

# Significance of ETV6 rearrangement in acute promyelocytic leukemia with t(15;17)/promyelocytic leukemia/retinoic acid receptor alpha

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**Abstract.** Acute promyelocytic leukemia (APL) is a common subtype of acute myeloid leukemia in China. Since the application of arsenic trioxide and all-trans retinoic acid in the treatment of APL, the prognosis has greatly improved. However, ~20% of patients with APL relapse upon completing chemotherapy. Decreasing the relapse rate and incidence of early mortality may pose the greatest challenges for the future management of APL. Recently, Ets variant 6 (ETV6) was reported to be involved in a variety of translocations associated with hematological malignancies of myeloid and lymphoid origin. To date, little is known about the clinical implication of ETV6 rearrangement in APL. In the present study, ETV6 rearrangement was examined by split-signal fluorescence *in situ* hybridization in 258 adults with APL, and its association with the clinical features and outcomes of the patients was analyzed. The data suggested that ETV6 rearrangement may be an independent unfavorable prognostic factor for overall survival in APL patients.

## Introduction

Acute promyelocytic leukemia (APL) is a distinct subtype of myeloid leukemia with particular clinical and biological characteristics, including a high mortality rate in newly diagnosed patients and the presence of t(15;17)(q22;q12-21), a chromosome reciprocal translocation between the long arms of chromosomes 15 and 17 (1,2). APL is currently considered to be a highly curable disease. However, decreasing

early mortality (EM) prior or during induction therapy, and improvement of long-term stabilization and cure of APL patients remain major causes of treatment failure in APL (3). The current risk-stratification system for APL is mainly based on the white blood cell (WBC) and platelet counts (4). There are numerous reports about the prognostic impact of additional molecular biomarkers, including Fms-related tyrosine kinase 3 (FLT3), c-Kit and nucleophosmin, in APL (5,6). FLT3 mutations have been confirmed to be a poor prognostic molecular marker (5,7).

The Ets variant 6 (ETV6) gene, which is mapped to 12p13, is a transcription factor of the E26 transformation-specific (ETS) family that plays a versatile role in hematological malignancies (8). ETS family members are essential for hematopoietic processes, including cell proliferation, differentiation, migration and apoptosis, as well as tissue remodeling, angiogenesis and hematopoiesis (9). High expression of members of the ETS family of transcription factors is closely associated with poor disease-free survival and overall survival (OS) in various hematological malignancies (8). Chromosomal translocations involving the ETV6 gene have been identified in multiple hematological malignancies characterized by the fusion of ETV6 to different partner genes, including Abelson murine leukemia viral oncogene homolog 1 (ABL1), acute myeloid leukemia (AML)1, myelodysplasia syndrome (MSD)1, meningioma (disrupted in balanced translocation) 1, 1-aminocyclopropane-1-carboxylate synthase 2, Janus kinase 2, six-twelve leukemia and neurotrophic tyrosine kinase, receptor, type 3 (10-17).

The present study investigated the presence of ETV6 rearrangement in 258 patients with APL using split-signal fluorescence *in situ* hybridization (FISH), and explored its prognostic impact. The results identified abelson-related gene (ARG, also known as ABL2) as an ETV6 fusion partner by reverse transcription-polymerase chain reaction (RT-PCR) analysis in 1 case of APL. The present study is the second to report an APL patient with ETV6/ARG rearrangement, following the first case reported by Iijima *et al* (18). To the best of our knowledge, the present study is the first to address the prognostic implication of ETV6 involvement in patients with APL.

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**Key words:** acute promyelocytic leukemia, Ets variant 6 gene, split-signal FISH, rearrangement, prognosis

## Materials and methods

**Patients and samples.** The present study was based on data collected from 258 patients with newly diagnosed APL at Binzhou Medical University Hospital (Binzhou, China) from May 2000 to August 2011, who had complete clinical data and sufficient cryopreserved bone marrow samples for the study. The follow-up deadline was August 2014, with a median follow-up time of 89.5 months (range, 3-199 months). The cohort included 154 males and 104 females (median age, 36.88 years; range, 13-72 years). Diagnosis of APL was established according to the French-American-British Cooperative Group criteria (19) and World Health Organization classification (1). The bone marrow samples were collected at the time of diagnosis. A total of 30 normal marrow donors were also enrolled in the study for comparison purposes. All patients provided informed consent for the use of their laboratory data in the present study, which was approved by the ethics committee of Binzhou Medical University Hospital.

**Bone marrow cell culture and cytogenetic study.** Bone marrow specimens were acquired from patients in the absence of stimuli caused by drugs such as colony stimulating factor, and cultivated for 16-24 h prior to harvesting the cells. Bone marrow cell chromosomes were conventionally prepared and analyzed by R-banding (20). Karyotype abnormalities were identified and described according to the International System for Human Cytogenetic Nomenclature (1995) (21).

**Split-signal FISH analysis.** Split-signal FISH analysis was applied to the chromosome samples of the aforementioned 258 APL patients, according to the manufacturer's protocol. Briefly, bacteria artificial chromosome (BAC) clones (RP11-434C1 and RP11-525I3) containing the ETV6 gene (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were amplified by PCR (15), and DNA was extracted using a plasmid DNA extraction kit (Qiagen GmbH, Hilden, Germany). Selected BAC sequences on either side of ETV6 were used as probes, and labeled with DIG-Nick Translation Mix (Roche Diagnostics, Basel, Switzerland) and Biotin-Nick Translation Mix (Roche Diagnostics). The labeled probes (termed DIG525I23 and Bio407P10, respectively) were then purified with Quick Spin Columns (Roche Diagnostics), and produced red and green fluorescence signals, respectively, under a fluorescence microscope (Axio Imager.A1; Zeiss GmbH, Jena, Germany). All subsequent hybridization procedures were performed as previously described (15).

**Flow cytometry immunophenotyping.** Of the 258 patients with APL, 228 bone marrow samples were sent to Guangzhou Jinyu Medical Science Inspection Center (Guangzhou, China) for flow cytometry immunophenotyping analysis, while the remaining samples were analyzed at the Central Laboratory of Binzhou Medical University Hospital. Bone marrow samples from APL patients were collected at the time of diagnosis in tubes containing heparin (Taixing Biological Chemical Co., Ltd., Shijiazhuang, China) to avoid coagulation. Flow cytometry analysis of the bone marrow specimens was performed with a flow cytometer (FACSCalibur, BD Biosciences, Franklin

Lakes, USA), according to standard immunofluorescence methods (22). Briefly, fluorescein and phycoerythrin-labeled mouse anti-human monoclonal antibodies (LSBio; LifeSpan Biosciences, Inc., Seattle, WA, USA) against myeloperoxidase (MPO), cluster of differentiation (CD)33, CD13, CD117, CD34 and human leukocyte antigen-antigen D related (HLA-DR) (5-10  $\mu$ l) were mixed with heparin-anticoagulated bone marrow samples (~50  $\mu$ l) and incubated at 4°C for 30 min, prior to the addition of 2 ml cell lysis solution (Shanghai Weiao Biotech Ltd., Shanghai, China). The mixture was placed at room temperature for 10 min upon being subjected to vibration, and then washed with distilled water and phosphate-buffered saline (PBS). Next, 0.5 ml PBS was added to the samples, which were subsequently analyzed by flow cytometry.

**RT-PCR.** The ETV6/ARG fusion gene was detected by RT-PCR in patients with ETV6 rearrangement. Total RNA was extracted from mononuclear cells isolated from bone marrow samples of patients with APL using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Complementary DNA was synthesized by RT using a reaction mixture that consisted of total RNA, random hexamer primer, 5X RT buffer, deoxyribonucleotide triphosphates (provided in the RT-PCR kit; Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) and M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) on a PCR system. Nested PCR was performed for amplification of the ETV6/ARG fusion gene, with specific forward primers for ETV6 (F1 5'-ATCGGGAAGACCTGGCTTACA-3' and F2 5'-TGAAGAGCACGCCATGCCCATG-3') and specific reverse primers for ARG (R1 5'-TGCCTGGGGTTCAACATCAC-3' and R2 5'-TA-CTGCCTCCAGTCTTGTCT-3'). In the first step of the reaction, the above primers, which were used as outer primers, may bind to alternative, similar primer binding sites, potentially resulting in multiple products; however, only one of those would carry the intended sequence. In the second step of the reaction, inter primers were used, which were designed according to the sequences of the preceding primers. The inter primers (ETV6 F1 5'-GGGAAGACCTGGCTT-3' and F2 5'-AGAGCACGCCATGCCCA-3'; and ARG R1 5'-CTGGGGTTCAACAT-3' and R2 5'-TGCCTCCAGTCTTG-3') were shorter than the outer primers. Primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China), and the reaction was conducted in a 9700 Applied Biosystems thermocycler (Thermo Fisher Scientific, Inc.), using the following cycling conditions: Incubation at 94°C for 4 min, followed by 36 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and polymerization at 72°C for 45 sec. In the second step, the PCR products from the first reaction were subjected to a second PCR with a different set of primers, using the following cycling conditions: Incubation at 95°C for 2 min, followed by 36 cycles of denaturation at 95°C for 45 sec, annealing at 60°C for 45 sec and polymerization at 72°C for 30 sec. The amplified products were subjected to electrophoresis in 1.5% agarose gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing ethidium bromide (Sangon Biotech Co., Ltd.), and visualized under ultraviolet light in a gel documentation system (Invitrogen; Thermo Fisher Scientific, Inc.). The electrophoresis equipment, buffers and markers used were obtained from Invitrogen (Thermo Fisher Scientific, Inc.).

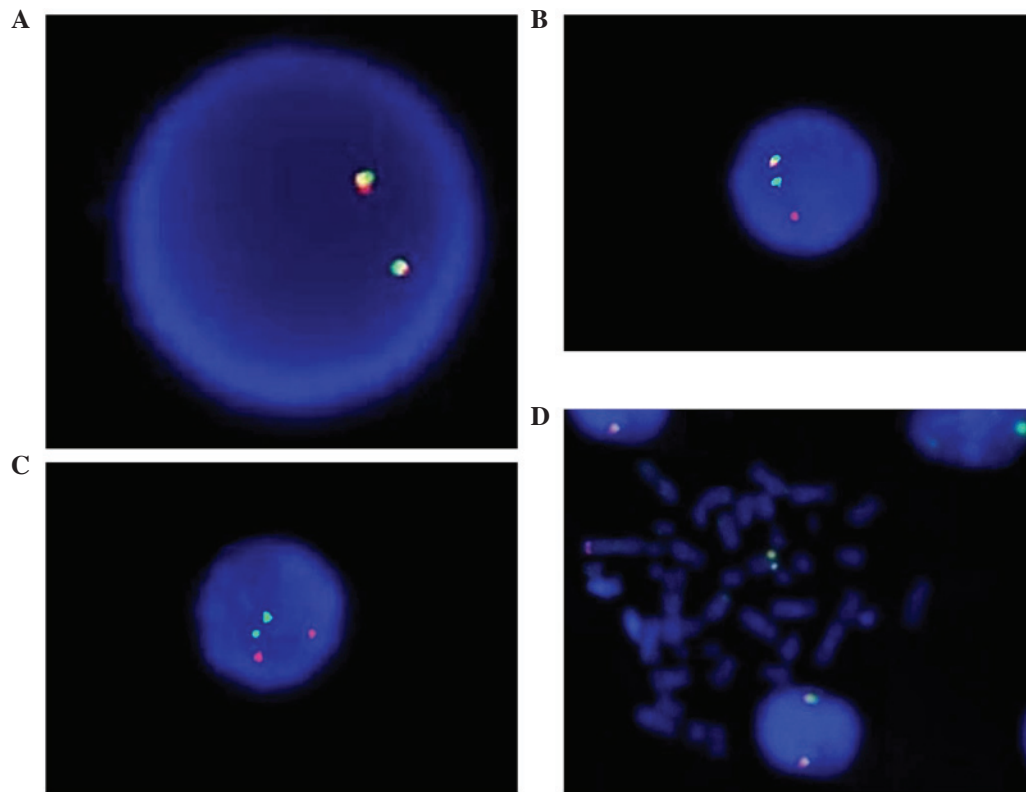


Figure 1. Split-signal fluorescence *in situ* hybridization analysis of ETV6 rearrangement in patients with acute promyelocytic leukemia. Red and green fluorescence signals correspond to the labeled probes DIG525I23 and Bio407P10, respectively. (A) Red and green fluorescence signal co-localization suggested no ETV6 rearrangement. (B) Separation of one red and one green fluorescence signal suggested ETV6 rearrangement. (C) Separation of both red and green signals suggested ETV6 rearrangement on homologous chromosomes. (D) Metaphase chromosomes were clearly recognizable. Both chromosomes 12p and 1q exhibited fluorescence signals, suggesting that there was translocation on homologous chromosomes between 12p and 1q (magnification, x1,000). ETV6, Ets variant 6.

**Statistical analyses.** APL samples were divided into two groups, namely ETV6 rearrangement-positive and ETV6 rearrangement-negative groups. Continuous and discrete patients' variables were compared using Mann-Whitney U test or Wilcoxon rank sum test and  $\chi^2$  test, respectively.  $P < 0.05$  was considered to indicate a statistically significant difference. OS was measured from the date of first diagnosis to mortality from any cause or last follow-up. Relapse was defined as a reappearance of t(15;17)/promyelocytic leukemia/retinoic acid receptor alpha. Patients who succumbed to the disease within 15 days of diagnosis were defined as early mortality (EM). To exclude confounding influence of different treatment regimens, all patients were included in the analysis of the association between ETV6 expression and clinical characteristics. By contrast, only those patients receiving conventional standard chemotherapy [daunorubicin (Zhejiang Haizheng Pharmaceutical Co. Ltd. (Zhejiang, China); 40 mg/m<sup>2</sup>/day on days 1-3; and cytarabine (Zhejiang Haizheng Pharmaceutical Co. Ltd.); 100 mg/m<sup>2</sup>/day on days 1-7] were included in the analyses of treatment effects and survival rates. Kaplan-Meier estimation was adopted to plot the corresponding survival curves, and log-rank tests were used to examine the differences between groups. Cox proportional hazards regression models were used to determine independent risk factors associated with survival in multivariate analyses. All statistical analyses were conducted with SPSS version 16 software (SPSS, Inc., Chicago, IL, USA).

## Results

**Identification of ETV6 rearrangement.** ETV6 rearrangements were detected in 8/258 (3.10%) cases by split-signal FISH analysis (Fig. 1). Of these, 1 case exhibited abnormal karyotype with 46,XY,t(15;17)(q22;q21)t(1;12)(q25;p12). However, traditional cytogenetic analysis did not identify any ETV6 rearrangement, with the exception of t(15;17). ETV6 rearrangement was not detected in any of the 30 healthy controls.

**Correlation of ETV6 rearrangement with clinical features and laboratory data in the cohort.** All patients with ETV6 rearrangement displayed higher WBC counts than patients who were negative for ETV6 rearrangement ( $P < 0.001$ ). The mean age of the patients was significantly different between the two groups ( $P = 0.017$ ). Compared with patients without ETV6 rearrangement, those with ETV6 rearrangement exhibited significantly more extramedullary infiltration. Gender ratio, platelet counts and hemoglobin levels did not differ significantly between the different subgroups ( $P > 0.05$ ). The expression of CD34, CD117, MPO and HLA-DR was higher in patients with ETV6 rearrangement than in ETV6 rearrangement-negative patients ( $P < 0.05$ ). The comparison of the clinical characteristics and laboratory data of the patients with positive and negative ETV6 rearrangement is presented in Table I.

Table I. Comparison of clinical features and clinical outcomes between Ets variant 6 gene rearrangement-positive and negative groups.

Variable	Total (n=258)	Positive group <sup>a</sup> (n=8)	Negative group <sup>a</sup> (n=250)	P-value
Gender, n (%)				0.869
Male	154	5 (3.25%)	149 (96.75%)	
Female	104	3 (2.88%)	101 (97.12%)	
Age, years (range)	36.88 (13-72)	45.63±5.38	36.60±0.65	0.017
Clinical manifestation				
Hepatosplenomegaly	52 (20.16%)	5 (62.50%)	47 (18.80%)	0.010
Lymphadenectasis	11 (4.26%)	2 (25.00%)	9 (3.60%)	0.039
Laboratory data (normal range)				
WBC count, cells/ $\mu$ l	4,349 (480-38,010)	6,540±1,362	4,279±313	0.027
Hemoglobin, g/dl	8.01 (4.40-13.10)	6.75±0.48	7.86±0.11	0.074
Platelet count, 10 <sup>3</sup> cells/ $\mu$ l	32.42 (3.00-152.00)	22.13±4.20	32.77±1.53	0.217
CD34, %	3.03 (0.00-80.00)	7.00±4.88	2.90±0.60	0.004
CD117, %	7.58 (0.00-80.00)	15.62±4.76	7.32±0.74	0.012
MPO, %	22.31 (0.00-80.00)	41.25±9.53	21.70±1.23	0.030
CD13, %	75.33 (5.00-100.00)	71.25±7.18	75.46±1.35	0.452
CD33, %	91.98 (60.00-100.00)	91.25±2.95	92.00±0.64	0.595
HLA-DR, %	2.44 (0.00-80.00)	10.62±6.16	2.18±0.46	0.018
Early mortality, n (%)	20 (7.75)	1 (12.50)	19 (7.60)	>0.999
CR rate, n (%) <sup>b</sup>	167 (88.83)	5 (71.43)	162 (89.50)	0.380

<sup>a</sup>Data are presented as the mean  $\pm$  standard deviation. <sup>b</sup>Among patients who received conventional standard chemotherapy. WBC, white blood cell; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-antigen D related; MPO, myeloperoxidase; CR, complete remission.

Table II. Multivariate analysis (Cox proportional hazards regression model) of overall survival in patients with acute promyelocytic leukemia.

Variables	Wald $\chi^2$	Exponential coefficient ( $\beta$ )	95% CI for $\beta$	P-value
WBC count <sup>a</sup>	10.081	1.127	1.047-1.213	0.001
Platelet count <sup>a</sup>	5.454	0.980	0.963-0.967	0.020
ETV6 rearrangement <sup>a</sup>	4.068	0.381	0.149-0.973	0.044
Hemoglobin levels	-	-	-	0.077
Age	-	-	-	0.113

<sup>a</sup>Only variables of those patients receiving all trans-retinoic acid plus conventional standard chemotherapy were incorporated into the multivariate Cox proportional hazards regression analysis. CI, confidence interval; WBC, white blood cell; ETV6, Ets variant 6.

*Effect of ETV6 rearrangement on the response to therapy and prognosis.* The 258 APL patients were immediately treated with all-trans retinoic acid [ATRA; Shandong Liangfu Pharmaceutical Co., Ltd. (Shandong, China); 30 mg/m<sup>2</sup>/day orally from day 1 until complete remission (CR)] as induction therapy if APL was suspected, and the treatment lasted until achievement of CR. Conventional standard chemotherapy with daunorubicin and cytarabine, in addition to arsenic trioxide [As<sub>2</sub>O<sub>3</sub>; Beijing Shuanglu Pharmaceutical Co., Ltd. (Beijing, China); 10 mg/day, intravenous drip on days 1-8], were administered as soon as ATRA had exerted an effect. With the exception of 1 case, who succumbed to

disease within 15 days (EM), the other 7 patients with ETV6 rearrangement received traditional standard chemotherapy based on ATRA, of whom, 5 cases experienced CR. In the ETV6 rearrangement-negative group, 19 patients succumbed to disease within 15 days (EM) and 181 cases received traditional standard chemotherapy, including 162 cases of CR. There was no obvious difference in EM rates between the two groups (12.5 vs. 7.6%;  $P>0.999$ ). CR rates did not exhibit significant differences between the two groups receiving standard chemotherapy or ATRA (71.43 vs. 89.50%;  $P=0.380$ ). However, it is worth mentioning that 4 patients with ETV6 rearrangement relapsed within 1 year following CR. The



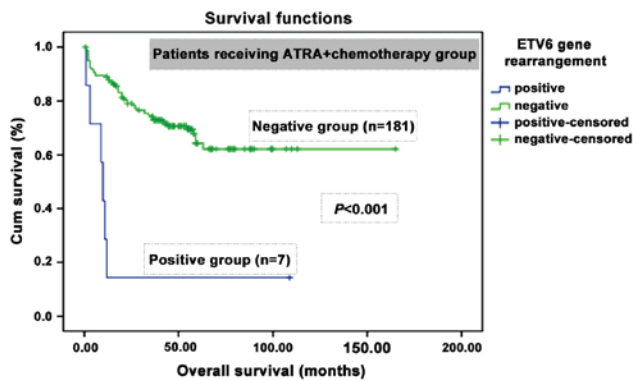


Figure 2. Kaplan-Meier estimates for ETV6 rearrangement-positive and negative groups. Median survival times were ~10 and >200 months for ETV6 rearrangement-positive and negative patients, respectively. Log-rank test revealed that there was a significant difference between the two survival curves, and the survival rate of the group with ETV6 rearrangement was remarkably lower than that of the group without ETV6 rearrangement ( $\chi^2=21.67$ ,  $P<0.001$ ). Cum, cumulative; ATRA, all trans-retinoic acid.

relapse rate within 1 year was markedly different between the two groups once CR was achieved (80.00 vs. 11.11%;  $P<0.001$ ). The association between ETV6 rearrangement and clinical outcome is presented in Table I. The Kaplan-Meier curve for OS revealed that the patients with ETV6 rearrangement had a significant shorter OS ( $P<0.001$ ) than those without ETV6 rearrangement after a median follow-up time of 89.5 months (Fig. 2). Cox proportional hazards regression models revealed that WBC and platelet counts, in addition to ETV6 rearrangement, were independent prognostic factors for OS in APL patients (Table II). These findings indicated that ETV6 rearrangement was an independent unfavorable prognostic factor for APL patients ( $P=0.044$ ).

**Identification of ETV6/ARG fusion gene by RT-PCR and clinical implication.** The fusion partner of ETV, ARG, was identified by RT-PCR in 1 of 8 cases. RT-PCR detected ETV6/ARG fusion gene products, 2 of which were the result of an alternative splicing event in the ARG gene (Fig. 3A). ETV6/ARG fusion gene products were not detected in the other 7 cases or healthy donors. The patient exhibiting ETV6/ARG fusion gene was a 56-year-old man, who was hospitalized at the Department of Hematology of Binzhou Medical University Hospital for 'fever, weakness' in August 2009. Physical examination on admission revealed multiple superficial lymphadenopathies and hepatosplenomegaly. Blood assay demonstrated markedly increased leukocytes levels, including WBC count of 14,860 cells/ $\mu$ l (normal range, 4,000-10,000 cells/ $\mu$ l), hemoglobin levels of 10.8 g/dl (normal range, 11.0-15.0 g/dl) and platelet count of  $5 \times 10^3$  cells/ $\mu$ l (normal range, 100-300 cells/ $\mu$ l), which was accompanied by 40% abnormal promyelocytes and an obviously abnormal coagulation index, including a prothrombin time of 15.6 sec (normal range, 10.0-14.0 sec), fibrinogen content of 0.8 g/l (normal range, 2.0-4.0 g/l) and D-dimer content of 3.7 mg/l (normal range, 0.0-0.7 mg/l). with 40% abnormal promyelocytes and an obviously abnormal coagulation index. Marrow aspiration disclosed that blasts comprised 95% of the myeloid cells, and the blasts were of median size and full of fine particles in the cytoplasm. Flow

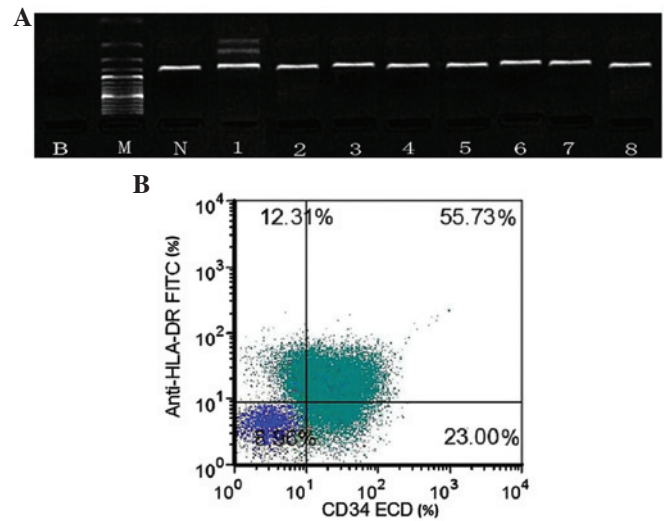


Figure 3. (A) Reverse transcription polymerase chain reaction analysis of ETV6/ARG fusion products. Patient samples (1-8) exhibited ETV6 rearrangement. In case 1, two specific bands corresponding to different ETV6/ARG fusion products were detected, which were the result of an alternative splicing event in the ARG gene. (B) Flow cytometry analysis of bone marrow specimens revealed abnormal promyelocytic cells positive for cluster of differentiation 34 and human leukocyte antigen-antigen D related. B, blank control using double distilled H<sub>2</sub>O; M, DNA marker DL2000; N, normal healthy donor; HLA-DR, human leukocyte antigen-antigen D related FITC, fluorescein isothiocyanate; CD, cluster of differentiation; ECD, electron coupled dye; ETV6, Ets variant 6; ARG, abelson-related gene.

cytometry analysis of the bone marrow revealed the following cellular characteristics: CD34<sup>+</sup>, HLA-DR<sup>+</sup> (Fig. 3B), CD33<sup>+</sup>, CD13<sup>+</sup> and CD117<sup>+</sup>. Cytogenetic analysis revealed an abnormal karyotype with 46,XY,t(15;17)(q22;q21)t(1;12)(q25;p12) by FISH. Diagnosis of M3v AML was considered. The patient was treated with ATRA upon diagnosis, while hydroxycarbamide [Shandong Qilu King-Phar Pharmaceutical Co., Ltd. (Shandong, China); 2 g/day, orally from day 1 until WBC count <10,000 cells/ $\mu$ l] and dexamethasone [Shanghai Tongyong Pharmaceutical Co., Ltd. (Shanghai, China); 5 mg/day, intravenous drip on days 1-10]. were used to prevent differentiation syndrome. However, his WBC count rapidly increased following oral administration of the drugs, and the patient succumbed to hematencephalon within 10 days of admission to hospital.

## Discussion

The ETV6 gene (also known as TEL), which is located on chromosome 12p13 and possesses a total length of 300 kb, encoding for 452 amino acids, is a member of the ETS family of transcription regulators, and is important in hematopoietic processes and hematological malignancies (23). ETV6 participates in the regulation of cell growth and differentiation, and stimulates erythroid differentiation of murine erythroid leukemia cells (24). Previous studies reported that chimeric mice with ETV6 knockout embryonic stem cells exhibited defective hematopoiesis within the first week of birth (25). ETV6 contains an ETS DNA-binding domain near its carboxy-terminus and a helix-loop-helix (HLH) domain near its amino-terminus, which mediates homotypic or heterotypic oligomerization with other ETV6 molecules or

transcription factors, respectively (23,26). Fusions between ETV6 and a number of different partner genes, mainly those coding for tyrosine kinases or transcription factors that are important for the initiation, progress and prognosis of disease, have been observed in various hematological malignancies (11-13). However, little is known about its prognostic implication in APL.

APL is the most common type of adult leukemia, and is currently considered to be a highly curable disease (4). Since the application of As<sub>2</sub>O<sub>3</sub> and ATRA in the treatment of APL, the prognosis has improved greatly (27). However, improving the relapse rate and EM incidence remain the greatest challenges for the future management of APL (28,29). The current risk-stratification system used for APL is mainly based on the WBC and platelet counts (4). Previous studies have indicated that certain chromosome abnormalities may be considered as important indicators for prognosis (30). Novel molecular biomarkers may aid to improve the risk stratification of APL and to identify those patients with a particularly poor prognosis (31,32). In the present study, a high expression of myeloid blast-associated antigens was identified in the ETV6 rearrangement-positive group, whereas the levels of promyelocyte-specific antigens were not significantly different between the ETV6 rearrangement-positive and the ETV6 rearrangement-negative groups. Although the EM and CR rates between the two groups were not significantly different, the rate of relapse within 1 year was significantly higher in the ETV6 rearrangement-positive group than in the ETV6 rearrangement-negative group. Therefore, ETV6 rearrangement expression in the bone marrow may serve as a novel prognostic biomarker for risk stratification in patients newly diagnosed with APL.

The occurrence of ETV6 rearrangement is common in childhood lymphocytic malignancies (33); however, only a few rare or sporadic case reports exist concerning adult AML (34,35). The expression of ETV6 mutations in myeloid malignancies has been widely reported (36,37). However, little is known about the clinical significance of ETV6 rearrangement in APL. In the present study, ETV6 rearrangement was detected in a large cohort of 258 APL patients, of which 8 cases were positive for ETV6 rearrangement. ETV6 rearrangement is an independent unfavorable prognostic factor for OS in APL, which is secondary only to the white blood cell and platelet counts. Despite the lower overall expression rate of ETV6 rearrangement in APL, compared with other leukemia subtypes such as acute lymphoblastic leukemia (ALL), MDS or non-M3 AML, ETV6 fusion was highly expressed (5/57 patients, 8.77%) in those patients with a WBC >5,000 cells/ $\mu$ l. The detection of ETV6 mutation is important in patients with a WBC >5,000 cells/ $\mu$ l, since the loss of tumor suppressor activity is possibly the pathogenic mechanism of the mutation. ETV6 rearrangement may inhibit the growth and normal differentiation of hematopoietic progenitor cells; thus, cells remain in a primitive stage (38).

The present study also confirmed the existence of ETV6/ARG in 1 of 8 APL patients. The patient, who exhibited a high leukocyte count, significant extramedullary infiltration features and was not sensitive to chemotherapy, succumbed to cerebral hemorrhage shortly after diagnosis. The present study is the second report of an APL patient with ETV6/ARG

rearrangement. Previously, Griesinger *et al* (39) reported ETV6/ARG rearrangement in a case of T-cell ALL. The ARG gene is a non-receptor tyrosine kinase that, together with ABL1, is characterized by a high homology with ABL1 in its protein tyrosine kinase (PTK), Src homology (SH)2 and SH3 domains (18). Aberrant activation of ABL and ARG downstream of several oncogenic growth factor receptors are associated with cancer progression, metastasis, drug resistance and poor prognosis (40). Imatinib, as a tyrosine kinase inhibitor specifically targeting kinases of the ABL family, is able to inhibit cell growth via inhibition of ARG in the APL cell line HT93A (41). As an oncogene, the ETV6/ARG fusion gene may depend on the oncogenic activity of chimeric PTK proteins by forming HLH domain-dependent homo-oligomers that result in the activation of tyrosine kinases in a ligand-independent manner (8). The ETV6/ARG fusion protein may induce progenitor cells to differentiate into myeloid cells rather than lymphoid cells (42). A previous *in vitro* study demonstrated that the ETV6/ARG oncoprotein contributes to autonomous cell growth through activating phosphorylated signal transducer and activator of transcription, leading to the upregulation of c-Myc oncogene expression (43). The present authors have also detected ETV6 rearrangements in other hematological malignancies such as AML (non M3), lymphadenoma and multiple myeloma by split-signal FISH (44-47).

In conclusion, ETV6 rearrangement is a reproducible event in hematological malignancies, which has been detected by different investigators under different conditions in various hematological malignancies, and is closely associated with poor patient prognosis. Thus, ETV6 rearrangement may become a molecular target in the treatment of hematological malignancies. In future studies, gene sequencing, transfection, RNA interference technology and animal models could be applied to reveal the pathogenic mechanism of ETV6 gene rearrangement.

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## References

1. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J and Vardiman JW (eds): WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Vol 2. 4th edition. IARC Press, Lyon, pp112-114, 2008.
2. Tallman MS and Kwaan HC: Reassessing the hemostatic disorder associated with acute promyelocytic leukemia. *Blood* 79: 543-553, 1992.
3. Cull EH and Altman JK: Contemporary treatment of APL. *Curr Hematol Malig Rep* 9: 193-201, 2014.
4. Jun M; Chinese Society of Hematology, Chinese Medical Association & Chinese Society of Hematologist, Chinese Medical Doctor Association: Guidelines for diagnosis and treatment of acute promyelocytic leukemia (2014). *Zhonghua Xue Ye Xue Za Zhi* 35: 475-477, 2014 (In Chinese).
5. Schnittger S, Bacher U, Haferlach C, Kern W, Alpermann T and Haferlach T: Clinical impact of FLT3 mutation load in acute promyelocytic leukemia with t(15;17)/PML-RARA. *Haematologica* 96: 1799-1807, 2011.

6. Yin J, Sun AN, Tian XP, Tian H, Wang RX, Yang Z, Wang XL, Wu DP, Qiu HY, Pan JL, *et al*: Clinical significance of common leukemia gene mutations in patients with acute promyelocytic leukemia. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 21: 39-44, 2013 (In Chinese).
7. Liu J, Zou XL, Liu TT, Jiang M and Niu T: FLT3 mutation in acute promyelocytic leukemia patients with extramedullary relapse. *Sichuan Da Xue Xue Bao Yi Xue Ban* (45): 670-674, 2014 (In Chinese).
8. Maki K, Arai H, Waga K, Sasaki K, Nakamura F, Imai Y, Kurokawa M, Hirai H and Mitani K: Leukemia-related transcription factor TEL is negatively regulated through extracellular signal-regulated kinase-induced phosphorylation. *Mol Cell Biol* 24: 3227-3237, 2004.
9. Akagi T, Kuure S, Uranishi K, Koide H, Costantini F and Yokota T: ETS-related transcription factors ETV4 and ETV5 are involved in proliferation and induction of differentiation-associated genes in embryonic stem (ES) cells. *J Biol Chem* 290: 22460-22473, 2015.
10. Yamamoto K, Yakushijin K, Nakamachi Y, Miyata Y, Sanada Y, Tanaka Y, Okamura A, Kawano S, Hayashi Y, Matsuoka H and Minami H: Extramedullary T-lymphoid blast crisis of an ETV6/ABL1-positive myeloproliferative neoplasm with t(9;12)(q34;p13) and t(7;14)(p13;q11.2). *Ann Hematol* 93: 1435-1438, 2014.
11. Cuneo A, Agostini P, Vitale A, Foà R and Castoldi G: Frequency of ETV6/AML1 fusion in adult acute lymphoblastic leukemia. *Leukemia* 17: 476-477, 2003.
12. Achkar WA, Aljapawe A, Liehr T and Wafa A: *De novo* acute myeloid leukemia subtype-M4 with initial trisomy 8 and later acquired t(3;12)(q26;p12) leading to ETV6/MDS1/EVI1 fusion transcript expression: A case report. *Oncol Lett* 7: 787-790, 2014.
13. Nofrini V, Berchicci L, La Starza R, Gorello P, Di Giacomo D, Arcioni F, Pierini V, Crescenzi B, Romoli S, Matteucci C and Mecucci C: MN1-ETV6 fusion gene arising from MDS with 5q-. *Leuk Res* 35: e123-e126, 2011.
14. Yagasaki F, Jinnai I, Yoshida S, Yokoyama Y, Matsuda A, Kusumoto S, Kobayashi H, Terasaki H, Ohyashiki K, Asou N, *et al*: Fusion of TEL/ETV6 to a novel ACS2 in myelodysplastic syndrome and acute myelogenous leukemia with t(5;12)(q31;p13). *Genes Chromosomes Cancer* 26: 192-202, 1999.
15. Ding BT, Guo NJ, Sun JZ, Gao HM, Wang YS and Chen Y: Analysis and clinical significance of ETV6 rearrangement in myelodysplastic syndromes patients. *Zhonghua Xue Ye Xue Za Zhi* 28: 804-807, 2007 (In Chinese).
16. Suto Y, Sato Y, Smith SD, Rowley JD and Bohlander SK: A t(6;12)(q23;p13) results in the fusion of ETV6 to a novel gene, STL, in a B-cell ALL cell line. *Genes Chromosomes Cancer* 18: 254-268, 1997.
17. Lambros MB, Tan DS, Jones RL, Vatcheva R, Savage K, Tamber N, Fenwick K, Mackay A, Ashworth A and Reis-Filho JS: Genomic profile of a secretory breast cancer with an ETV6-NTRK3 duplication. *J Clin Pathol* 62: 604-612, 2009.
18. Iijima Y, Ito T, Oikawa T, Eguchi M, Eguchi-Ishimae M, Kamada N, Kishi K, Asano S, Sakaki Y and Sato Y: A new ETV6/TEL partner gene, ARG (ABL-related gene or ABL2), identified in an AML-M3 cell line with a t(1;12)(q25;p13) translocation. *Blood* 95: 2126-2131, 2000.
19. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR and Sultan C: Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med* 103: 620-625, 1985.
20. Schreck RR, Warburton D, Miller OJ, Beiser SM and Erlanger BF: Chromosome structure as revealed by a combined chemical and immunochemical procedure. *Proc Natl Acad Sci USA* 70: 804-807, 1973.
21. Mitelman F (ed): *ISCN 1995: An International System for Human Cytogenetic Nomenclature*. Karger, Basel, pp1-114, 1995.
22. Chen SS: Flowcytometric-immunophenotyping for leukemia needs to be standardized. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 10: 1-5, 2002 (In Chinese).
23. Bohlander SK: ETV6: A versatile player in leukemogenesis. *Semin Cancer Biol* 15: 162-174, 2005.
24. Takahashi W, Sasaki K, Kvomatsu N and Mitani K: TEL/ETV6 accelerates erythroid differentiation and inhibits megakaryocytic maturation in a human leukemia cell line UT-7/GM. *Cancer Sci* 96: 340-348, 2005.
25. Wang LC, Swat W, Fujiwara Y, Davidson L, Visvader J, Kuo F, Alt FW, Gilliland DG, Golub TR and Orkin SH: The TEL/ETV6 gene is required specifically for hematopoiesis in the bone marrow. *Genes Dev* 12: 2392-2402, 1998.
26. Hart SM and Foroni L: Core binding factor genes and human leukemia. *Haematologica* 87: 1307-1323, 2002.
27. Tomita A, Kiyoi H and Naoe T: Mechanisms of action and resistance to all-trans retinoic acid (ATRA) and arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) in acute promyelocytic leukemia. *Int J Hematol* 97: 717-725, 2013.
28. He B, Hu S, Qiu G and Gu W: Clinical characteristics of acute promyelocytic leukemia manifesting as early death. *Mol Clin Oncol* 1: 908-910, 2013.
29. Xu F, Yin CX, Wang CL, Jiang XJ, Jiang L, Wang ZX, Yi ZS, Huang KK and Meng FY: Immunophenotypes and immune markers associated with acute promyelocytic leukemia prognosis. *Dis Markers* 2014: 421906, 2014.
30. Lazarevic V, Hörstedt AS, Johansson B, Antunovic P, Billström R, Derolf A, Hulegårdh E, Lehmann S, Möllgård L, Nilsson C, *et al*: Incidence and prognostic significance of karyotypic subgroups in older patients with acute myeloid leukemia: The Swedish population-based experience. *Blood Cancer J* 4: e188, 2014.
31. Won D, Shin SY, Park CJ, Jang S, Chi HS, Lee KH, Lee JO and Seo EJ: OBFC2A/RARA: A novel fusion gene in variant acute promyelocytic leukemia. *Blood* 121: 1432-1435, 2013.
32. Albano F, Zagaria A, Anelli L, Orsini P, Minervini CF, Impera L, Casieri P, Coccato N, Tota G, Brunetti C, *et al*: Lymphoid enhancer binding factor-1 (LEF1) expression as a prognostic factor in adult acute promyelocytic leukemia. *Oncotarget* 5: 649-658, 2014.
33. Eguchi-Ishimae M, Eguchi M, Ishii E, Miyazaki S, Ueda K, Kamada N and Mizutani S: Breakage and fusion of the TEL (ETV6) gene in immature B lymphocytes induced by apoptogenic signals. *Blood* 97: 737-743, 2001.
34. Kralik JM, Kranewitter W, Boesmueller H, Marschon R, Tschurtschenthaler G, Rumpold H, Wiesinger K, Erdel M, Petzer AL and Webersinke G: Characterization of a newly identified ETV6-NTRK3 fusion transcript in acute myeloid leukemia. *Diagn Pathol* 6: 19, 2011.
35. Cazzaniga G, Tosi S, Aloisi A, Giudici G, Daniotti M, Pioltelli P, Kearney L and Biondi A: The tyrosine kinase abl-related gene ARG is fused to ETV6 in an AML-M4Eo patient with a t(1;12)(q25;p13): Molecular cloning of both reciprocal transcripts. *Blood* 94: 4370-4373, 1999.
36. Murati A, Brecqueville M, Devillier R, Mozziconacci MJ, Gelsi-Boyer V and Birnbaum D: Myeloid malignancies: Mutations, models and management. *BMC Cancer* 12: 304, 2012.
37. Walz C, Erben P, Ritter M, Bloor A, Metzgeroth G, Telford N, Haferlach C, Haferlach T, Gesk S, Score J, *et al*: Response of ETV6-FLT3-positive myeloid/lymphoid neoplasm with eosinophilia to inhibitors of FMS-like tyrosine kinase 3. *Blood* 118: 2239-2242, 2011.
38. Van Vlierberghe P, Ambesi-Impimbato A, Perez-Garcia A, Haydu JE, Rigo I, Hadler M, Tosello V, Della Gatta G, Paietta E, Racevskis J, *et al*: ETV6 mutations in early immature human T cell leukemias. *J Exp Med* 208: 2571-2579, 2011.
39. Griesinger F, Janke A, Podleschny M and Bohlander SK: Identification of an ETV6-ABL2 fusion transcript in combination with an ETV6 point mutation in a T-cell acute lymphoblastic leukaemia cell line. *Br J Haematol* 119: 454-458, 2002.
40. Hossain S, Dubielecka PM, Sikorski AF, Birge RB and Kotula L: Crk and ABI1: Binary molecular switches that regulate abl tyrosine kinase and signaling to the cytoskeleton. *Genes Cancer* 3: 402-413, 2012.
41. Nishimura N, Furukawa Y, Sutheesophon K, Nakamura M, Kishi K, Okuda K, Sato Y and Kano Y: Suppression of ARG kinase activity by ST1571 induces cell cycle arrest through up-regulation of CDK inhibitor p18/INK4c. *Oncogene* 22: 4074-4082, 2003.
42. Iijima Y, Okuda K, Tojo A, Tri NK, Setoyama M, Sakaki Y, Asano S, Tokunaga K, Kruh GD and Sato Y: Transformation of Ba/F3 cells and Rat-1 cells by ETV6/ARG. *Oncogene* 21: 4374-4383, 2002.
43. Iriyama N, Hatta Y and Takei M: ETV6/ARG oncoprotein confers autonomous cell growth by enhancing c-Myc expression via signal transducer and activator of transcription 5 activation in the acute promyelocytic leukemia cell line HT93A. *Leuk Lymphoma* 56: 2416-2423, 2015.

44. Naiel A, Vetter M, Plekhanova O, Fleischman E, Sokova O, Tsaour G, Harbott J and Tosi S: A novel three-colour fluorescence in situ hybridization approach for the detection of t(7;12)(q36;p13) in acute myeloid leukaemia reveals new cryptic three way translocation t(7;12;16). *Cancers (Basel)* 5: 281-295, 2013.
45. Otsubo K, Kanegane H, Eguchi M, Eguchi-Ishimae M, Tamura K, Nomura K, Abe A, Ishii E and Miyawaki T: ETV6-ARNT fusion in a patient with childhood T lymphoblastic leukemia. *Cancer Genet Cytogenet* 202: 22-26, 2010.
46. Najfeld V, Cozza A, Berkofsky-Fessler W, Prchal J and Scalise A: Numerical gain and structural rearrangements of JAK2, identified by FISH, characterize both JAK2617V>F-positive and -negative patients with Ph-negative MPD, myelodysplasia, and B-lymphoid neoplasms. *Exp Hematol* 35: 1668-1676, 2007.
47. Kwon WK, Lee JY, Mun YC, Seong CM, Chung WS and Huh J: Clinical utility of FISH analysis in addition to G-banded karyotype in hematologic malignancies and proposal of a practical approach. *Korean J Hematol* 45: 171-176, 2010.