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Original Research

All-trans retinoic acid inhibits HOXA7 expression in leukemia cell NB4

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Abstract: Leukemia is a malignant proliferative disease of blood system, which is caused by hyperplasia of white blood cells and infiltration into other tissues and organs with blood flow, leading to a series of clinical manifestations. In this study, we detected the expression of HOXA7 gene in human acute promyelocytic leukemia cell line NB4. The expression level of HOXA7 decreased in the presence of ATRA, which was able to inhibit the proliferation of NB4 cells. Furthermore, ATRA altered the morphology of NB4 cells. The study suggested that HOXA7 might be a new gene candidate that influences the maturation of acute myeloid leukemia, and provided the molecular basis for the treatment for acute promyelocyticleukemia.

Key words: NB4 cell line, HOXA7 gene, All-trans retinoic acid, acute promyelocytic leukemia.

Introduction

Leukemia is a malignant proliferative disease of blood system, which is caused by hyperplasia of white blood cells and infiltration into other tissues and organs with blood flow, leading to a series of clinical manifestations. Leukemia can occur in any age, and with the highest incidence in children, especially in preschool and school-age children (1, 2). The incidence rate was about 3-4/100000 in children less than 10 years old. In which, more than 90% leukemia were acute leukemia and few were chronic leukemia (3). According to the main infected cells, acute leukemia is divided into acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL).

Homeobox (HOX) gene was first discovered in Drosophila (4), which was also found in human body, and located on four chromosomes: No.7, 17, 12 and 2, thereby designated as A, B, C, D clusters (5, 6). Each cluster was consisted of multiple gene fragments with different size; in turn they were named numerically. More than thirty HOX genes were found, and most of them contained homeobox fragments of 180 nucleotides and transcribed sequences with 60 amino acids; which was called homeodomain with high conservatism.

HOX gene plays a regulatory role in growth and development, and its abnormal expression may lead to many diseases (7). Meanwhile, HOX gene plays an important role in the regulation of proliferation and differentiation of hematopoietic cells, which could participate in the development of the hematopoietic system and promote HSPC differentiate into erythroid, myeloid, lymphoid, etc (8, 9). HOX gene was not only expressed in normal hematopoietic cells, but also expressed in leukemia cells, and resulting in occurrence of leukemia by combing other transcription factors or other mutant gene (10, 11). In recent years, it has been found that all-trans retinoic acid (ATRA) could induce HOX gene expression *in vivo* and *in vitro* (12, 13). Further research found that the main inducers were retinoic acid receptor (RAR) (14). Retinoic acid (RA) and its receptor was combined with retinoic acid response element (RARE), leading to the activation or inhibition of gene transcription. RARE was present in the promoter region of HOXA1, A7, A9 genes (15). Treatment of acute promyelocytic leukemia (M3 type) using RA was the success cases of molecular targeted cancer therapy (16). Treatment of leukemia with ATRA had been confirmed to be associated with regulation of ATRA and HOX genes (17). However, few studies reported whether the HOXA7 gene was associated with leukemia and whether ATRA could regulate the human acute promyelocytic leukemia NB4 cell proliferation and the expression of HOXA7 gene in NB4 cells. In this study, we detected the effect of ATRA on NB4 cells, and the dynamic expression alteration of HOXA7 gene in the NB4 cells with the treatment of ATRA.

Materials and Methods

NB4 cell culture

The NB4 cell line was purchased from DSMZ. NB4 cells were cultured in RPMI1640 medium with 10% fetal bovine serum, added with 100 μg/ml penicillin-streptomycin, and placed in 5% CO_2incubator at 37°C.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) was used to test the cell proliferation. The kit was purchased from Beyotime Biotechnology, Shanghai, China. The protocol was strictly followed by the Kit manual. Briefly, 10μl CCK-8 solution was added into each well. The cells were incubated for 1 hour, and analyzed by a microplate reader (Bio-Rad). The cells were treated with ATRA to determine the optimal concentration. The OD values at 450nm were measured.

ATRA treatment

NB4 cells were divided into four groups. 1) Control

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group: without the ATRA treatment; 2) Group 4 h: 1 μmol/L ATRA were added into cells for 4 h; 3) Group 48 h: 1 μmol/L ATRA were added into cells for 48 h; 4) Group 96 h: 1 μmol/L ATRA were added into cell for 96 h.

Wright Giemsa staining

The Wright-Giemsa Stain (Sigma-Aldrich) was placed upon the cells to cover the entire surface for 3 minutes. Then, the cells were rinsed with PBS until the edges show faintly pinkish red. The slides were allowed to dry in the air, and mounted with mounting medium. The cell differentiation and apoptosis were identified by morphological changes.

Immunocytochemistry

The cells were fixed in 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature. PBS containing 0.1% Triton X-100 was used to permeabilize the cells for 10 min, and incubated in H2O2 for 10 min. Then, the cells were incubated with 1% bovine serum albumin (BSA) for 30 min to block unspecific binding of the antibodies, with diluted primary rabbit anti-HOXA7 antibody (1:1000, Abcam) overnight at 4°C.The slides were incubated in the secondary antibody for 1hour at room temperature, then in the staining solution for 5 min to develop the color. Finally, the slides were mounted in resinene.

Real Time PCR

The protocol for Real Time PCR was reported previously (18). Briefly, the total RNA was extracted by Trizol (Life Technologies). Synthesis of cDNA was performed by using one-step RT-PCR kit from Takara. A mock control was carried out to exclude DNA contamination. Primers used are as follows: HOXA7, 5'-CCCCTGGATGCGGTCTT-3' and 5'-CCTTCGTTATGCTCTTTC-3'. GAPDH, 5'-ATGCTGGCGCTGAGTACGTC-3' and 5'-GGTCATGAGTCCTTCCACGATA-3'. SYBR Green (Toyobo) RT-PCR amplification and real time fluorescence detection were performed using the PRISM 7300 sequence detection system (Applied Biosystems). Relative gene expression was calculated by the ΔΔCt method. The calibrator was the unstimulated control sample. At the end of the experiments, the products were analyzed by gel electrophoresis to confirm the presence and assess the purity of the amplicons of interest. Each sample was analyzed in triplicate.

Western Blotting

The protocol for Western Blotting was reported previously (19). Briefly, 2 μg cell lysates were loaded on each lane of 10% polyacrylamide gel, and then blotted onto a polyvinylidenedifluoride (PVDF) membrane. After blocking with a PBST containing 5% nonfat dry milk, the blots were incubated with antibodies against HOXA7 (1:500, Abcam). Peroxidase-linked anti rabbit IgG (Invitrogen) were used as secondary antibodies. These proteins were visualized by using an ECL western blotting detection kit (Amersham Biosciences).

Statistical analysis

All data were expressed as mean±standard deviation ($\bar{x}$ ±s) and analyzed with ANOVA by SPSS17.0 software. The normalized density for immunocytochemistry and western blotting results were analyzed by Image J. The normalized density for cell staining = total intensity/cell number. The normalized density for western blotting band = total intensity/area of selected region. Statistical differences were analyzed with LSD pairwise comparisons. A $P<0.05$ was considered as statistically significant.

Results

Treatment of ATRAaltered the morphology of the NB4 cells

First, we used the CCK-8 to determine the optimal ATRA concentration. As shown in the Figure.1, 10 and 5μmol/L ATRA showed high cytotoxicity to NB4, while 1,0.5,0.1μmol/L did not have apparent cytotoxicity. Since the lower concentration may not have the biological effect on NB4 cells, we used the 1μmol/L ATRA in the following experiments.

After the treatment of 1μmol/L ATRA, we observed the apparent morphological alteration in the NB cells. Compared to the normal NB cells with round and large nuclei (Fig.2A), the cellular nuclei started to shrink and size of cytoplasm was increasing at 4 hours after ATRA treatment (Fig.2B). 24 hours after ATRA treatment, apparent changes were observed in the nuclei and cytoplasm (Fig.2C). The nuclei continued to shrink, while the cytoplasm expanded further. The edge between nuclei and cytoplasm became obvious, and nuclei-cy-

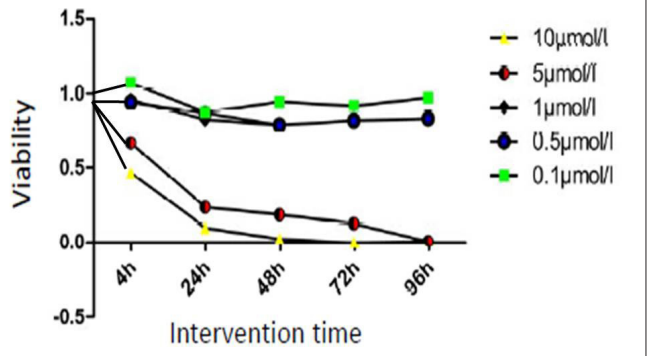

Figure 1. The effect of ATRA at different concentrations on the viability of NB4 cells.

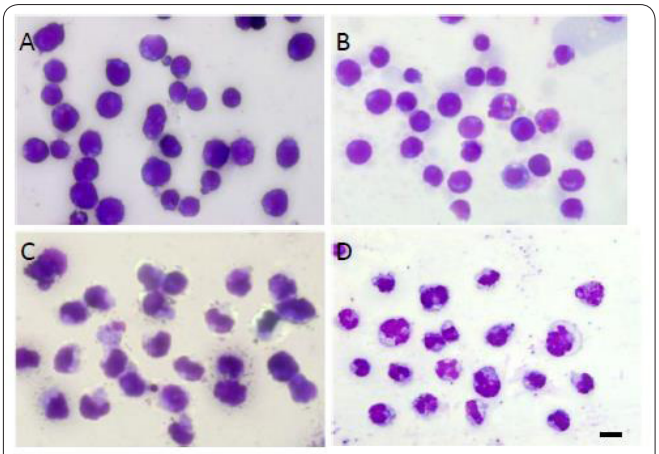

Figure 2. Treatment of ATRA altered the morphology of the NB4 cells by Giemsa staining. (A) NB4 cells without the treatment of ATRA. (B-D) The NB4 cells were treated with ATRA for 4h, 24h, and 96h. Scale bar, 10 μm.

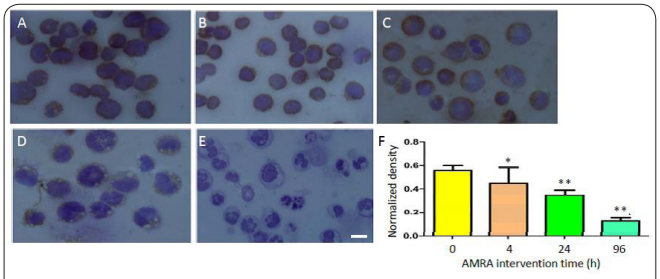

Figure 3. Treatment of ATRA decreased the expression of HOXA7 detected by immunocytochemistry. (A) NB4 cells without the treatment of ATRA. (B-D) The NB4 cells were treated with ATRA for 4h, 24h, and 96h. (E) Negative control without the incubation of primary antibody. (F) Statistical analysis for the immunocytochemistry data. *p<0.05, **p<0.01. Scale bar, 10 μm.

toplasm ratio decreased significantly compared to the control after the ATRA treatment for 96 hours (Fig.2D).

ATRA treatment decreased the HOXA7 expression in NB4 cells

Next, we elucidated the effect of ATRA on the HOXA7 protein expression in the NB4 cells. In the NB4 cells without the treatment of ATRA, the HOXA7 expression was strong, and the positive signal was dense and heavy (Fig.3A). 4 hours after ATRA treatment, HOXA7 expression level was decreased by 19% compared to that in the untreated cells ($p<0.05$, Fig.3B). The expression level of HOXA7 was decreased by 38% and 77% at 24 and 96 hours after ATRA treatment ($p<0.01$, Fig.3C, 3D). There was weak signal around the cell membrane.

Similar result was obtained when we tested the mRNA level of HOXA7. The real time PCR result indicated that the HOXA7 mRNA level decreased by 37% at 4 hours after ATRA treatment compared to the untreated cells ($p<0.05$). 24 and 96 hours after treatment, HOXA7 mRNA level was decreased by 74%and 96%, respectively ($p<0.01$, Fig.4).

The change in the HOXA7 protein level was also detected by Western Blotting. In the presence of ATRA for 4 hours, the protein level of HOXA7 was decreased by 15% compared to the control ($p<0.05$, Fig.5). Then, the protein level of HOXA7 was further decreased by 18% and 97% at 24 and 96 hours after treatment ($p<0.05$, $p<0.01$, Fig.5).This data demonstrated that HOXA7 was expressed in the leukemia cells NB4, and the expression level was decreased with the treatment of ATRA.

Discussion

Acute leukemia is a common cancer in children. At present, the basic treatment was chemotherapy, while the peripheral blood stem cell transplant, cord blood stem cell transplant, gene therapy are emerging. Although these methods have been used in clinic, the prognosis of leukemia is still not ideal with high recurrence rate. In order to overcomeand cure leukemia, numerous studies have been made in the pathogenesis and treatment of leukemia.

In our previous experiments, we also studied the expression of HOX gene in blood system. Liu found that HOXB8were expressed in human umbilical cord blood stem cells, and the expressions would be increased

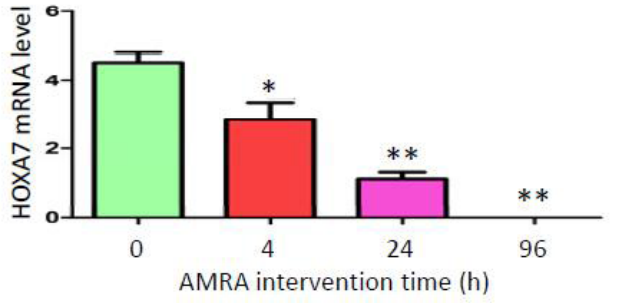

Figure 4. Statistical analysis for the change in HOXA7 mRNA level in the presence of ATRA.*p<0.05, **p<0.01.

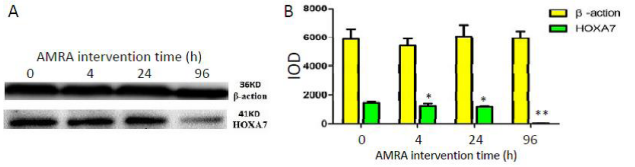

Figure 5. Treatment of ATRA decreased the HOXA7 protein level in the NB4 cells. (A) Western blotting analysis indicated HOXA7 expression level was decreased in the presence of ATRA. (B) Statistical analysis for the western blotting data. *p<0.05, **p<0.01.

treating with ATRA by FQ-RT-PCR and Western blot, respectively (20). However, the function of some HOX genes was still unclear; our study investigated the expression of HOXA7 gene in NB4 cells and the expression changes treating with ATRA, in order to explore the relationship between HOXA7 gene and acute promyelocytic leukemia.

More than 10 types of HOXA and HOXB genes were highly expressed in leukemia cells. Alharbi RA *et al* found that HOX gene could promote cell differentiation in early embryonic development, and played important roles in development (21). The expression imbalance of HOX gene was associated with AML, ALL and other malignant diseases. Jia*et al*detected the expression of HOXA9 gene in 46 cases of children with acute leukemia (including AML and ALL) and 15 cases of children with congenital thrombocytopenia purpura, and found that the HOXA9 gene was highly expressed in acute leukemia (AML was higher than ALL), and the higher expression of HOXA9, the worse the prognosis (22). Strakova J *et al* found that HOXA7-10, HOXA13 and HOXB2-4 were highly expressed in leukemia cells, and there was difference about the expression of HOXA7 and HOXB3-4 between B lymphocytic leukemia and T lymphocytic leukemia (23). Bach et al studied all HOXA clusters genes in hematopoietic progenitor cells, and reversed HOXA genes into backbone tissues, lastly the proliferation of hematopoietic progenitor cells were detected (10). The results showed that except HOXA2 and HOXA5 genes, other HOXA genes could inhibit and delay the differentiation of hematopoietic cells, leading to colony formation; and HOXA7-9 could promote the formation of permanent cell line, which played an important role in carcinogenesis of the hematopoietic system. Soulier J *et al*believed that the abnormal expression of HOXA cluster genes played a decisive role in occurrence of T lymphocytic leukemia (24). Eklundestablished different subtypes of AML mouse models to

analyze the gene expression, and found that the expression of HOXB3, HOXB4, HOXA7-11 were increased, suggesting that disorders of HOX genes might be related to the incidence of AML (11).

Using FQ-RT-PCR, Western blot, immunohistochemistry and other techniques, human acute promyelocytic leukemia NB4 cells as leukemia model, ATRA as inducer, we found that 1 μmol/L ATRA could promote NB4 cell differentiation and maturation, HOXA7 gene and protein were stably expressed in NB4 cells, and ATRA could decrease the expression of HOXA7 gene and protein (P<0.05), which was consistent with previous studies. The expression of HOXA7 was increased in AML and down-regulated treating with ATRA, suggesting that the molecular mechanisms of treatment of leukemia with ATRA might be related to regulation of HOXA7 expression.

In summary, the molecular mechanisms of treatment of leukemia with ATRA might be related to regulation of HOXA7 gene and protein expression. Our study showed that treatment of promyelocytic leukemia with ATRA might be via down-regulation of HOXA7 gene. Studies also suggested that ATRA was an effective drug in treatment of acute promyelocyticleukemia. ATRA could reduce the proliferation of cancer cells and promote their differentiation to block the proliferation of malignant diseases. Thereby we speculate that ATRA can be used to treat leukemia through intervention of HOXA7 gene expression.

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