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# Comparative Proteomic Analysis of Human Leukemic Cells with and without Inducible Expression of Leukemogenic AML1-ETO Protein

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## Abstract

**AML1-ETO is a leukemogenic fusion protein generated by chromosomal translocation t(8; 21) (q22; q22), one of the most frequent chromosomal abnormalities in acute myeloid leukemia. The fusion protein has been shown to present dichotomous functions on leukemic cells: growth arrest versus differentiation block. However, their precise molecular mechanisms are not completely clear. In this work, we try to explore potential AML1-ETO-targeted proteins through comparing two-dimensional electrophoresis (2DE)-based global protein expression profiles of leukemic U937 cells with and without inducible expression of AML1-ETO. As a result, we identified 14 unique proteins deregulated in AML1-ETO-carrying leukemic cells, including 3 up-regulated such as hairy and enhancer of split 5 (HES5) and 11 down-regulated such as MAT1 (menage a trois-1) and mitogen-activated protein kinase organizer 1 (MORG1). These proteins were widely involved in stem cell maintenance, cell cycle, signal transduction and transcription. The further investigation on their roles in leukemic cells will uncover new clues to understanding leukemogenic effects of AML1-ETO fusion protein.**

**Key Words: leukemia, AML1-ETO, proteomics, MORG1, MAT1**

## Introduction

Acute myeloid leukemia (AML), a prevalent hematopoietic malignancy, is characterized with complete or partial block of the maturation/ differentiation of myeloid progenitor cells at different stages. Pathogenesis of AML has been attributed to acquired genetic changes, especially different types of specific reciprocal chromosome translocations, most of which cause the production of abnormal fusion proteins invariably involving transcription factors, often fusing a DNA-binding domain of a transcriptional activator to a transcriptional repressor (15, 30). As a consequence, AML-associated fusion proteins function as aberrant transcriptional regulators with the potential to interfere with the processes of myeloid differentiation.

As a crucial transcription factor for definitive hematopoiesis, acute myeloid leukemia 1 (AML1), also known as runt-related transcription factor 1

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(RUNX1) or core-binding factor (CBF) α2, is a common target for chromosomal translocations involved in hematopoietic malignancies (19, 29). In addition to translocation t(12;21) in approximately 25% of childhood pre-B-cell acute lymphoblastic leukemias, t(16;21)(q24;q22) associated with therapy-related myeloid malignancies and t(3;21) in lymphoblast crisis evolving from chronic myelogenous leukemia, which respectively generate *TEL-AML1*, *AML1-MTG16* and *AML1-EVI1* fusion genes. The most frequent AML-associated chromosomal translocation involved in AML1 gene is the t(8;21)(q22;q22), which is detected in about 40% patients with $M_2$-type AML of French-American-British (FAB) classification and 10-20% AML patients (15). This translocation fuses *AML1* on chromosome 21q22 with *ETO* (also called *MTG8*) gene on chromosome 8q22, generating a chimeric protein AML1-ETO (11, 13, 20).

It has been demonstrated that ectopic expression of AML1-ETO inhibits leukemic cell differentiation towards granulocytic, monocytic or erythroid cells as well as induces NIH 3T3 cell transformation (1, 12, 14, 21, 23), while AML1-ETO suppression by small interfering RNAs supports myeloid differentiation of t(8;21)-positive leukemic cells (10). On the other hand, inducible expression of AML1-ETO was also demonstrated to induce growth arrest of leukemic cells (6), possibly *via* up-regulating connexin-43 expression (Li *et al.*, in submission), and to increase the sensitivity of leukemic cells to apoptosis (Lu *et al.*, in submission). The dichotomous functions of AML1-ETO on leukemic cells make its leukemogenic mechanisms more complicated (6). As a matter of fact, AML1-ETO-associated leukemogenesis may also require a secondary event or "hit" for progression to a frank leukemia, because AML1-ETO presented in newborn infant umbilical bloods at a frequency 100-fold greater than the corresponding leukemic risk (18). More importantly, AML1-ETO alone is not sufficient to induce leukemia in several AML1-ETO-expressing transgenic mice, except the use of powerful mutagen N-ethyl-N-nitrosourea or co-expression of TEL-PDGFRB and AML1-ETO, as recently reviewed by Kurokawa and Hirai (13). In spite of these facts, AML1-ETO was commonly believed to occupy a dominant position in the pathogenesis of leukemia, at least as an accomplice (33).

Transcription function of AML1-ETO and its wide type protein AML1 have been intensively investigated. AML1-ETO protein encompasses the N terminus of AML1 and nearly intact ETO protein lacking only the first 30 amino acid residues. Correspondingly, the fusion protein lacks the transcriptional activation domain of AML1 but carries the *Drosophila Melanogaster* protein runt homology domain (RHD), which mediates AML1 binding to the DNA consensus sequence TGT/cGGT and complexes with CBFβ to regulate transcription of AML1-targeted genes including interleukin-3, granulocyte- macrophage colony-stimulating factor, myeloperoxidase and neutrophil elastase. The ETO protein interacts with nuclear co-repressors N-CoR and mSin3A to recruit active histone deacetylases (HDACs) and thus to cause lower levels of histone acetylation and less-accessible chromatin (3, 16). Therefore, most studies have focused on the function of ETO in the context of the AML1-ETO fusion protein that causes aberrantly transcriptional regulation of genes usually activated by AML1. By physical protein-protein interaction, moreover, AML1-ETO can also interfere with other myeloid differentiation-related transcription factors such as CCAAT/enhancer binding protein α (4), PU.1 (31), and myeloid ELF-like factor (17, 25). It should be noted that more is involved than just the dominant inhibitory effect on the functions of AML1-ETO. For example, AML1-ETO was shown to synergistically transactivate the M-CSF receptor promoter with AML1, for which the region of ETO is necessary (22).

Due to complicated cellular and molecular effects of AML1-ETO on leukemic cells, some groups have performed gene expression profile analyses by oligonucleotide microarray to explore global effects of inducible AML1-ETO expression on a murine myeloid progenitor L-G cell line (24) and human leukemic U937 cell line (2). It has been widely known that mRNA expression is not parallel to protein expression. Hence, this work tried to use two-dimensional electrophoresis (2DE) coupled with mass spectrometry to compare proteomic pattern of leukemic U937 cell line with and without induced expression of AML1-ETO protein by ecdysone inducible system.

## Materials and Methods

### *Cell Culture and Treatment*

U937-A/E 9/14/18 cell line with conditional AML-ETO expression was generated by the ecdysone inducible system from parental U937 cells as described previously (8, 12) and provided by Dr. M. Lübbert in the Department of Medicine, University of Freiburg Medical Center (Freiburg, Germany) (8). The cell line was cultured in RPMI-1640 (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD, USA) and maintained in 5% $CO_2$, 95% humidified air at 37°C. For the induction of AML1-ETO expression in the cell line, 5 μM ponasterone A (Invitrogen, Groningen, Netherlands) in absolute alcohol was added to the

medium for 24 hours and western blots were performed to confirm AML1-ETO expression. The cells with treatment of equivalent absolute alcohol for 24 hours were used as control. Over 98% cells were maintained alive, as determined by the trypan-blue exclusion assay.

*Sample Preparation for 2DE*

U937-A/E 9/14/18 cells (about $8 \times 10^7$) with treatment of ponasterone A or absolute alcohol were washed for 3 times with cooled Tris-buffered sucrose solution (250 mM sucrose, 10 mM Tris · Cl, pH 7.0), which was followed by incubation with hypotonic solution A [10 mM HEPES, 1 mM $MgCl_2$, 10 mM KCl, 0.4 mM dithiothreitol (DTT), pH 7.9] for 10 min on ice. Then, cell pellets were resuspended in solution A with 0.2% NP-40 and centrifuged (3,000 × g, 1 min) at 4°C. Afterwards, precipitates (nuclei and cellular membrane) were rinsed with cooled Tris-buffered sucrose solution for 3 times to remove excessive ions and were dissolved in lysis solution [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT, 40 mM Tris · Cl, 0.2% Bio-lyte (pH 3-10), 10% isopropanol, 12.5% water-saturated isobutanol] for 1 hour at room temperature. Finally, samples were collected and quantitated by protein assay kit (Bio-Rad, Hercules, CA, USA).

*Two-Dimensional Electrophoresis*

About 100 μg protein in 300 μl rehydration solution [8 M urea, 2% (w/v) CHAPS, 0.2% Bio-lyte (pH 3-10), 18 mM DTT and 0.002% bromophenol blue] was loaded onto 17 cm immorbilized pH gradient (IPG) strip with a non-linear range of pH 3-10 (Bio-Rad, Hercules, CA, USA) and underwent isoelectric focusing electrophoresis on a Protean IEF Cell system (Bio-Rad, Hercules, CA, USA) at 20°C with following schedules: 50 V for 12 hours, 200 V for 1 hour, 500 V for 1 hour, 1,000V for 2 hours, 10,000V for 2 hours and followed by 10,000V for 40,000 V · hours. The gel strip was then incubated sequentially in equilibration solutions using the same stock solution [6 M urea, 30% (v/v) glycerol, 2% SDS, 0.002% bromophenol blue and 50 mM Tris · Cl, pH 8.8] supplemented freshly with 1% (w/v) DTT (step I) and 4% (w/v) iodoacetamide (IAA) (step II) each for 15 min at room temperature with gentle shake, placed onto the top of 12.5% continuous vertical polyacrylamide gel, sealed with 0.5% (w/v) low melting point agarose (Promega, Madison, WI, USA) and performed the second dimensional SDS electrophoresis in PROTEAN II xi 2-D cell (Bio-Rad, Hercules, CA, USA) under 16 mA per gel for 30 minutes and then 32 mA per gel until the dye front reached the gel bottom.

*Silver Staining and Scanning of 2DE Gels*

The slab gels were rinsed with Milli-Q water, fixed in fixation fluid (40% methanol, 10% glacial acetic acid) overnight. Having been immerged into sensitizing solution [0.2% (w/v) sodium thiosulfate, 6.8% (w/v) anhydrous sodium acetate and 30% methanol] for 30 min, the gels were rinsed with Milli-Q water 5 min for 3 times, and were then incubated with 0.25% (w/v) silver nitrate for 20 min. Afterwards, the gels were treated with 2.5% (w/v) exsiccated sodium carbonate and 0.04% (v/v) formaldehyde. After protein spots on the gels were well developed, 1.46% (w/v) EDTA was used to end further reaction. The whole process of silver staining was performed under gentle shake. Three pairs of silver-stained gels were scanned with GS-800 calibrated imaging density meter (Bio-Rad, Hercules, CA, USA). Differential analysis was performed with the assistant of PDQuest Image Analysis Software (version 7.2, Bio-Rad, Hercules, CA, USA). Total density on each gel was used as loading control. Stably altered spots among gels were excised for further mass spectrometric (MS) identification.

*Mass Spectrometry Analysis*

Differential proteins were manually excised into ZipPlate micro-SPE plate (Millipore, Billerica, MA, USA) wells by spot and undergone in-gel tryptic digestion. Briefly, after washed in Milli-Q water for 3 times, gel pieces were incubated with silver destaining solution (30 mM potassium ferricyanide and 100 mM sodium thiosulfate mixed with equal volume) in the dark for 20 min at room temperature. Rinsed the gel pieces with Milli-Q water twice, incubated them in turn with 25 mM ammonium bicarbonate/5% acetonitrile, 25 mM ammonium bicarbonate/50% acetonitrile and pure acetonitrile till they were dehydrated, shrunk and whitened. Desiccated them with vacuum, then added 10 μl trypsin solution (Trypsin Gold, mass spectrometry grade, Promega, Madison, WI, USA, 10 ng/μl in 25 mM ammonium bicarbonate) per well and placed the plate in humidified incubator at 37°C overnight. Peptide fragments were extracted and desalted in turn with 0.2% trifluoroacetic acid. Centrifuged the plate at 1,750 × g for 15 min to elute the peptide fragment with 5 μl 50% acetonitrile/0.1% trifluoroacetic acid, and then vacuum dehydrated them. Resuspended the peptides with 2 μl matrix solution (10 mg/ml α-cyano-4-hydroxy cinnamic acid dissolved in 50% acetonitrile/ 0.1% trifluoroacetic acid), then spotted 0.7 μl/sample onto MALDI (matrix-assistant laser desorption ionization) target and submitted into 4700 proteomics analyzer (Applied Biosystems, Foster City, CA, USA) for acquiring MS and tandem MS data.

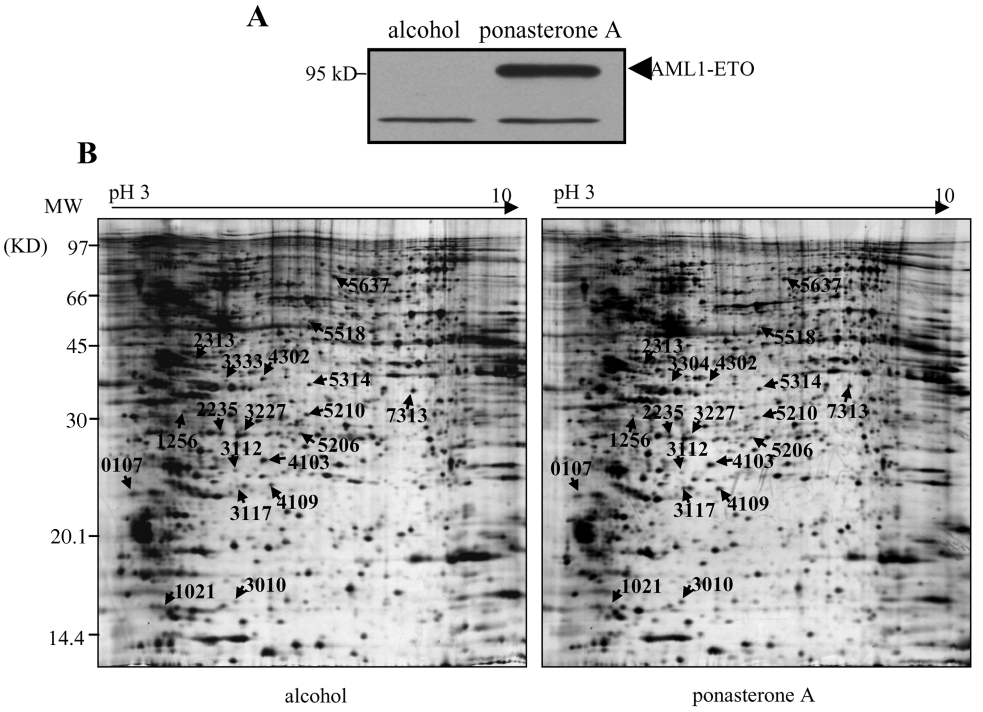


Fig. 1. The representative 2-DE maps of U937 cells with and without AML1-ETO expression. U937-A/E 9/14/18 cells were treated with 5 μM ponasterone A and absolute alcohol for 24 hours. AML1-ETO protein was detected by Western blot (A). Then, 100 μg of each protein samples as described in Materials and Methods was loaded onto corresponding IPG strip, followed by the second dimension electrophoresis on a continuous vertical polyacrylamide gel (12.5%). A pair of representative 2DE images by silver staining was shown (B). Significantly deregulated protein spots were labeled in numbers. All experiments were repeated for 3 times with the similar results.

*Database Searching*

Mass spectra data were imported into integrated GPS software (Global Protein Server, Applied Biosystems, Foster City, CA, USA) and were matched to records in NCBI protein non-redundant (Nr) database for identification with searching engine of MASCOT. Searching parameter of mass tolerance was limited to 100 ppm. The results from both the MS and MS/MS spectra were accepted as good identification when the GPS score confidence was higher than 95%.

*Western Blot*

Protein samples were equally loaded on 8% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane blot (Bio-Rad, Hercules, CA, USA) on mini gel apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk for 1 hour, and was probed with anti-ETO or anti-MAT1 antibody (both 1 : 1000, Santa Cruz Biotech, Santa Cruz, CA, USA) and corresponding horseradish peroxidase (HRP) linked secondary antibody (Dako Cytomation, Denmark). The signal was detected with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction.

*Statistic Analysis*

The volume of differential spots on corresponding gels was expressed as mean ± SD. The paired Student's *t* test was used for statistical analysis between two groups. Significant level was set at $P < 0.05$.

## Results

As reported previously (8, 12), ponasterone A treatment for 24 hours significantly induced AML1-ETO protein expression, while alcohol-treated cells did not (Fig. 1A). To identify AML1-ETO-modulated protein, equal amount of enriched nuclear extracts from these ponasterone A and alcohol-treated U937-A/E 9/14/18 cells were separated and compared by 2DE. Fig. 1B showed a pair of representative silver stained images in one experiment. The three independent experiments showed the similar 2DE patterns, indicating the good stability of our test system (Fig. 2, upper panels). As analyzed by PDQuest software, total 1159 ± 46 spots

were detected. With the spot volume, which was estimated and normalized by total density on the corresponding gel, significantly increased and decreased spots in AML1-ETO-expressing cells compared with alcohol-treated cells ($P < 0.05$) were judged as modulated (directly or indirectly) proteins. Nineteen out of such differential spots were found, among which 5 were up-regulated and 14 were down-regulated. An example was shown in Fig. 2, where spot 3117 was significantly up-regulated in ponasterone A treated U937-A/E 9/14/18 cells.

Then, these 19 differential spots were cut out for identification by MALDI-TOF- TOF mass spectrometry. The results demonstrated that 17 spots were successfully identified with MS and/or MS/MS analysis, in combination with NCBI Nr database searching. Fig. 3 gave a representative example for the identification of spot 3117. MAT1 (spot 5314, Fig. 4A), which was found down-regulated in the above analysis, was further validated by western-blot analysis (Fig. 4B), which showed that MAT1 was down-regulated significantly after ponasterone A treatment in a very similar manner to the alteration in 2DE. In addition, the time course of MAT1 expression demonstrated that its down-regulation was tightly followed the accumulation of AML1-ETO in U937A/E 9/14/18 cell line.

All identified spots were summarized in Table 1. They included 14 unique proteins, because spots 2235, 3227 and 5206, which were aligned in a horizontal manner on the gel image and decreased in very similar folds in AML1-ETO-expressing cells, were identified as splicing factor, arginine/serine-rich 9 (SFRS9) protein, and core-binding factor-beta (CBFβ) was also present on both spots 3117 and 4109, which were up- regulated in the similar folds. Notably, each of the experimental pI of spot 1021, 2235, 3227, 5210 and 5518 was far lower than their corresponding theoretical pI, which possibly indicated some post-translational modifications such as phosphorylation, happened to these proteins and resulted in the changed pI. All identified proteins are involved in a variety of cellular functions such as cell cycle, signal transduction, transcription and nucleotide metabolism.

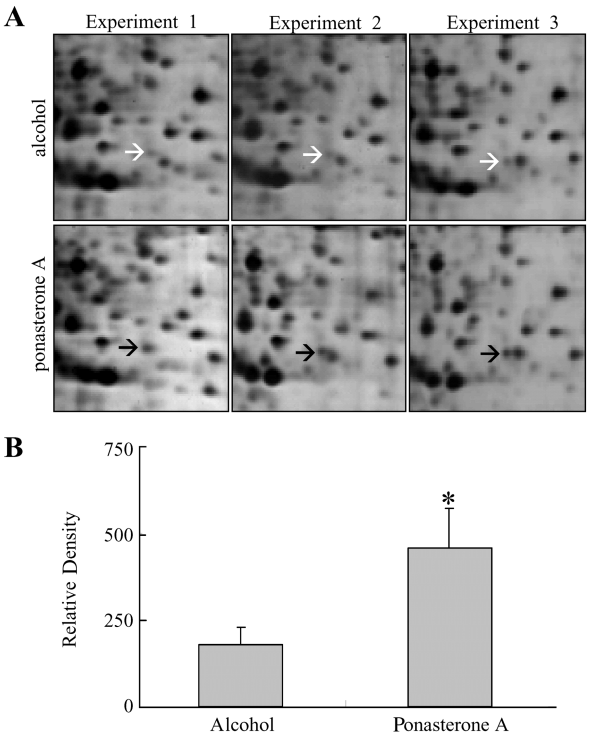


Fig. 2. A representative example for deregulated protein in AML1-ETO- expressing U937 cells. In each of three repeated experiments, spot 3117 was significantly up-regulated. The volume of the spot was shown in the lower panels, as indexed by mean ± SD of three independent experiments. ($P < 0.05$ *vs*. control)

## Discussion

In this work, we identified fourteen unique proteins that were responsive to AML1-ETO expression in leukemic cells. It is well known that CBFβ is a heterodimerizing partner of AML1 protein. In other words, it is essential for the transcriptional activity of AML1 where CBFβ is translocated into the nucleus and heterodimerizes with AML1 protein. Previously, it was shown that AML1-ETO protein is able to accumulate CBFβ in the nucleus more efficiently than wild-type AML1, probably because of the higher affinity of the chimeric protein for CBFβ than that of wild-type AML1 (26). Here, we also showed that there was increased CBFβ protein in the nucleus-enriched fraction of AML1-ETO-expressing U937 cells compared with AML1-ETO-minus U937 cells. The reproducible datum indicates the effectiveness of our proteomic analysis, although most proteins identified in this work remain to need further confirmation by one-to-one western blots. It is worthy noting that CBFβ protein was distributed on two spots with different pI (spot 3117 and 4109 respectively with pI 3.7 and pI 4.3, Table 1), which possibly suggests that CBFβ protein presents two different modified forms in the presence of over expression of AML1-ETO protein. As described above, it has been shown that AML1-ETO expression in leukemic cells also induces growth arrest at G1 phase (6). It is rational to speculate some complex mechanisms to be involved in this event, although effects of down-regulation of c-Myc and up-regulation of connexin-43 have been proposed (6).

As documented, the cyclin-dependent kinase (CDK) activating kinase (CAK) is composed of three subunits: CDK7, Cyclin H and MAT1 (menage a

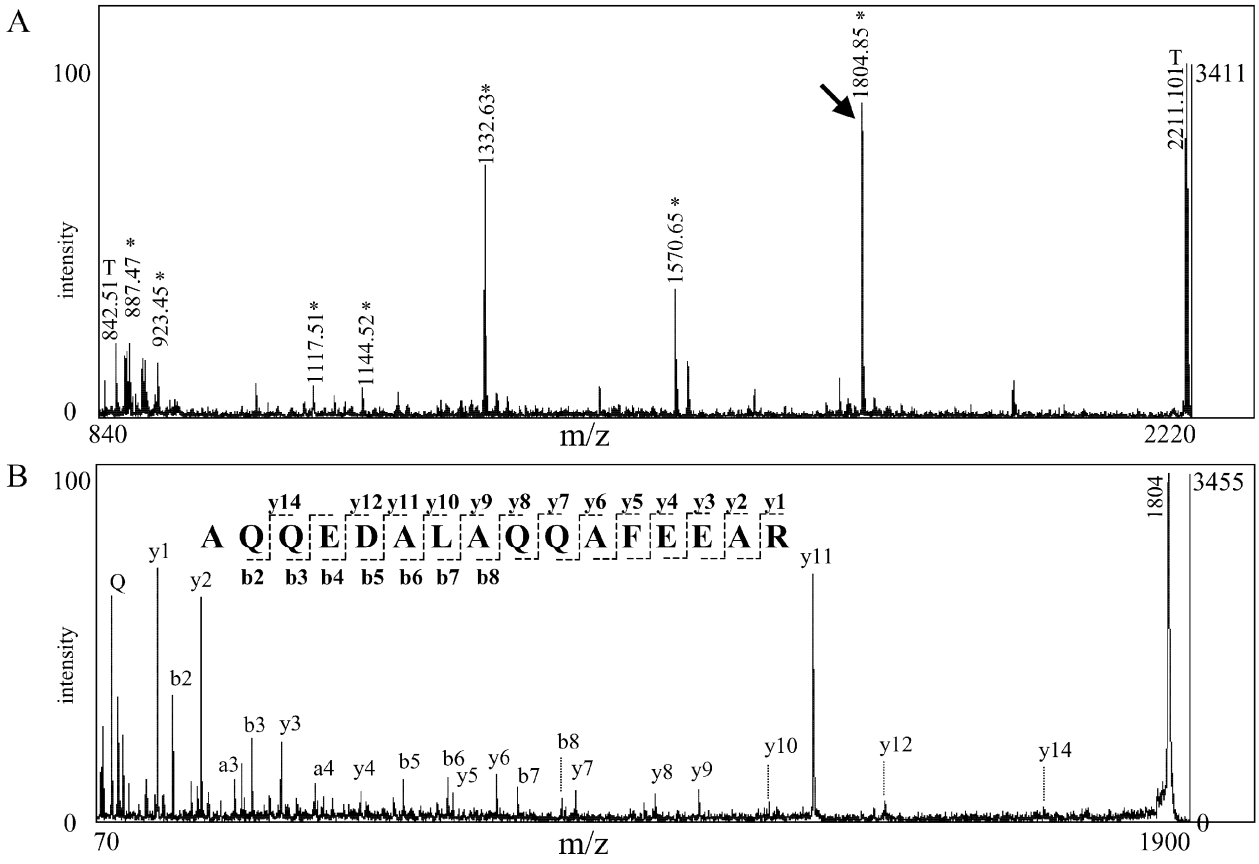


Fig. 3. Identification of spot 3117 by mass spectrometry. A. Peptide mass spectrum of spot 3117 with tryptic digestion. "T" and "*" respectively indicate trypsin autolytic peptides selected for internal calibration and peaks matched to CBFβ. The matched peptides occupy 47% sequence coverage and obtain 136 score. Arrow indicated ion at m/z = 1804.85 was selected for MALDI-TOF-TOF tandem MS analysis. B. Fragmentation spectrum for ion 1804.85. y-ions, b-ions as well as a-ions are denoted in the spectrum. The identified sequence is AQQEDALAQQAFEEAR interpreted as y and b series ions.

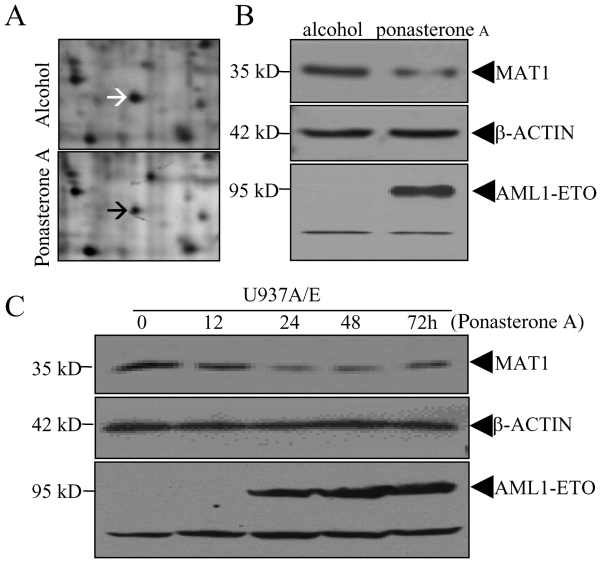


Fig. 4. The expression of MAT1 is decreased during the accumulation of AML1-ETO in U937A/E 9/14/18. A. MAT1 (spot 5314) decreased on 2D maps after treated with 5 μM ponasterone A for 24 hours. B. Western blot indicated that MAT1 down-regulated after the same treatment as A, with β-ACTIN serving as loading control. C. Time course of MAT1 with induced expression of AML1-ETO in U937A/E 9/14/18. When treated with 5 μM ponasterone A for different time as labeled, the expression of AML1-ETO was accumulated in U937A/E 9/14/18 cell line, while MAT1 gradually decreased.

**Table 1. The differential expressed proteins in U937 cells with and without AML1-ETO expression.**

| Spot No. | Protein | NCBI Accession No. | Mean fold P/C (n = 3) | pI (exptl. / theor.) | MW (exptl. / theor.) | PMF | | | PFF | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Peptide | Coverage | Score | Peptide | Score |
| 0107 | chain B, crystal structure of the human co-chaperone p23 | gi\|9257074 | 0.4 ± 0.2 | 3.0/5.09 | 19/15.1 | 6 | 64 | 187 | | |
| 1021 | hairy and enhancer of split 5 (Drosophila) | gi\|56204847 | 4.4 ± 0.8 | 3.2/9.52 | 16/18.3 | 5 | 47 | 110 | | |
| 1256 | unidentified | | 0.6 ± 0.1 | 3.4/- | 18.5/- | | | | | |
| 2235 | splicing factor, arginine/serine-rich 9 | gi\|4506903 | 0.6 ± 0.2 | 3.6/8.74 | 28/25.5 | 10 | 40 | 121 | 3 | 95 |
| 2313 | transformer-2 protein homolog (TRA-2 alpha) | gi\|9558733 | 2.8 ± 0.4 | 3.4/11.3 | 44/32.7 | | | | | |
| 3010 | unidentified | | 2.6 ± 0.8 | 4.0/- | 15/- | | | | | |
| 3112 | amyloid beta (A4) precursor protein-binding, family A, member 2 binding protein isoform 1 | gi\|38569413 | 0.7 ± 0.2 | 3.7/5.44 | 25/41.1 | 5 | 24 | 77 | | |
| 3117 | core-binding factor, beta subunit isoform 1 | gi\|13124881 | 2.3 ±0.9 | 3.7/5.47 | 23/22.0 | 8 | 47 | 136 | 2 | 127 |
| 3227 | splicing factor, arginine/serine-rich 9 | gi\|4506903 | 0.4 ± 0.2 | 4.2/8.74 | 27/25.6 | 10 | 46 | 73 | 2 | 53 |
| 3304 | mitogen-activated protein kinase organizer 1 | gi\|14150114 | 0.6 ±0.1 | 3.6/5.36 | 40/35.1 | 6 | 22 | 110 | | |
| 4103 | glutathione transferase (EC 2.5.1.18) | gi\|1575434 | 0.4 ±0.1 | 4.3/5.43 | 26/23.6 | 8 | 45 | 142 | 3 | 141 |
| 4109 | core-binding factor, beta subunit isoform 1 | gi\|13124881 | 2.1 ±0.9 | 4.3/5.47 | 23/22.0 | 13 | 50 | 148 | | |
| 4302 | inorganic pyrophosphatase (EC 3.6.1.1) | gi\|33150672 | 0.5 ± 0.2 | 4.3/5.54 | 38/33.1 | 14 | 59 | 179 | | |
| 5206 | splicing factor, arginine/serine-rich 9 | gi\|4506903 | 0.6 ±0.1 | 5.3/8.74 | 28/25.6 | 8 | 33 | 104 | 3 | 72 |
| 5210 | DUT protein | gi\|47123503 | 0.6 ± 0.2 | 5.5/9.46 | 30/26.8 | 5 | 28 | 99 | | |
| 5314 | menage a trois 1 (CAK assembly factor) | gi\|4505225 | 0.3 ±0.2 | 5.5/5.79 | 35/35.8 | 8 | 41 | 151 | 3 | 99 |
| 5518 | nucleolar protein NOP52 | gi\|4503247 | 0.5 ± 0.2 | 5.6/9.39 | 49/53.0 | 4 | 16 | 86 | | |
| 5637 | PKM2 protein | gi\|478822 | 0.6 ± 0.3 | 6.7/7.58 | 70/58.4 | 4 | 17 | 84 | | |
| 7313 | HuR RNA binding protein | gi\|1022961 | 0.7 ±0.2 | 8.4/9.23 | 40/36.2 | 9 | 27 | 115 | 2 | 40 |

The table lists the differential expressed proteins identified in ponasterone A treated (P) and absolute alcohol treated (C) U937, according to a software-aided spot volume ratio of these two treated cells. The experimental (exptl) isoelectric point (pI) and molecular weight (MW) are shown for each protein, together with theoretical (theor) pI and MW, which are derived from the amino acid sequences in NCBI. “peptide” means peptide counts matched in MS .

**Table 2. AML1 binding sites in the region of 5000 bp upstream of the first exon of identified protein-coded genes.**

| Spot No. | Protein | TGTGGT | TGCGGT |
|---|---|---|---|
| 0107 | chain b, crystal structure of the human co-chaperone p23 | 603 | |
| 2313 | transformer-2 protein homolog (TRA-2 alpha) | 410, 1747, 2139, 2316, 2644, 3572, 4860 | |
| 2235,3227& 5206 | splicing factor, arginine/serine-rich 9 | 1663, 3148, 3407 | 301 |
| 3117 | core-binding factor, beta subunit isoform 1 | 3500, 4780 | |
| 3304 | mitogen-activated protein kinase organizer 1 | 2066 | |
| 4103 | glutathione transferase (EC 2.5.1.18) | 2851, 3409, 3960, 4293, 4868 | 265 |
| 5210 | DUT protein | 3621 | 1108, 2639 |
| 7313 | HuR RNA binding protein | 1417, 3384 | |

trois-1). In this work, we showed that AML1-ETO expression significantly reduced MAT1 protein. Reduced MAT1 possibly causes the low activity of CAK, which is responsible for the activating phosphorylation of CDK1, CDK2, CDK4 and CDK6 and regulation of the cell cycle. Together with nine other subunits, furthermore, MAT1 also contributes to the formation of general transcription factor TFIIH, where it is involved in promoter clearance and progression of transcription from the preinitiation to the initiation stage (9, 28). Obviously, the role of MAT1 in AML1-ETO-induced growth arrest deserves further investigation.

We also found that mitogen-activated protein kinase organizer 1 (MORG1), a member of the WD-40 protein family that was isolated as a binding partner of the extracellular signal-regulated kinase (ERK) pathway scaffold protein MP1, was decreased when AML1-ETO over-expressed in U937 cells. More recently, MORG1 was reported to specifically associate with several components of the ERK pathway, including MP1, Raf-1, MEK, and ERK, and to facilitate ERK activation through stabilizing their assembly into an oligomeric complex (32). The ERK pathway phosphorylates AML1 and this ERK-induced phosphorylation is critical to AML1's normal transcriptional function (27). Moreover, phosphorylation of AML1 on specific serine/threonine residues also controls both transcriptional activity and rate of degradation (5). Based on the knowledge, we postulated that the decrease of MORG1 might reduce the activity of ERK and thus inhibit phosphorylational activation of AML1, which may become a way for the dominant-negative effect of AML1-ETO on wild type AML1 functions.

*HES* genes, members of the hairy and enhancer of split (*HES*) gene family encoding basic helix-loop-helix-type transcriptional repressors, are the immediate targets of NOTCH1. A wide role for NOTCH1 signaling has been shown in the pathogenesis of acute leukemia, especially T-acute lymphoblastic leukemia (T-ALL) (7). Here we showed that hairy and enhancer of split 5 (HES5) protein (spot 1021) was significantly up-regulated in AML1-ETO-expressing U937 cells. Given the role of HES5 in maintaining the renewal of neural stem cells, whether increased HES5 in AML1-ETO carrying cells contributes to stem cell-derived leukemogenesis may deserve close attention.

Finally, most out of 14 proteins we identified were found to be down-regulated in the enriched nuclear protein of U937-AE 9/14/18 with AML1-ETO expression, which is consistent with dominant-negative effects of AML1-ETO on transcriptional factor AML1, which binding to the DNA consensus sequence TGT/cGGT in the promoter regions of many target genes. Thus, we analyzed the sequence 5kb upstream to the first exon of 14 genes coding the altered proteins identified in this work. As shown in Table 2, 8 out of 14 deregulated proteins have at least one such site in their corresponding promoter regions. Whether these binding sites mediate their binding with AML1 and/or AML1-ETO remains to be illustrated in the future. In summary, with global proteomic analysis, we found some new potentially functional proteins, which were modulated in AML1-ETO expressing leukemic cells. Further knowledge about their roles in leukemic cells will provide new clues for better understanding of leukemogenetic effects exerted by AML1-ETO fusion protein.

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## References


1. Ahn, M.Y., Huang, G., Bae, S.C., Wee, H.J., Kim, W.Y. and Ito, Y. Negative regulation of granulocytic differentiation in the myeloid precursor cell line 32Dcl3 by ear-2, a mammalian homolog of Drosophila seven-up, and a chimeric leukemogenic gene, AML1/ETO. *Proc. Natl. Acad. Sci. USA*. 95: 1812-1817, 1998.
2. Alcalay, M., Meani, N., Gelmetti, V., Fantozzi, A., Fagioli, M., Orleth, A., Riganelli, D., Sebastiani, C., Cappelli, E., Casciari, C., Sciurpi, M.T., Mariano, A.R., Minardi, S.P., Luzi, L., Muller, H., Di Fiore, P.P., Frosina, G. and Pelicci, P.G. Acute myeloid leukemia fusion proteins deregulate genes involved in stem cell maintenance and DNA repair. *J. Clin. Invest*. 112: 1751-1761, 2003.
3. Amann, J.M., Nip, J., Strom, D.K., Lutterbach, B., Harada, H., Lenny, N., Downing, J.R., Meyers, S. and Hiebert, S.W. ETO, a target of t(8;21) in acute leukemia, makes distinct contacts with multiple histone deacetylases and binds mSin3A through its oligomerization domain. *Mol. Cell. Biol*. 21: 6470-6483, 2001.
4. Behre, G., Zhang, P., Zhang, D.E. and Tenen, D.G. Analysis of the modulation of transcriptional activity in myelopoiesis and leukemogenesis. *Methods* 17: 231-237, 1999.
5. Biggs, J.R., Zhang, Y., Peterson, L.F., Garcia, M., Zhang, D.E. and Kraft, A.S. Phosphorylation of AML1/RUNX1 regulates its degradation and nuclear matrix association. *Mol. Cancer Res*. 3: 391-401, 2005.
6. Burel, S.A., Harakawa, N., Zhou, L., Pabst, T., Tenen, D.G. and Zhang, D.E. Dichotomy of AML1-ETO functions: growth arrest versus block of differentiation. *Mol. Cell. Biol*. 21: 5577-5590, 2001.
7. Chiaramonte, R., Basile, A., Tassi, E., Calzavara, E., Cecchinato, V., Rossi, V., Biondi, A. and Comi, P. A wide role for NOTCH1 signaling in acute leukemia. *Cancer Lett*. 219: 113-120, 2005.
8. Fliegauf, M., Stock, M., Berg, T. and Lubbert, M. Williams-Beuren syndrome critical region-5/non-T-cell activation linker: a novel target gene of AML1/ETO. *Oncogene* 23: 9070-9081, 2004.
9. Giglia-Mari, G., Coin, F., Ranish, J.A., Hoogstraten, D., Theil, A., Wijgers, N., Jaspers, N.G., Raams, A., Argentini, M., van der Spek, P.J., Botta, E., Stefanini, M., Egly, J.M., Aebersold, R., Hoeijmakers, J.H. and Vermeulen, W. A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. *Nat. Genet.* 36: 714-719, 2004.
10. Heidenreich, O., Krauter, J., Riehle, H., Hadwiger, P., John, M., Heil, G., Vornlocher, H.P. and Nordheim, A. AML1/MTG8 oncogene suppression by small interfering RNAs supports myeloid differentiation of t(8;21)-positive leukemic cells. *Blood* 101: 3157-3163, 2003.
11. Hiebert, S.W., Lutterbach, B. and Amann, J. Role of co-repressors in transcriptional repression mediated by the t(8;21), t(16;21), t(12;21), and inv(16) fusion proteins. *Curr. Opin. Hematol*. 8: 197-200, 2001.
12. Jiang, Y., Xue, Z.H., Shen, W.Z., Du, K.M., Yan, H., Yu, Y., Peng, Z.G., Song, M.G., Tong, J.H., Chen, Z., Huang, Y., Lubbert, M. and Chen, G.Q. Desferrioxamine induces leukemic cell differentiation potentially by hypoxia-inducible factor-1 alpha that augments transcriptional activity of CCAAT/enhancer-binding protein-alpha. *Leukemia* 19: 1239-1247, 2005.
13. Kurokawa, M. and Hirai, H. Role of AML1/Runx1 in the pathogenesis of hematological malignancies. *Cancer Sci.* 94: 841-846, 2003.
14. Kurokawa, M., Tanaka, T., Tanaka, K., Ogawa, S., Mitani, K., Yazaki, Y. and Hirai, H. Overexpression of the AML1 proto-oncoprotein in NIH3T3 cells leads to neoplastic transformation depending on the DNA-binding and transactivational potencies. *Oncogene* 12: 883-892, 1996.
15. Look, A.T. Oncogenic transcription factors in the human acute leukemias. *Science* 278: 1059-1064, 1997.
16. Lutterbach, B., Westendorf, J.J., Linggi, B., Patten, A., Moniwa, M., Davie, J.R., Huynh, K.D., Bardwell, V.J., Lavinsky, R.M., Rosenfeld, M.G., Glass, C., Seto, E. and Hiebert, S.W. ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. *Mol. Cell. Biol*. 18: 7176-7184, 1998.
17. Mao, S., Frank, R.C., Zhang, J., Miyazaki, Y. and Nimer, S.D. Functional and physical interactions between AML1 proteins and an ETS protein, MEF: implications for the pathogenesis of t(8;21)-positive leukemias. *Mol. Cell. Biol*. 19: 3635-3644, 1999.
18. Mori, H., Colman, S.M., Xiao, Z., Ford, A.M., Healy, L.E., Donaldson, C., Hows, J.M., Navarrete, C. and Greaves, M. Chromosome translocations and covert leukemic clones are generated during normal fetal development. *Proc. Natl. Acad. Sci. USA*. 99: 8242-8247, 2002.
19. Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G. and Downing, J.R. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* 84: 321-330, 1996.
20. Peterson, L.F. and Zhang, D.E. The 8;21 translocation in leukemogenesis. *Oncogene* 23: 4255-4262, 2004.
21. Puccetti, E., Obradovic, D., Beissert, T., Bianchini, A., Washburn, B., Chiaradonna, F., Boehrer, S., Hoelzer, D., Ottmann, O.G., Pelicci, P.G., Nervi, C. and Ruthardt, M. AML-associated translocation products block vitamin D(3)-induced differentiation by sequestering the vitamin D(3) receptor. *Cancer Res*. 62: 7050-7058, 2002.
22. Rhoades, K.L., Hetherington, C.J., Rowley, J.D., Hiebert, S.W., Nucifora, G., Tenen, D.G. and Zhang, D.E. Synergistic up-regulation of the myeloid-specific promoter for the macrophage colony-stimulating factor receptor by AML1 and the t(8;21) fusion protein may contribute to leukemogenesis. *Proc. Natl. Acad. Sci. USA*. 93: 11895-11900, 1996.
23. Schwieger, M., Lohler, J., Friel, J., Scheller, M., Horak, I. and Stocking, C. AML1-ETO inhibits maturation of multiple lymphohematopoietic lineages and induces myeloblast transformation in synergy with ICSBP deficiency. *J. Exp. Med*. 196: 1227-1240, 2002.
24. Shimada, H., Ichikawa, H. and Ohki, M. Potential involvement of the AML1-MTG8 fusion protein in the granulocytic maturation characteristic of the t(8;21) acute myelogenous leukemia revealed by microarray analysis. *Leukemia* 16: 874-885, 2002.
25. Steffen, B., Serve, H., Berdel, W.E., Agrawal, S., Linggi, B., Buchner, T., Hiebert, S.W. and Muller-Tidow, C. Specific protein redirection as a transcriptional therapy approach for t(8;21) leukemia. *Proc. Natl. Acad. Sci. USA*. 100: 8448-8453, 2003.
26. Tanaka, K., Tanaka, T., Kurokawa, M., Imai, Y., Ogawa, S., Mitani, K., Yazaki, Y. and Hirai, H. The AML1/ETO(MTG8) and AML1/Evi-1 leukemia-associated chimeric oncoproteins accumulate PEBP2beta(CBFbeta) in the nucleus more efficiently than wild-type AML1. *Blood* 91: 1688-1699, 1998.
27. Tanaka, T., Kurokawa, M., Ueki, K., Tanaka, K., Imai, Y., Mitani, K., Okazaki, K., Sagata, N., Yazaki, Y., Shibata, Y., Kadowaki, T. and Hirai, H. The extracellular signal-regulated kinase pathway phosphorylates AML1, an acute myeloid leukemia gene product, and potentially regulates its transactivation ability. *Mol. Cell. Biol.* 16: 3967-3979, 1996.
28. Tassan, J.P., Jaquenoud, M., Fry, A.M., Frutiger, S., Hughes, G.J. and Nigg, E.A. *In vitro* assembly of a functional human CDK7-cyclin H complex requires MAT1, a novel 36 kDa RING finger protein. *EMBO. J.* 14: 5608-5617, 1995.
29. Tenen, D.G. Disruption of differentiation in human cancer: AML shows the way. *Nat. Rev. Cancer*. 3: 89-101, 2003.
30. Tenen, D.G., Hromas, R., Licht, J.D. and Zhang, D.E. Transcription factors, normal myeloid development, and leukemia. *Blood* 90: 489-519, 1997.
31. Vangala, R.K., Heiss-Neumann, M.S., Rangatia, J.S., Singh, S.M., Schoch, C., Tenen, D.G., Hiddemann, W. and Behre, G. The myeloid master regulator transcription factor PU.1 is inactivated by

AML1-ETO in t(8;21) myeloid leukemia. *Blood* 101: 270-277, 2003.

32. Vomastek, T., Schaeffer, H.J., Tarcsafalvi, A., Smolkin, M.E., Bissonette, E.A. and Weber, M.J. Modular construction of a signaling scaffold: MORG1 interacts with components of the ERK cascade and links ERK signaling to specific agonists. *Proc. Natl. Acad. Sci. USA.* 101: 6981-6986, 2004.
33. Wiemels, J.L., Xiao, Z., Buffler, P.A., Maia, A.T., Ma, X., Dicks, B.M., Smith, M.T., Zhang, L., Feusner, J., Wiencke, J., Pritchard-Jones, K., Kempski, H. and Greaves, M. In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. *Blood* 99: 3801-3805, 2002.