

PML(NLS⁻) protein: A novel marker for the early diagnosis of acute promyelocytic leukemia

ZHI-LING SHAN^{1,2*}, XIN-YU ZHU^{1,2*}, PENG-PENG MA^{1,2}, HUI WANG^{1,2},
JIANBIN CHEN³, JUN LI⁴, LIANG ZHONG² and BEI-ZHONG LIU^{1,2}

¹Department of Laboratory Medicine and Central Laboratory of Yong-chuan Hospital, Chongqing Medical University, Chongqing 402160; ²Key Laboratory of Laboratory Medical Diagnostics, Department of Laboratory Medicine, Ministry of Education, Chongqing Medical University, Chongqing 400016; ³Department of Hematology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 630014; ⁴Department of Clinical Laboratory, The Third People's Hospital of Chongqing, Chongqing 400014, P.R. China

Received May 17, 2016; Accepted June 15, 2017

DOI: 10.3892/mmr.2017.7272

Abstract. Promyelocyte leukemia-retinoic acid receptor α (PML-RAR α) is known as a fusion gene of acute promyelocytic leukemia (APL). Previous studies have reported that neutrophil elastase (NE) cleaves PML-RAR α in early myeloid cells, which leads to the removal of the nuclear localization signal (NLS) in PML and increases the incidence of APL. The resultant PML without the NLS is termed PML(NLS⁻). The aim of the present study was to verify the existence and location of the PML(NLS⁻) protein in NB4 cells. NB4 cells underwent electroporation with the pCMV-HA-NE plasmid to form NB4-HA-NE cells, which were then transplanted to produce tumors in nude mice and samples were collected from patients with APL. Western blot analysis, an immunofluorescence assay, confocal laser microscopy and immunohistochemistry were performed to detect the expression and localization of the PML(NLS⁻) protein. The findings demonstrated that PML(NLS⁻) was detectable in the cytoplasm of NB4-HA-NE cells, the tumors in nude mice and in neutrophils from patients with APL. This indicated that PML(NLS⁻) may be an effective and novel target for the diagnosis of APL.

Introduction

Acute promyelocytic leukemia (APL) is a type of acute myeloid leukemia (AML) with a high cure rate. However, delays in the diagnosis and treatment of APL are associated with increased morbidity and mortality (1). Bone marrow smearing for pathological examination is considered to be the gold standard in the diagnosis of APL; however, it is time-consuming and usually painful for the patients. Patients are treated with arsenic trioxide (ATO; As₂O₃) and all trans retinoic acid (ATRA), which are used in combination with chemotherapy. These treatments produced a marked effect in patients with APL; however, they also induced side effects. Thus, it is imperative to develop novel alternative methods for the rapid diagnosis and treatment of APL.

A previous study revealed that ~90% of APL cases have the promyelocyte leukemia-retinoic acid receptor α (PML-RAR α) fusion protein, a protein that interferes with the signal transduction of wild-type RAR α , and the normal structure and function of PML and its nuclear body (2); thus, it has an important role in the occurrence and development of APL. It has been previously reported that transgenic and knock-in animals expressing PML-RAR α in early myeloid cells developed APL (2-4); however, the APL phenotype was reversed when PML-RAR α was expressed in late myeloid cells (5). In addition, PML-RAR α does not act as a whole protein; it may be cleaved by neutrophil elastase (NE) into two variants, known as PML without a nuclear localization signal (NLS), the PML(NLS⁻) protein (~53 kDa), and the NLS-RAR α protein (~61 kDa) (6). Lane *et al* (7) demonstrated that following the introduction of PML-RAR α into early myelocytes without NE, the fusion gene was not cleaved and the probability of developing APL (2-3%) was lower than that observed in early myelocytes. In addition, Zhang *et al* (8) investigated the molecular mechanisms underlying the therapeutic effect of As₂O₃ on APL and reported that the occurrence of almost all tumors may be associated with the abnormal localization of PML. These findings indicate that the cleavage products of PML-RAR α may serve important roles in the occurrence and development of APL during the early phase. Thus, the aim of the present study was to investigate

Correspondence to: Ms. Liang Zhong, Key Laboratory of Laboratory Medical Diagnostics, Department of Laboratory Medicine, Ministry of Education, Chongqing Medical University, 1 Yixueyuan Road, Chongqing 400016, P.R. China
E-mail: 530659075@qq.com

Professor Bei-Zhong Liu, Department of Laboratory Medicine and Central Laboratory of Yong-chuan Hospital, Chongqing Medical University, 439 Xuanhua Road, Chongqing 402160, P.R. China
E-mail: liubeizhong@cqmu.edu.cn

*Contributed equally

Key words: acute promyelocytic leukemia, promyelocyte leukemia (nuclear localization signal) protein, NB4 cells, localization

whether the cleavage product of PML-RAR α , the PML(NLS⁻) protein, is localized in the cytoplasm of NB4-HA-NE cells, following the transplantation of tumors in nude mice and neutrophils from patients with APL. The present findings may provide novel methods for the early diagnosis of APL.

Materials and methods

Samples. The present study was approved by the Ethical Committee of Chongqing Medical University (Chongqing, China). The samples used in the present study were collected by the Department of Hematology of The Affiliated First Hospital of Chongqing Medical University (Chongqing, China). Blood samples (n=9) were obtained from 6 patients with APL (age, 30-55; 2 males, 4 females) and 3 non-M3-patients, including 2 M2 (age, 53-59; 1 male and 1 female) and 1 M6 (age, 67; female) patients, where M2, M3 and M6 refer to subtypes of leukemia. In addition, samples from 10 healthy subjects (age, 28-53; 4 males, 6 females) were collected as controls; subjects were characterized as healthy by obtaining normal results following general examination of their blood, urine, and liver and kidney function, and they also had no PML/RAR α fusion transcripts. A total of 20 ml blood was obtained from the elbow vein and all samples were collected between February and July 2013. All patients were of similar socioeconomic status and written informed consent was obtained from all participants.

Male BALB/c nude mice (n=9; age, 6 weeks; weight, 18-22 g) were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China) and housed in a specific pathogen free environment (22-25°C; 40-60%; 12-h light/dark cycle) with free access to food and water.

Cell culture. NB4 cells (Shanghai Institute for Biological Science, Chinese Academy of Sciences, Shanghai, China) were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO₂. K562 cells (Shanghai Institute for Biological Science, Chinese Academy of Sciences) were maintained in Hyclone RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% FBS (Hyclone; GE Healthcare Life Sciences) at 37°C and 5% CO₂. Passaging was performed once every 1-2 days and cells in the logarithmic growth phase were used in the subsequent experiments (9).

Electroporation. The pCMV-HA plasmid was obtained from Clontech Laboratories, Inc. (Mountainview, CA, USA). The pCMV-HA-NE plasmid was constructed based on the pCMV-HA plasmid and preserved in our lab with a titer of 1x10¹¹ PFU/ml. The pCMV-HA-NE plasmid was electroporated into NB4 cells (1x10⁶) using the Amaxa[®] Cell Line Nucleofector[®] kit V (Lonza Group, Ltd., Basel, Switzerland). Following incubation at 37°C for 24 h, observations were performed under an inverted fluorescence microscope.

Isolation of human peripheral neutrophils. Fresh anti-coagulated blood (20 ml; from patients and controls) was diluted with PBS at a ratio of 1:1. This mixture was added to 20 ml separation media (Tianjin Haoyang Biotech Co., Ltd., Tianjin, China) and then centrifugation was performed at 600 x g for

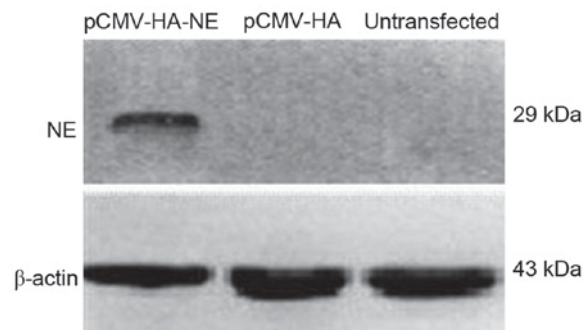


Figure 1. Western blot analysis of the NE levels in NB4-HA-NE cells. NB4 cells were transfected with either pCMV-HA-NE or pCMV-HA plasmids, or were left untransfected. β -actin was used as the loading control. NE was only expressed in the NB4-HA-NE cells, and was undetectable in the NB4-HA and NB4 cells. NE, neutrophil elastase; HA, human influenza hemagglutinin.

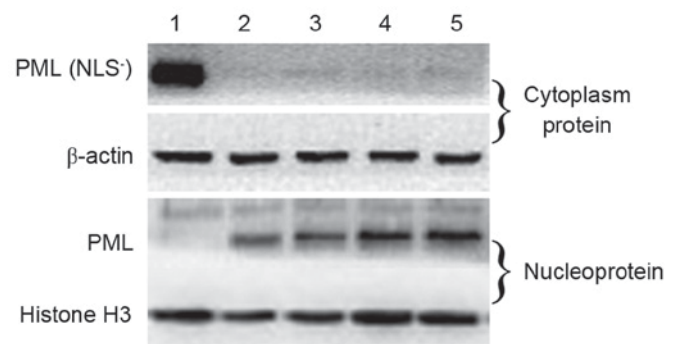


Figure 2. Western blot analysis of PML(NLS⁻) protein expression. β -actin and Histone H3 were used as loading controls. Lane 1, pCMV-HA-NE; lane 2, pCMV-HA; lane 3, untransfected group; lane 4, K562 cells; lane 5, normal group. PML(NLS⁻) expression was high in the cytoplasm of NB4-HA-NE cells and the expression was reduced in the other cell lines. The PML protein was primarily expressed in the nuclei of the other cells. PML(NLS⁻), promyelocyte leukemia-retinoic acid receptor α ; NE, neutrophil elastase; HA, human influenza hemagglutinin.

25 min at 20°C. Subsequently, 4 layers were produced: The first layer was plasma, the second layer was mononuclear cells, the third layer was separation media rich in neutrophils and the fourth layer was red blood cells. The first and second layers were removed and the third and fourth layers were transferred into a tube containing 10 ml of PBS; this was followed by centrifugation at 600 x g for 30 min at 20°C. Neutrophils were then treated with red blood cell lysis buffer (Tianjin Haoyang Biotech Co., Ltd., Tianjin, China) thrice and then washed 3 times with PBS. Following a final centrifugation at 250 x g for 10 min at 20°C, the remaining neutrophils were collected.

Protein extraction. Cells were washed 3 times with PBS. Following centrifugation at 2,000 x g for 3 min at 4°C, the supernatant was removed and the cells were harvested for use. A total of 20 μ l cell suspension was mixed with 200 μ l of cytoplasmic protein extraction reagent A (Beyotime Institute of Biotechnology, Shanghai, China) containing 2 μ l of PMSF (a protease inhibitor). Following vortexing for 5 sec, cells were re-suspended and placed on ice for 15 min. Cytoplasmic protein extraction reagent B (10 μ l; Beyotime Institute of Biotechnology) was then applied and placed on ice for a further 1 min. Following

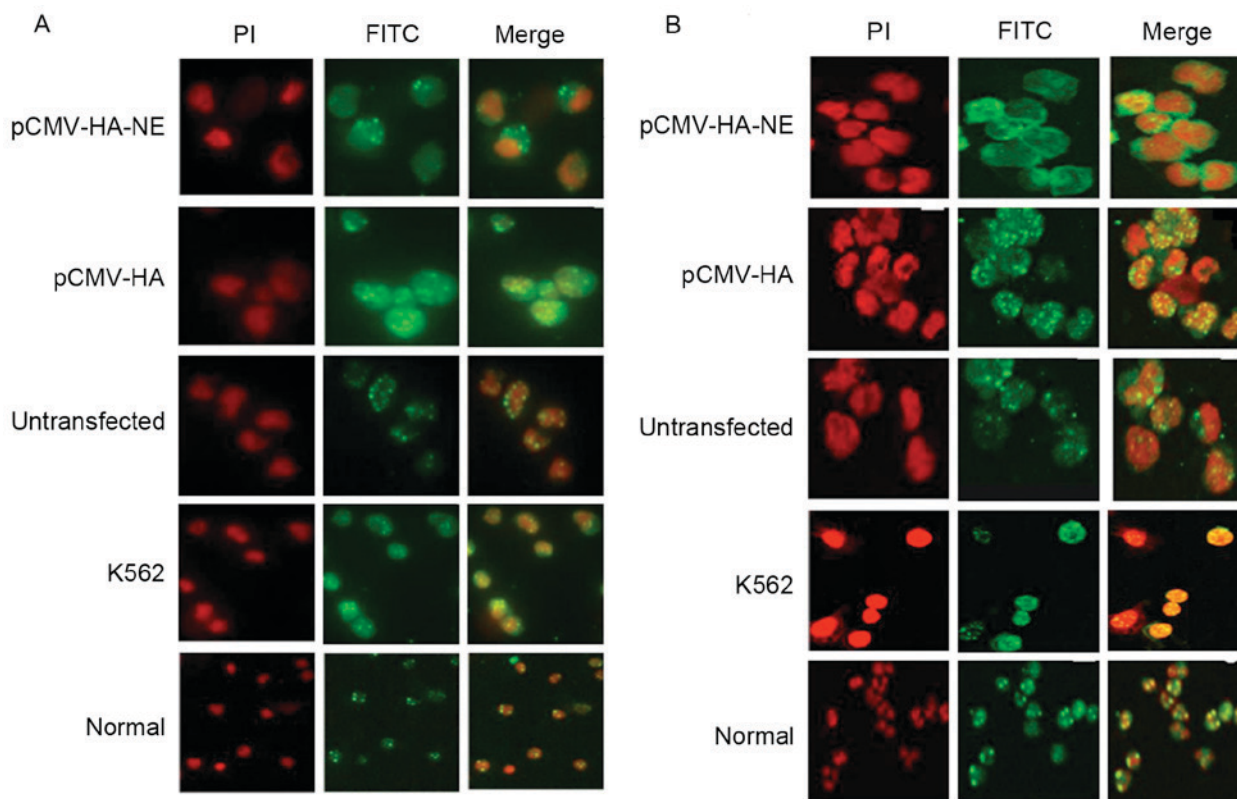


Figure 3. PI and FITC staining of the different cell groups. PML(NLS⁻) and PML proteins were stained with FITC (green staining) and the nuclei were stained with PI (red staining). Images were photographed at the same exposure time with a magnification of x400. The results of (A) immunofluorescence staining and (B) confocal laser microscopy indicated that the PML(NLS⁻) protein was localized in the cytoplasm of NB4-HA-NE cells, whereas PML protein was localized in the nuclei of the other types of cells. PI, propidium iodide; PML(NLS⁻), promyelocyte leukemia-retinoic acid receptor α ; NE, neutrophil elastase; HA, human influenza hemagglutinin.

vortexing for 5 sec, centrifugation was performed at 4°C for 5 min at 13,000 x g. The supernatant (cytoplasmic protein) was collected and transferred to a cold Eppendorf tube. The nuclear protein extraction reagent (50 μ l; Beyotime Institute of Biotechnology) containing 2 μ l of PMSF was added and placed on ice for 2 min following vortexing for 15 sec. This process was repeated for 30 min, then centrifugation was performed at 4°C for 10 min at 16,000 x g. The supernatant (nuclear protein) was collected and transferred to a cold Eppendorf tube.

Western blot assay. Proteins were quantified using the bicinchoninic acid method. Then, 50 μ g protein/lane was subjected to electrophoresis in a 5% stacking gel and 12% separation gel and were then transferred onto a PVDF membrane. The membrane was blocked in 5% non-fat milk at room temperature for 2 h and then treated with rabbit anti-human PML polyclonal antibody (cat. no. ab53773; 1:1,000), rabbit anti-human NE polyclonal antibody (cat. no. ab68672; 1:1,000; both Abcam, Cambridge, MA, USA), rabbit anti-human Histone H3 polyclonal antibody (cat. no. P30266; 1:5,000; Abmart, Shanghai, China) and mouse anti-human β -actin antibodies (cat. no. BM0627; 1:4,000; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) in 5% non-fat milk at 4°C overnight. Following washing in TBST twice (10 min for each) and in TBS for 10 min, the membrane was treated with goat anti-rabbit (cat. no. ZB-2301) or goat anti-mouse (cat. no. ZB-2305) IgG horseradish peroxidase (HRP)-linked secondary antibodies (Beijing Zhongshan

Golden Bridge Biotechnology Co., Ltd.; 1:1,000, in 5% non-fat milk) at 37°C for 1 h. The membrane was then washed again as described above. Visualization was performed as described previously (10,11).

Immunofluorescence staining. Cells (1×10^3) in the logarithmic growth phase were washed in PBS 3 times and then placed on slides. They were then fixed in 4% paraformaldehyde for 20 min at room temperature and washed three times with PBS. Transparentization was performed with 0.1% Triton-X-100 for 15 min, which was followed by blocking in 10% goat serum (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at room temperature for 30 min. Cells were then incubated with rabbit anti-human PML antibody (cat. no. ab53773; 1:300; Abcam) in blocking serum (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) at 4°C overnight. Following 3 washes in PBS, cells were treated with FITC-conjugated secondary antibody (cat. no. ZF-0311; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) in blocking serum (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; 1:300) at 37°C for 1 h. Following 3 washes with PBS, cells were incubated with propidium iodide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 5 min and then washed 3 times in PBS. Mounting was performed using 70% glycerol and observations were completed under a fluorescence microscope. Images were processed with NIS-Elements Basic Research software F Package (version 4.00; Nikon Corporation, Tokyo, Japan). A total of 10 fields of view were

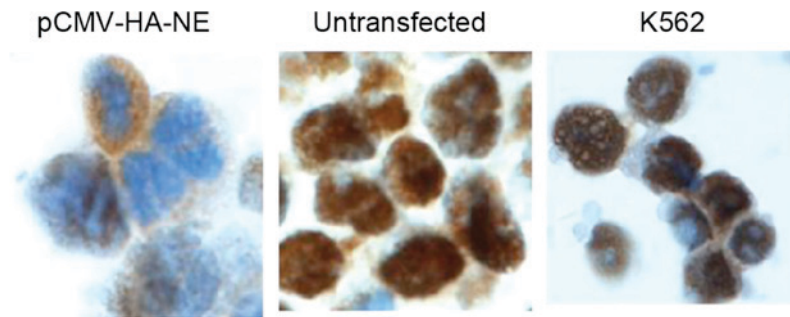


Figure 4. Immunohistochemistry analysis of PML(NLS⁻) protein localization (magnification, x400). The results showed that the PML(NLS⁻) protein was expressed in the cytoplasm of the NB4-HA-NE transfected cells; however, the PML(NLS⁻) protein was observed in the nuclei of the other cell groups. PML(NLS⁻), promyelocyte leukemia-retinoic acid receptor α ; NE, neutrophil elastase; HA, human influenza hemagglutinin.

analyzed. The red fluorescence represents nuclei, and green fluorescence indicates PML protein.

Confocal laser microscopy. Cells were observed under a confocal laser microscope (TCS-SP2; Leica Microsystems, Inc., Buffalo Grove, IL, USA) and the protein expression of PML(NLS⁻) in the cytoplasm was determined. The red and green fluorescence channel was set. The excitation wavelength of green fluorescence was 488 nm and observation was performed at >600 nm. The excitation wavelength of red fluorescence was 540 nm and observation was performed at >600 nm. A total of 10 fields with the highest fluorescence intensity were observed; red fluorescence represents nuclei and green fluorescence is PML protein.

Tumor growth in nude mice and immunohistochemistry. Logarithmic growth phase cells (1×10^7) were added to 100 μ l of serum-free RPMI-1640 medium. This 100 μ l cell suspension was applied to the back of 9 nude mice on the right hand-side by subcutaneous injection to produce a tumor xenograft. One week later, animals were sacrificed and the tumor was harvested. Approximately 100 mg of the tumor was digested in 0.25% trypsin at 37°C for 3 min in order to collect cells. The remaining tumor was fixed at 4°C in 4% paraformaldehyde for 24 h, then dehydrated, embedded in paraffin and sectioned (12). Tissue sections (5 μ m) were maintained at 60°C overnight prior to deparaffinization, hydration and washing in distilled water. Antigen retrieval was performed using citrate solution by heating in a microwave for 7 min on a medium heat setting. Six min later, heating was performed at a medium heat for 7 min. The sections were allowed to cool to room temperature and were then washed in PBS three times (3 min for each). The sections were dried, circled with a wax-pen and then treated with 3% H₂O₂ (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd) at room temperature for 10 min; sections were then washed 3 times with PBS (3 min for each). PBS was subsequently removed and sections were blocked in goat serum at 37°C for 30 min. Rabbit anti-human PML antibody (cat. no. ab53773; Abcam) in PBS (1:300) was then applied at 4°C in a humidified environment overnight. Following 3 washes with PBS (3 min for each), sections were treated with biotin-conjugated secondary antibody (cat. no. SPN-9001; 1:300; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd) at room temperature for 30 min.

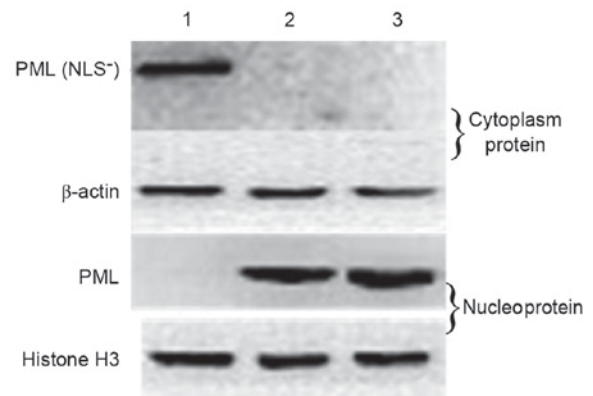


Figure 5. Western blot analysis of PML(NLS⁻) protein expression in transplanted tumors in nude mice. β -actin and Histone H3 were used as the loading controls. Lane 1, pCMV-HA-NE; lane 2, untransfected group; lane 3, K562 cells. PML(NLS⁻) protein in nude mice was expressed and localized in the cytoplasm of NB4-HA-NE cells, whereas PML was localized primarily in the nuclei of NB4 and K562 cells. PML(NLS⁻), promyelocyte leukemia-retinoic acid receptor α ; NE, neutrophil elastase; HA, human influenza hemagglutinin.

Following 3 washes with PBS (3 min for each), sections were incubated with HRP-conjugated streptavidin working solution (cat. no. SPN-9001; 1:300; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd) at room temperature for 1 h, followed by 3 washes in PBS (3 min for each). Visualization was performed using 3,3'-diaminobenzidine (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd) at room temperature for 2 min and sections were observed under a light microscope. When completed, the reaction was stopped by washing with tap water. Counterstaining was performed with hematoxylin at room temperature for 13 sec, which was followed by treatment with lithium carbonate solution for 10 sec. Following washing with tap water and PBS 3 times (3 min for each), sections were dried and transparentization was performed with xylene for 30 min. Sections were then mounted with gum and the cells were observed using fluorescence microscopy.

Results

NE protein expression in the NB4-HA-NE cells. Western blotting was performed to evaluate the expression of the NE protein in NB4-HA-NE cells. As presented in Fig. 1, the specific band was detected at 29 kDa in the pCMV-HA-NE group; however,

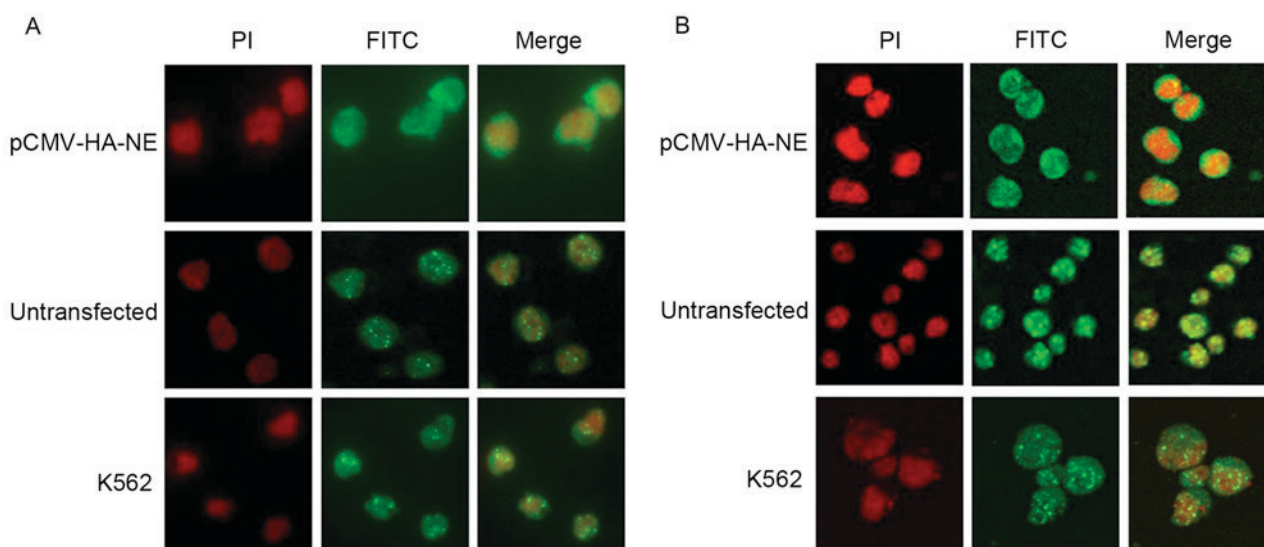


Figure 6. PI and FITC staining in transplanted tumors in nude mice. PML(NLS⁻) and PML proteins were stained with FITC (green staining) and the nuclei were stained with PI (red staining). Images were photographed at the same exposure time with a magnification of x400. The results of (A) immunofluorescence assay and (B) confocal laser microscopy indicated that the PML(NLS⁻) in nude mice was localized in the cytoplasm of NB4-HA-NE cells, whereas PML was localized primarily in the nuclei of the remaining cell lines. PI, propidium iodide; PML(NLS⁻), promyelocyte leukemia-retinoic acid receptor α ; NE, neutrophil elastase; HA, human influenza hemagglutinin.

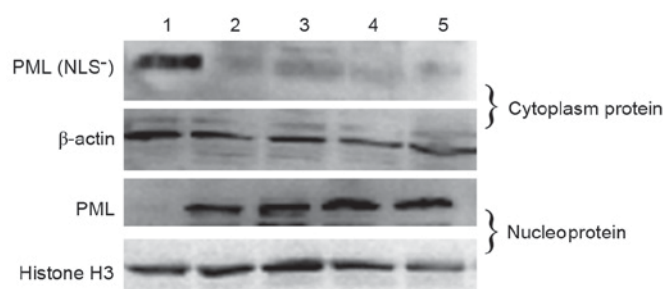


Figure 7. Western blot analysis of PML(NLS⁻) protein expression in neutrophils from patients with APL. β -actin and Histone H3 were used as the loading controls. Lane 1, neutrophils from patients with APL; lane 2, non-M3-1; lane 3, non-M3-2; lane 4, non-M3-3; lane 5, healthy controls. PML(NLS⁻) was expressed and localized in the cytoplasm of neutrophils in M3. PML(NLS⁻) was undetectable in neutrophils from non-M3 samples and healthy subjects and PML protein was primarily localized in the nucleus. PML(NLS⁻), promyelocyte leukemia-retinoic acid receptor α ; APL, acute promyelocytic leukemia.

no bands were observed in the pCMV-HA and untransfected groups. The present findings indicated that the NE protein was successfully expressed in the pCMV-HA-NE group.

PML(NLS⁻) protein expression and localization in different cells. PML(NLS⁻) protein expression was determined by a western blot assay. The expression of the PML(NLS⁻) protein in different cells was evaluated in order to identify the differences between the PML(NLS⁻) and PML proteins.

As presented in Fig. 2, in the pCMV-HA-NE group (lane 1) a specific band was observed at 53 kDa for PML(NLS⁻); however, no band was evident at 98 kDa for PML. The present results indicated that nuclear PML may be transported into the cytoplasm due to the absence of the NLS and the PML(NLS⁻) protein may be localized in the cytoplasm. Conversely bands were observed at 98 kDa for PML, however, no bands were identified at 53 kDa for PML(NLS⁻). This indicated that the PML(NLS⁻) protein was primarily expressed in the cytoplasm

of NB4-HA-NE cells transfected with the pCMV-HA-NE plasmid.

Localization of the PML(NLS⁻) protein in different cells measured by immunofluorescence staining and confocal laser microscopy. In the pCMV-HA-NE group, the green fluorescing PML(NLS⁻) protein was evident in the cytoplasm, with a patchy distribution; however, it was not observed in the nucleus. In the remaining 4 groups, the green fluorescing PML protein was observed in nuclei and not in the cytoplasm. These findings indicated that the PML-RAR α fusion protein was successfully cleaved by NE and nuclear PML was transported into cytoplasm due to the absence of the NLS (Fig. 3).

Detection of PML(NLS⁻) protein expression and localization in xenografts from nude mice using immunohistochemistry. To examine PML(NLS⁻) protein expression *in vivo*, NB4, K562 and NB4-HA-NE cells were expressed in a xenograft from nude mice. As presented in Fig. 4, the PML(NLS⁻) protein was observed in the cytoplasm and not in the nuclei of the pCMV-HA-NE group. However, PML was observed in the nuclei of the remaining cell treatment groups. This indicated that the PML(NLS⁻) protein was detectable in the cytoplasm of NB4-HA-NE cells.

Detection of PML(NLS⁻) protein expression by western blot assay. Western blot analysis revealed a band at 53 kDa for PML(NLS⁻) and no band at 98 kDa for PML in the pCMV-HA-NE group. The present results indicated that nuclear PML was transported into the cytoplasm due to the absence of the NLS and that the PML(NLS⁻) protein was localized to the cytoplasm. However, in the other groups, bands were observed at 98 kDa for PML and not at 53 kDa for PML(NLS⁻). The results demonstrated that PML protein expression was primarily in the nuclei, which was markedly higher than that observed in NB4 cells transfected with pCMV-HA-NE (Fig. 5).

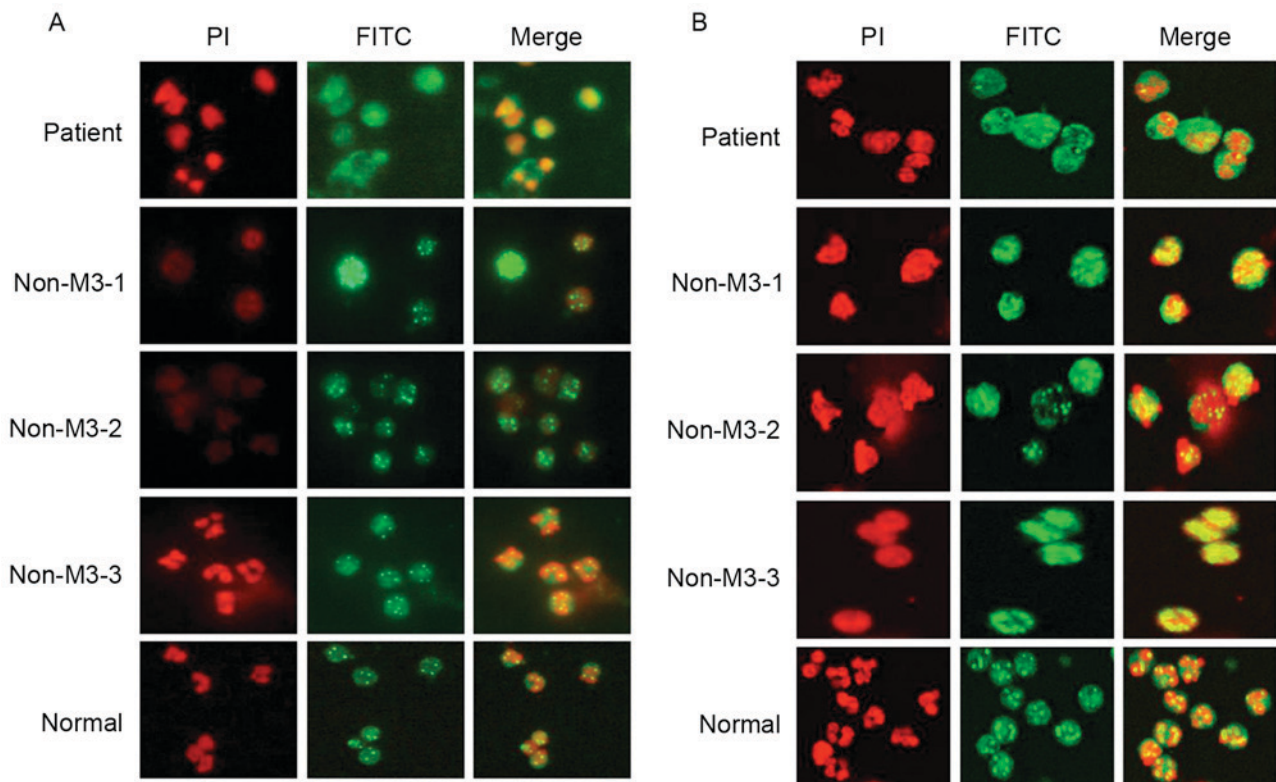


Figure 8. PI and FITC staining in neutrophils from patients with APL. PML(NLS⁻) and PML proteins were stained with FITC (green staining) and the nuclei were stained with PI (red staining). Images were photographed at the same exposure time with a magnification of x400. The results of (A) immunofluorescence assay and (B) confocal laser microscopy indicated that the PML(NLS⁻) protein was localized in the cytoplasm of neutrophils in patients with APL. PML(NLS⁻) was undetectable in neutrophils from non-M3 samples and healthy subjects and PML was primarily localized in the nucleus. PI, propidium iodide; APL, acute promyelocytic leukemia; PML(NLS⁻), promyelocyte leukemia-retinoic acid receptor α .

Detection of PML(NLS⁻) protein localization by immunofluorescence staining and confocal laser microscopy. In the pCMV-HA-NE group, green PML(NLS⁻) protein was evident in the cytoplasm with a patchy distribution, while PML was not observed in the nuclei. However, in the other groups, green PML protein was observed with an uneven distribution in the nuclei, and the expression was higher than that observed in the pCMV-HA-NE group. These findings indicated that the nuclear PML was transported into cytoplasm due to the absence of NLS (Fig. 6).

PML(NLS⁻) protein expression and localization in neutrophils collected from patients with APL. PML(NLS⁻) protein expression was evaluated by western blotting analysis. As shown in Fig. 7, a band was detected at 53 kDa for PML(NLS⁻); however, no obvious band was observed at 98 kDa for PML in neutrophils obtained from patients with APL. The results indicated that the nuclear PML was transported into the cytoplasm due to the absence of NLS, and the PML(NLS⁻) protein was localized in the cytoplasm. By contrast, bands were only detected at 98 kDa for PML, not at 53 kDa, in the other groups. These results demonstrated that PML protein expression was primarily in the nucleus, which was observed in markedly higher levels in the control subjects when compared with that in the neutrophils from patients with APL.

Detection of PML(NLS⁻) protein in neutrophils by immunofluorescence staining and confocal laser microscopy.

In the neutrophils obtained from patients with APL, green PML(NLS⁻) protein was observed in the cytoplasm with an uneven distribution, and no PML expression was identified in the nuclei. However, when compared to neutrophils from patients with APL, there was markedly more PML protein in the nuclei of neutrophils from healthy subjects and non-M3 patients. These results indicated that the PML(NLS⁻) protein was detectable in the cytoplasm of neutrophils from patients with APL (Fig. 8).

Discussion

APL is a subtype of AML that is clinically characterized by a tendency towards severe hemorrhage and a susceptibility to disseminated intravascular coagulation, which results in high levels of morbidity and mortality (13). Treatment with ATRA and ATO significantly improves the outcome of APL by increasing the remission rate and reducing the number of therapy-associated mortalities (14). Although advancements have been achieved in the clinical treatment of APL, 12-30% of patients may still develop recurrence and resistance to ATRA therapy (15). Thus, further investigation of the molecular pathogenesis of APL and the potential diagnostic and therapeutic targets is important for the targeted therapy of APL. Previous studies investigating APL have focused on the detection of the PML-RAR α fusion gene or protein, and the effect of ATRA and ATO on apoptosis and differentiation in leukemia cells, and on the PML-RAR α fusion gene (15,16).

However, the methods for the early diagnosis and targeted therapy of APL have not been significantly improved. Notably, Lane *et al* (7) revealed that the PML-RAR α fusion protein may be cleaved into two variants (~53 and 61 kDa) by NE, which are also termed the PML(NLS⁻) and NLS-RAR α proteins. In addition, they also demonstrated that NE cleavage served an important role in the occurrence and development of APL.

Our previous study successfully constructed the PML/PML(NLS⁻) and the RAR α /NLS-RAR α eukaryotic expression vectors (17,18). These vectors were transfected into NB4 cells, and indirect immunofluorescence staining was used to localize these proteins. The previous findings revealed that the localization of PML and RAR α was altered significantly: RAR α in the cytoplasm was transported into the nuclei due to the presence of the NLS, and nuclear PML was transported into the cytoplasm due to the absence of the NLS. In the present study, the pCMV-HA-NE plasmid was used for transfection with NB4 cells and western blot analysis, immunofluorescence staining, immunohistochemistry and confocal laser microscopy were performed to detect the expression and localization of the PML cleavage product, PML(NLS⁻). The current results revealed that PML(NLS⁻) was present in the cytoplasm of the NB4-HA-NE cells. In addition, neutrophils were collected from patients who were diagnosed with APL and the analysis revealed that the PML(NLS⁻) protein was localized in the cytoplasm of these neutrophils. There is previous evidence demonstrating that human wild-type PML may act as a cell-growth suppressor (19-22), with different characteristics to PML(NLS⁻). Interfering with PML(NLS⁻) protein expression may attenuate the proliferation of HL-60 cells and promote their apoptosis (17). This suggests that PML(NLS⁻) may promote the development of APL.

Collectively, the results of the present study demonstrated that NB4-HA-NE cells express the PML(NLS⁻) protein in the cytoplasm, which was confirmed in the neutrophils of patients diagnosed with APL. These findings provide a novel method for the early diagnosis of APL and the identification of APL recurrence and also offer a novel strategy for the investigation of APL pathogenesis. The collection of bone marrow is not necessary for the diagnosis of APL, which reduces the patients' pain and provides a novel approach for its treatment. The normal PML was expressed in the nuclei and PML was expressed in low levels in the cytoplasm; the expression of the PML(NLS⁻) protein in the cytoplasm alone is not sufficient to confirm its localization. Thus, future studies will focus on the NLS of PML using larger sample sizes.

In conclusion, the PML(NLS⁻) protein was localized in the cytoplasm of NB4-HA-NE cells, transplanted tumors in nude mice and in neutrophils from patients with APL. The present results indicate that PML(NLS⁻) may be an effective novel target for the diagnosis and treatment of APL.

Acknowledgements

The authors would like to thank Professor Jianbin Chen and Professor Jun Li for providing the patient samples. The present study was supported by the National Natural Science Foundation of China (grant no. 81171658) and the Key Program of Natural Science Foundation of Chongqing (grant no. 2011BA5037).

References

- Mohamed M, Dun K and Grabek J: Atypical features in a patient with acute promyelocytic leukaemia: A potential diagnostic pitfall. *BMJ Case Rep* 2013; pii: bcr2013200152, 2013.
- Grisolano JL, Wesselschmidt RL, Pelicci PG and Ley TJ: Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR alpha under control of cathepsin G regulatory sequences. *Blood* 89: 376-387, 1997.
- He LZ, Tribioli C, RivR, Peruzzi D, Pelicci PG, Soares V, Cattoretti G and Pandolfi PP: Acute leukemia with promyelocytic features in PML/RARalpha transgenic mice. *Proc Natl Acad Sci USA* 94: 5302-5307, 1997.
- Westervelt P, Lane AA, Pollock JL, Oldfather K, Holt MS, Zimonjic DB, Popescu NC, DiPersio JF and Ley TJ: High-penetrance mouse model of acute promyelocytic leukemia with very low levels of PML-RARalpha expression. *Blood* 102: 1857-1865, 2003.
- Early E, Moore MA, Kakizuka A, Nason-Burchenal K, Martin P, Evans RM and Dmitrovsky E: Transgenic expression of PML/RARalpha impairs myelopoiesis. *Proc Natl Acad Sci USA* 93: 7900-7904, 1996.
- Lane AA and Ley TJ: Neutrophil elastase is important for PML-retinoic acid receptor alpha activities in early myeloid cells. *Mol Cell Biol* 25: 23-33, 2005.
- Lane AA and Ley TJ: Neutrophil elastase cleaves PML-RARalpha and is important for the development of acute promyelocytic leukemia in mice. *Cell* 115: 305-318, 2003.
- Zhang XW, Yan XJ, Zhou ZR, Yang FF, Wu ZY, Sun HB, Liang WX, Song AX, Lallemand-Breitenbach V, Jeanne M, *et al*: Arsenic trioxide controls the fate of the PML-RARalpha oncoprotein by directly binding PML. *Science* 328: 240-243, 2010.
- Gao Y, Wang S, Liu B and Zhong L: Roles of GINS2 in K562 human chronic myelogenous leukemia and NB4 acute promyelocytic leukemia cells. *Int J Mol Med* 31: 1402-1410, 2013.
- Zhou Y, Liu XH, Qu SD, Yang J, Wang ZW, Gao CJ and Su QJ: Hyperbaric oxygen intervention on expression of hypoxia-inducible factor-1 α and vascular endothelial growth factor in spinal cord injury models in rats. *Chin Med J (Engl)* 126: 3897-3903, 2013.
- Fuchs NV, Loewer S, Daley GQ, Izsvák Z, Löwer J and Löwer R: Human endogenous retrovirus K (HML-2) RNA and protein expression is a marker for human embryonic and induced pluripotent stem cells. *Retrovirology* 10: 115, 2013.
- Sun L, Zhu X, Xu L, Wang Z, Shao G and Zhao J: Antitumor effects of 32P-chromic-poly (L-lactide) brachytherapy in nude mice with human prostate cancer. *Oncol Lett* 6: 687-692, 2013.
- Borowska A, Stelmaszczyk-Emmel A and Pawelec K: Central nervous system haemorrhage causing early death in acute promyelocytic leukaemia. *Cent Eur J Immunol* 40: 486-488, 2015.
- Xin L, Wan-jun S, Zeng-jun L, Yao-zhong Z, Yun-tao L, Yan L, Chang-chun W, Qiao-chuan L, Ren-chi Y, Ming-zhe H, *et al*: A survival study and prognostic factors analysis on acute promyelocytic leukemia at a single center. *Leuk Res* 31: 765-771, 2007.
- Laurenzana A, Pettersson F and Miller WH: Role of PML/RAR α in the pathogenesis of APL. *Drug Discov Today: Dis Mech* 3: 499-505, 2006.
- Lallemand-Breitenbach V, Zhu J, Chen Z and de Thé H: Curing APL through PML/RAR α degradation by As2O3. *Trends Mol Med* 18: 36-42, 2012.
- Zhu HH, Qin YZ, Lai YY, Shi HX, Liu YR, Jiang B and Huang XJ: Expression of PML-RAR α is Up-regulated during ATRA and arsenic combined induction without influence on long-term prognosis of acute promyelocytic leukemia. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 21: 872-878, 2013.
- Hu XX, Zhong L, Zhang X, Gao YM and Liu BZ: NLS-RAR α promotes proliferation and inhibits differentiation in HL-60 cells. *Int J Med Sci* 11: 247-254, 2014.
- Mu ZM, Chin KV, Liu JH, Lozano G and Chang KS: PML, a growth suppressor disrupted in acute promyelocytic leukemia. *Mol Cell Biol* 14: 6858-6867, 1994.
- Ahn MJ, Langenfeld J, Moasser MM, Rusch V and Dmitrovsky E: Growth suppression of transformed human bronchial epithelial cells by all-trans-retinoic acid occurs through specific retinoid receptors. *Oncogene* 11: 2357-2364, 1995.
- Koken MH, Linares-Cruz G, Quignon F, Viron A, Chelbi-Alix MK, Sobczak-Thépot J, Juhlin L, Degos L, Calvo F and de Thé H: The PML growth-suppressor has an altered expression in human oncogenesis. *Oncogene* 10: 1315-1324, 1995.
- Le XF, Yang P and Chang KS: Analysis of the growth and transformation suppressor domains of promyelocytic leukemia gene, PML. *J Biol Chem* 271: 130-135, 1996.