection

The murine pro-B cell line Ba/F3 was cultured in RPMI-1640 medium (Hyclone, Thermo Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA), 10 ng/ml murine interleukin 3 (IL3) and 1% penicillin and streptomycin. Generation of Ba/F3-FLT3-ITD cells was described previously [23]. FLT3-ITD-transfected Ba/F3 cells were then further transfected with the pMSCV-neo-HOXB2 or pMSCV-neo-HOXB3 construct or empty pMSCV-neo vector. Cells were selected with 0.8 mg/ml G-418 for 2 weeks. Transfected cells were maintained in Ba/F3 medium as previously described [24]. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Immunoprecipitation and western blotting

Cells were starved of serum and cytokines for 4 h and were washed once with cold PBS before lysis with Triton X-100 based lysis buffer. Cell lysates were mixed with SDS and DTT containing loading buffer in a 1:1 ratio and boiled before separation by SDS-PAGE. For immunoprecipitation 1 μg anti-STAT5 antibody was added to cell lysates and was kept for 1 h on ice followed by purification on protein G Dynabeads and SDS-PAGE analysis.

2.4. Apoptosis, cell proliferation, and colony formation assay

Cells were washed three times to remove cytokine before all experiments. Annexin V and 7-aminoactinomycin D (7-AAD) apoptosis kit (BD Biosciences) was used to measure apoptosis in cytokine-depleted cells. Cells positive for annexin V and both annexin V/7-AAD were counted as apoptotic cells. To measure cell proliferation, 10,000 cells were seeded in each well of a 96-well plate and incubated for 48 h. AlamarBlue (Molecular Probe) was used to measure cell viability. Semisolid methylcellulose medium (Stem Cell Technologies) was used for colony formation assay. Around 500 cells were seeded and cultured for seven days before counting colonies.

2.5. Microarray data analysis

The data set GSE14468 was used which was generated from a cohort of 598 newly diagnosed AML patients [25]. Gene expression was compared using significance analysis of microarrays (SAM) tools [26] and gene set enrichment analysis (GSEA) [27]. One-way ANOVA was used for statistical analysis. In statistical significance tests, "ns" represents not significant, "*" represents $p < 0.05$, "**" represents $p < 0.01$, and "***" represents $p < 0.001$.

3. Results

3.1. HOXB family proteins are upregulated in FLT3-ITD positive AML

To understand the molecular difference between oncogenic FLT3-ITD positive and negative AML, we analyzed gene expression data of AML patients. We used expression data from bone marrow aspirates or peripheral blood samples of 598 cases of de novo AML. Using SAM tool we checked the differential gene expression. We observed that several HOXB family genes were upregulated in FLT3-ITD positive patients (Table S1). HOXB2 displayed 2.3-fold upregulation, HOXB3 4.4-fold, HOXB5 1.4-fold and HOXB6 2.2 fold upregulation (Fig. S1A). Therefore, our data suggest that expression of several HOXB genes is deregulated in FLT3-ITD-driven AML.

3.2. HOXB2 and HOXB3 are independent prognostic markers in AML

The HOXB family includes 10 genes, HOXB1-9 and 13. Since several HOXB-family genes were deregulated in FLT3-ITD driven AML, we checked whether expression of HOXB-family genes has any prognostic significance in AML. We transformed relative expression values to the Z-score and divided patients into two groups depending on higher or lower HOXB genes expression. We observed that higher expression of either HOXB2 (Fig. 1A) or HOXB3 (Fig. 1B) but not HOXB5 (Fig. S1B) correlated with poor prognosis compared to lower HOXB2 ($P = 0.0053$) and HOXB3 ($P = 0.0147$) expression. This also holds true for higher expression of HOXB6 (Fig. S1C), HOXB7 (Fig. S1D), HOXB8 (Fig. S1E) and HOXB9 (Fig. S1F). Lower HOXB2 (Fig. 1C) and HOXB3 (Fig. 1D) expression further correlated with better event-free survival ($P = 0.0234$ and $P = 0.0432$ respectively). Although HOXB2 and HOXB3 expression levels displayed prognostic significance in the total patient group independent of FLT3 mutations, we were unable to show any prognostic significance in only FLT3-ITD-dependent AML (data not shown) probably due to limited number of patient samples in each group. Since both HOXB2 and HOXB3 genes expression were upregulated in FLT3-ITD-dependent AML and since both genes expression profiles displayed independent prognostic significance, we checked whether HOXB2 and HOXB3 expression levels correlate with each other. We observed a strong correlation in between expression of the two genes ($r^2 = 0.8633$) suggesting that patients having higher HOXB2 expression will also have a higher HOXB3 expression and *vice versa* (Fig. 1E). In addition to FLT3-ITD positive AML patients (Fig. 1F), HOXB2 and HOXB3 expression was upregulated in patients carrying the NPM1 mutation (Fig. 1G). Although HOXB2 and HOXB3 expression correlated with FLT3-ITD and NPM1 mutations, expression neither correlated with patients age (Fig. S1G and S1H) nor with the patients sex (Fig. S1I). However, expression of both HOXB2 (Fig. S1J) and HOXB3 (Fig. S1K) was significantly downregulated in the FAB M3 group patients.