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Original Research

The expression of Slit2 and Robo1 increased during retinoic acid syndrome in acute promyelocytic leukemia and impacted differentiated cell migration

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ABSTRACT

Retinoic acid syndrome (RAS) is a serious complication developed during the induction therapy of acute promyelocytic leukemia (APL). Cytokines and differentiated cells migration play important roles in the development of RAS. Slit guidance ligand 2 (Slit2) and roundabout 1 (Robo1) involve in cell migration. Our study aimed to investigate the expression of Slit2 and Robo1 in APL and check whether they affected promyelocytes migration. 62 cases of newly diagnosed APL patients were involved and received all-trans retinoic acid (ATRA) and arsenic trioxide as induction therapy. Bone marrow cells (BMCs) were obtained on days 0 and 28, and promyelocytes and plasma were collected from day 1 to day 21. The expression of Robo1 in promyelocytes, and that of Slit2 and cytokines, including IL-8,IL-1β and others, in serum were monitored. 20 healthy individuals donated their cells as control. Of the 62 APL patients, 16 (25.81%) patients developed RAS. The expression of Robo1, Slit2 and IL-8 increased significantly with the development of RAS. In the 16 patients with RAS, levels of Slit2, Robo1 and IL-8 were higher during the development of RAS than before or after the RAS ($P < 0.05$). RhSlit2-N and rhIL-8 induced cells migration, and the migration induced by IL-8 was not inhibited by rhSlit2-N. Elevated Slit2 and Robo1 levels might be useful markers for the diagnosis and treatment of RAS. The levels of Slit2, Robo1 and IL-8 showed a positive correlation with the severity of RAS. Slit2 and IL-8 promoted the migration of differentiated cells.

Introduction

Acute promyelocytic leukemia (APL) is a special subtype of acute myeloid leukemia (AML) with malignant atypical promyelocytes and t (15;17) translocation which means that the promyelocytic leukemia (PML) gene on chromosome 15 was fused to the retinoic acid receptor alpha (RARα) gene on chromosome (17). The rate of complete remission (CR) is about 80% after treatment with all-trans retinoic acid (ATRA)-, arsenic trioxide (ATO)-, and anthracycline-based chemotherapy [1]. Unfortunately, the rate of the retinoic acid syndrome (RAS), a life-threatening therapeutic complication, accounts for about 25% [1,2]. RAS is characterized by unexplained fever, dyspnea, pleural effusion, unexplained hypotension, weight gain and renal insufficiency [3]. RAS only occurred in APL patients during induction therapy, and not in other types of AML patients. Differentiated cells release a large number of inflammatory cytokines and adhesion molecules that induce the migration and adhesion of differentiated cells [4,5]. Although corticosteroids can effectively inhibit the secretion of cytokines, they cannot inhibit the migration of cells. A study reported that corticosteroids inhibited the development of RAS; however, the preventive use of dexamethasone does not decrease RAS-related deaths [4].

Signs and symptoms of RAS were regarded as like the characters of acute respiratory distress syndrome (ARDS) and the inflammatory cascade. Therefore, differentiated cells are faced with the challenge of traversing fast-flowing blood vessels and migrating to such locations. Slit2 was reported to be involved in the migration and infiltration of leukocytes by binding to its receptor Roundabout-1 (Robo1) [6]. Certain studies showed that Slit2 was highly expressed in the brain, kidneys, and lungs [7,8]. Others found that Slit2 was upregulated in the brain during inflammation [9]. In 2001, Wu et al. found, using the anti-Robo1 antibody, that Robo1 was expressed in the neutrophils differentiated from HL-60 cells [8]. Slit2 was shown to inhibit chemotaxis of leukocytes induced by the stromal-derived factor (SDF)-1 by binding to the Robo1 receptor [10]. Subsequent studies found that Slit2/Robo1 inhibited the

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migration of neutrophils, lymphocytes, and dendritic cells induced by cytokines [10–12]. However, another study showed that Slit2/Robo1 participated in promoting the chemotaxis of eosinophils during allergic airway inflammation and vascular endothelial cells [12]. Another study showed that Robo1 promoted inflammation and Robo4 inhibited it by regulating the secretion of cytokines [13]. Studies have further shown that the migration by Slit2/Robo1 was related to the concentration of Slit2, where the migration increased from low concentration to high [14, 15]. In 2008, Jones et al. found that Slit2 maintained vascular integrity and inhibited the cytokine storm via the Robo4 receptor [16]. In 2011, London proved that Slit2 inhibited the inflammatory response through the Robo4 receptor, not Robo1, by promoting vascular endothelial stability [17].

The development of RAS was always accompanied by cytokine secretion and promyelocyte migration. The expression of Slit2 and Robo1 in APL patients with RAS has not been reported and whether Slit2 affects the migration of differentiated cells in APL is not known. This is the first time that the expression of Slit2 and Robo1 in APL patients with RAS and whether they influence the migration of promyelocytes or the chemotaxis induced by cytokines is evaluated. In the current study, 62 newly diagnosed APL patients were involved and we detected the expression of Slit2, Robo1 and cytokines and examined their functions using NB4 cells.

Methods

Patients and cell lines

There were 62 newly diagnosed APL patients admitted to our hospital from October 2015 to July 2019, including 24 males and 38 females (ratio of males to females was 1:1.58) ranging from 16 to 66 years (median age 37). APL was diagnosed based on the results of morphological, immunological, cytogenetic, and molecular biology tests. All the patients received 25 mg/m^2/day ATRA accompanied by chemotherapy, including cytarabine and daunorubicin, as induction treatment. The diagnosis of RAS was made according to Larson and Tallman [18]. APL patients with RAS belonged to the experimental group. The control group included APL patients without RAS and healthy people, including 10 donors of hematopoietic stem cell transplantation and 10 healthy volunteers from our hospital. The cells of APL patients were extracted from the bone marrows (BMs) before treatment, and peripheral blood (PB) was acquired during the induction treatment on days 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 21.

The non-resistant NB4 cell line was provided by Dr. Liang of the Department of Hematology, Zhongshan University, and the A549 cell line by Dr. Wang of the Department of thoracic surgery, Xiangya Hospital. The cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Corning, USA) and Penicillin-Streptomycin (Sigma, USA) at 37 °C in the presence of 5% CO_2. NB4 cells in the logarithmic growth phase were seeded in 6-well plates and treated with 1 uM all-trans retinoic acid (ATRA) and 100 uM N-Formyl-Met-Leu-Phe (fMLP) for 48 h.

The peripheral blood mononuclear cells (PBMCs), hematopoietic stem cells (HSCs), and NB4 cells were used to detect the expression of Robo1, and the serum of patients and supernatant of NB4 and A549 cells were separated to measure the expression of cytokines and Slit2, respectively. All the specimens from the patients and volunteers were obtained by abiding by the guidelines by the Helsinki declaration (revised 1975 edition, 2008) and the medical ethics committee of the First Affiliated Hospital of Guangxi Medical University.

Reagents

Mouse anti-human Robo1 primary antibody (Mouse IgG1 Clone #770,502, R&D, USA) and PE-conjugated fluorescent-labeled secondary antibody (F0102B, R&D Biotechnology, USA) for measuring the levels of Robo1 using flow cytometry. FITC-conjugated Goat anti-Mouse IgG (H + L) secondary antibody (#GAM001, MultiSciences Biotech Co., Ltd, USA) was used as a reference for Immunofluorescence assay. Mouse monoclonal primary antibody against Robo1 (2 μg/mL, R&D #770,506), human GAPDH(R&D Catalog #AF5718, USA) and anti-mouse IgG secondary antibody (#HAF007, R&D, US) were used for western blotting. PrimeScript™ RT reagent Kit with gDNA Eraser (Cat. RR047A, TaKaRa, Japan) and GoTaq® qPCR Master Mix (#A6002, Promega Corporation, USA) were used to analyze the RNA levels using quantitative real-time PCR. All-trans retinoic acid (ATRA) (R 2625, Sigma Prod., USA) was obtained. Slit2 Quantitative ELISA kit (E-EL-H0931c, Elabscience, China), IL-8 ELISA kit (#Q8000B, R&D, USA), and IL-1β ELISA kit (#DLB50, R&D, USA) were used to measure the levels of Slit2, IL-8, and IL-1β, respectively. rhIL-8 (200–08 M, Peprotech, USA) and rhSlit2-N (150-11, Peprotech, USA) were used to perform the migration assay using transwell.

Flow cytometry to test the expression of Robo1

BMCs were extracted from APL patients before treatment and PBMCs were collected from day 0 to day 21. NB4 cells treated with ATRA (1 uM) and fMLP (100 nM) were harvested for further use. Cells were centrifuged and resuspended in 0.5% BSA solution with a concentration of 2×10^6 / ml. Then, they were co-incubated with monoclonal mouse anti-human Robo1 antibodies (#770,502, R&D biotechnology, USA) with a concentration of 2.5 μg/10^6 cells for 2 h at room temperature (RT). After washing three times, a PE-conjugated fluorescent dye-labeled secondary antibody (F0102B, R&D Biotechnology, USA) was added, and the cells were incubated for 30 min at RT followed by washing three times. The expression of Robo1 was detected and analyzed using FACS Calibur (BD Biosciences, CA, USA). There were three control samples, including blank control without antibody, negative control with only secondary antibody, and IgG isotype control with IgG isotype antibody. Data acquisition was performed using the Cell Quest software.

Immunofluorescence test to measure Robo1 expression

Cells were taken and fixed in 4% phosphate-buffered paraformaldehyde overnight, then washed twice with phosphate-buffered saline (PBS), blocked with 3% goat Serum for 30 min, and labeled with mouse anti-human Robo1 primary antibody (Mouse IgG1 Clone #770,502, R&D, USA) at a 15 μg/mL dilution in 0.1% BSA buffer with PBS at 4 °C overnight. After incubation, the cells were washed 3 times with PBS and incubated with FITC-conjugated Goat anti-Mouse IgG (H+L) secondary antibody (#GAM001, MultiSciences Biotech Co, Ltd, USA) at a 1:1000 concentration at RT for 2 h in the dark. Then, the cells were washed and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min followed by examination and imaging using an inverted fluorescence microscope (IX71; Olympus, Japan) equipped with a SPOT Insight QE CCD camera and a laser-scanning confocal microscope. Scores of Robo1 expression by immunofluorescence were calculated according to the Fromowitz et al. method [20].

Western blotting analysis of Robo1

Cell proteins were extracted with RIPA buffer and the concentration was measured using the Pierce BCA kit. A total of 50 μg of protein from each sample was resolved on an SDS-PAGE and transferred to a nitrocellulose membrane, which was incubated in 5% milk in PBS containing 0.3% Tween20 overnight at 4 °C. After electrophoresis and membrane transfer, the membranes were incubated with mouse monoclonal Robo1 primary antibody (2 μg/mL, #770,506, R&D) and mouse GAPDH monoclonal antibody (1 μg/mL, #AF5718, R&D, USA) in Tris-buffered saline with Tween-20(TBST)at 4 °C overnight. Further, membranes were incubated with a secondary antibody (#HAF007, R&D, USA) at a 1:1000 dilution for 1 h at 25 °C. After washing three times with TBST,

the membranes were developed using the ECL plus Western Blotting Detection system (Thermo Fisher Scientific, USA). Membranes were exposed to radiographic film and the expression of targeted proteins was quantified by detecting specific radiographic bands using the BandScan. Immunoreactive bands were visualized using enhanced chemiluminescence (Life Technologies, Grand Island, NY).

Real-time quantitative polymerase chain reaction

The mRNA was extracted from ATRA-treated NB4 cells and PBMCs of APL patients using the total RNA extraction kit (#Z3100, Promega Corporation, USA) according to the manufacturer's instructions. The amount and purity of RNA were measured using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). A total reaction volume of 20 μl of reverse-transcribed cDNA was obtained from 2 ug of total mRNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (#RR047A, TaKaRa, Japan). The quantitative real-time PCR was subsequently performed with GoTaq® qPCR Master Mix (#A6002, Promega Corporation, USA) and 0.2 uM of primers using an ABI 7000VI sequence detection system (Applied Biosystems). The primer sequences of the target genes for qRT-PCR were as follows: Robo1 forward - 5′-CTTACACCCGTAAAAGTGACGC-3′ and reverse - 5′-TGGTCTTCTAAGACAGTCAGC-3′; β-actin forward - 5′-TGACGTGGACATCCGCAAAG-3′ and reverse - 5′-CTGGAAGGTGGACAGCGAGG-3′; IL-1β forward - 5′-TTGTTGCTCCATATCCTGTCC-3′ and reverse - 5′-CACATGGGATAACGAGGCTT-3′; IL-8 forward - 5′-CTCTTGGACGCCTTCCTGATT-3′ and reverse - 5′- TATGCACTGACATCTAAGTTCTTTAGC-3′; IL-6 forward - 5′-GTGAAAGCAGCAAAGAGGC-3′ and reverse - 5′-TGGGTCAGGGGTGGTTAT-3′; TNF-α forward - 5′-CCACCCATGTGCTCCTCA-3′ and reverse - 5′-CTGGCAGGGGCTCTTGAT-3′; CXCR1 forward - 5′-CCTGGATGCCACTGAGATTC-3′ and reverse - 5′-GCCAAGAACTCCTTGCTGAC-3′; CXCR2 forward - 5′-CACATGGGGCAGAAGCAC-3′ and reverse - 5′-GCAGGACCAGGTTGTAGGG-3′; CXCR3 forward - 5′-GGTGGTGCTGGTGGACAT-3′ and reverse - 5′-CCCCTACAAAGGCATAGAGC-3′; CXCR4 forward - 5′-GGCTGCCTTACTACATTGGG-3′ and reverse - 5′-AGCTAGGGCCTCGGTGAT-3′. Primer sequences of Slit2 were designed and synthesized by Genecopoeia biotechnology (#HQP022584, Primer ID: Hs-QRP-33,031, NM_004787, PCR size: 124 bp, Tm: 60 °C). The cycling parameters of RT-qPCR were: Hot-start activation at 95 °C for 2 min, 40 cycles at 95 °C (15 s) and 60 °C (60 s) followed by a melting curve analysis. Annealing/extension of 40 cycles at 95 °C for 15 s and 60 °C for 60 s followed by a melt curve analysis. Data were normalized against the amplification of beta-2-microglobulin as an endogenous control transcript and analyzed using the comparative threshold cycle method. The experiments were repeated 3 times, and the mRNA expression was calculated using the 2-ΔΔ CT method.

Enzyme-linked immunosorbent assay

Serum and the supernatant were separated and samples were prepared. Cytokines were quantified using quantitative ELISA kits for Slit2 (E-EL-H0931c, Elab-Science, China), IL-8 (#Q8000B, R&D, USA), and IL-1β (#DLB50, R&D, USA) according to the manufacturer's instructions. Data were analyzed using ELIASA (M200Pro, TECAN Group Ltd. Austria).

Transwell migration assay performed to evaluate the effect of IL-8 and Slit2-N on NB4 cell migration

A 24-well transwell plate (Corning, USA) was used to observe the NB4 cell migration induced by Slit2 and IL-8. Based on our experimental results and literature review, NB4 cells were treated with ATRA and fMLP for 48 h. About 0.2 ml of freshly isolated treated NB4 cells (2×10^6 / ml) resuspended in RPMI1640 supplemented with 10% heat-inactivated FBS were transferred to the upper layer of the insert. The upper inserts were placed into the 0.2 ml medium containing NB4 cells treated with 1 uM ATRA and fMLP for 48 h, and then in the RPMI 1640 medium with 0.1% BSA at 37 °C for 2 h. According to previously known results and those from our experiments, the optimum concentration of rh-Slit2 required was 0.4 mg/ml [19] and that of rhIL-8 was 20 pg/ml. rh-Slit2 (150–11, Peprotech, USA) and rhIL-8(200–08 M, Peprotech, USA) were placed in the lower layer and co-cultured in 5% CO_2 at 37 °C for 24 h. Cells that migrated through the filter into the lower wells were counted.

Statistical methods

Differences between groups were compared using one-way ANOVA with Dunnet's post-test for normally distributed data, and by the Kruskal-Wallis test followed by Dunn's multiple comparisons test for data showing non-Gaussian distribution. Incidences were compared using the Chi-square test and analyzed using Pearson or Fisher's exact test according to the minimum expected count, which was more or less than 5. The relativity of data was analyzed by Spearman's test. A value of $P < 0.05$ was considered statistically significant. All analysis was performed using the SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and the GraphPad InStat software (GraphPad Software, Inc., San Diego, CA, USA).

Results

The characterization of RAS

Of the 62 cases, complete remission was observed in 57 patients (91.94%) and the median time to complete remission was 28 days (18–38 days). Hemorrhage was the initial symptom observed in 45 patients, accounting for about 72.58%, and other symptoms included dizziness and fatigue, pain and anemia. Of the 62 APL patients, 14 patients showed a high risk (WBC $> 10 \times 10^9$/L) and 48 patients showed low and moderate risks (WBC $< 10 \times 10^9$/L). Of the 62 cases,16 patients developed RAS (25.81%) and 46 patients did not develop RAS which were incorporated into the non-RAS groups. Median cumulative dosage of retinoic acid to the development of RAS was 280 mg (180–350 mg). Of the 16 patients with RAS, 5 were male and 11 were female (male to female ratio was 1:2.2), 10 patients developed severe RAS (≥4 symptoms of RAS), including 3 patients that died, and 6 patients developed mild and moderate RAS (≤3 symptoms of RAS). Of the 16 patients, 10 patients were at high risk, 4 patients showed moderate risk and 2 patients showed low risk. Of the 10 patients with high risk, 8 patients developed severe RAS (≥4 symptoms of RAS) and 2 patients developed moderate RAS (3 symptoms of RAS). Median time to development of RAS from the start of ATRA administration was 10 days (range 3–15 days). A total of 13 of the 16 RAS patients (81.25%) achieved remission under close observation and prompt treatment, and 3 patients (18.75%) having a high risk died. We found that age and sex showed no significant differences between RAS and non-RAS groups ($p > 0.05$). However, the frequency of high-risk APL in the RAS group was significantly more common ($P < 0.001$), showing a positive correlation between the degree of risk and RAS. (Table 1).

The expression of Robo1 increased in RAS

The expression of Robo1 was compared across the different groups, including APL patients with RAS (called RAS group), APL patients without RAS (called non-RAS group), and people in the control group. Comparisons were also made across Robo1 expressions before, during, and after the occurrence of RAS.

The results of immunofluorescence showed that Robo1 was expressed in differentiated cells as RAS developed, and the score was 3–4 according to the Fromowitz et al. method [20]. There was no expression (score: 0–1) in non-RAS, control, and RAS groups after the

Table 1
The characteristics of the 62 cases of APL.

Item		RAS(No.) Positive (16)	Negative (46)	*P*
Sex	Male	5	19	0.561
	Female	11	27	
Age (year)	16–35	5	13	0.487
	36–60	8	29	
	>60	3	4	
Risk stratification	High	10	4	0.000
	Middle	4	30	
	Low	2	12	
Preventive use of dexamethasone	Yes	1	8	0.425
	No	15	38	
IL-8 upregulation	Yes	16	4	0.000
	No	0	42	
Slit2 upregulation	Yes	16	2	0.000
	No	0	44	
Robo1 upregulation	Yes	16	2	0.000
	No	0	44	

Note: The upregulation of IL-8 and Slit2 and Robo1 means the range of values above that tested in the control group in our article. IL-8 and Slit2 were 0.72–1.16 0pg/ml and 71.68–181.82 pg/ml by enzyme-linked immunosorbent assay method, Robo1 was 2.40–4.18% by flow cytometry in the control group.

alleviation of RAS (Fig. 1 A1–4). Similarly, in the RAS group, the expression of Robo1, as measured using flow cytometry, in differentiated cells was only 2–3% before treatment, which rose to about 11–30% when RAS developed, and decreased to about 3–5% after RAS alleviated, and the differences were significant ($P < 0.001$). The expression was 2–5% in the non-RAS and control groups ($P < 0.001$). The severity of RAS positively correlated with the expression of Robo1 by the method of Spearman's coefficient ($r = 0.893$, $P < 0.001$) (Fig. 2, Tables 2–4). We detected the expression of Robo1 using the western blotting and RT-PCR method (Fig. 1C). Similarly, the mRNA and protein levels of Robo1 were upregulated only when RAS developed in the RAS group, showing a positive correlation with RAS. There was no Robo1 expression in NB4 cells and that in ATRA-treated NB4 cells was lower than ATRA- and fMLP-treated NB4 cells by RT-PCR method.

The upregulation of Slit2 in serum was correlated to RAS

The gene expression of Slit2 by RT-PCR in APL cells and NB4 cells was significantly lower than that in the control group ($P < 0.001$), and there was no significant increase as RAS developed. However, it was significantly higher in A549 cells treated with ATRA than that in NB4 and APL-patient cells ($P < 0.001$), and it was higher than that in A549 cells without ATRA ($P < 0.001$).

In the APL patients, the level of Slit2 in serum examined by ELISA rose to about 1.55 ug/ml when RAS developed, which was higher ($P < 0.001$) than that in the other two groups (about 0.05–0.16 ug/ml). The level of Slit2 was only 0.04–1.05 ug/ml in the RAS group before the development and after the alleviation of RAS. Also, the higher the Slit2 expression, the more severe was RAS, as calculated using Spearman's rank-order coefficient ($r = 0.808$, $P = 0.001$) (Tables 2–4). The high level of Slit2 (about 2 ug/ml) was detected in the supernatant of ATRA-treated A549 cells but was only 0.05 ug/ml in ATRA-treated NB4 cells.

The level of cytokines in RAS

The gene expression of cytokines and cytokine receptors, including IL-8, IL-1β, TNF-a, IL-6, CXCR1,CXCR2,CXCR3 and CXCR4, was detected in differentiated cells and ATRA-treated NB4 cells using RT-PCR. The results showed that the gene expression of Il-8 upregulated at 48 h in ATRA-treated NB4 cells, which was ten times that at 0 h ($r = 0.732$, $P < 0.001$). The expression of Il-1β was four times that at 0 h ($P < 0.001$). TNF-a and IL-6 showed no obvious changes in ATRA-treated NB4 cells ($P > 0.05$). The gene expression of CXCR1, CXCR2 and CXCR3 increased gradually and that of CXCR4 changed slightly with the induction treatment. We detected the gene expression of cytokines in 16 APL patients with RAS. The results showed that only IL-8 rose to about 20–30 times when RAS developed among the detected cytokines. It increased significantly compared to that in the non-RAS and control groups (Fig. 3) and showed a positive correlation with RAS ($r = 0.560$, $P < 0.001$). IL-8 and IL-1β might be two important factors in RAS.

We detected the levels of IL-8 and IL-1β in the serum of APL patients and supernatant of NB4 cells using ELISA method. In the APL patients, the concentration of IL-8 in the serum in the RAS group was about 7 pg/ml, which was higher than that in the control and non-RAS groups ($P < 0.001$). The IL-8 level increased with the development of RAS and decreased as RAS alleviated, showing a positive association with the severity of RAS using Spearman's test ($r = 0.783$, $P < 0.001$) (Table 4). The level of IL-1β in the RAS group was about 4–6 pg/ml, which was a higher than that in the other groups ($P < 0.001$); however, there was no obvious relationship between the level of IL-1β and the degree of RAS ($r = 0.363$, $P = 0.246$) (Tables 2–4). The level of IL-8 increased obviously in the supernatant of NB4 cells after the treatment with ATRA ($P < 0.001$) and there was a slight increase in the level of IL-1β in the supernatant of NB4 treated by ATRA ($P = 0.008$).

The migration of NB4 cells induced by rhSlit2-N and rhIL-8

NB4 cells were transferred in the upper chamber after treatment with ATRA and fMLP for 48 h followed by starvation for 12 h. rhSlit2 (0.40 g/L) was added to the lower layer of the transwell. The results showed that the frequency of migration of NB4 cells to rhSlit2 was 23.21%, higher than that to FBS 9.64% ($P = 0.002$). The frequency of migration of NB4 cells was only 12.60% ($P = 0.044$) when Robo1 antibody (2 ug/10^5 cells) was added to the upper layer of the transwell (Fig. 1B, D). The rate of NB4 cell migration toward ATRA-treated A549 cells was 23.47% at 2 h, higher than that towards FBS 5.02% ($P < 0.001$) (Fig. 1D).

Similarly, treated NB4 cells starved for 12 h in the upper layer of the transwell preferred to migrate to rhIL-8 (15 ng/L) in the lower layer, and the percentage of cells was about 19.83% at 2 h, higher than that with FBS in the lower layer (7.86%, $P < 0.001$). The frequency of migration (8.11%) was not different even when the Robo1 antibody (2 ug/10^5 cells) was added to the upper layer of the transwell. The frequency of NB4 cells migration towards A549 cells added with rhIL-8 was higher than that to rhIL-8 or A549 cells alone (Fig. 1D).

Slit2/Robo1 did not inhibit the chemotaxis of NB4 cells induced by rhIL-8

We determined whether the migration of NB4 cells induced by IL-8 is affected by Robo1 and Slit2. When rh-IL8 was added to the lower layer and Slit2-N to the upper and lower layers separately, the frequencies of migration were 19.85%, 23.22%, and 45.67%, respectively. Although there was a decrease in the number of cells migrating with Slit2-N in the upper layer and an increase with Slit2-N in the lower layer, the difference was not significant ($P > 0.05$). The results indicated that cell migration induced by rhIL-8 was not inhibited by Slit2-N.

Discussion

RAS is an important cause of death in the induction therapy of APL. The exact mechanisms underlying which were unclear and the diagnosis depends on clinical observation. In the current study, RAS developed in 16 of the 62 patients, accounting for 25.81%. Of the 16 patients 3 (18.75%) patients died. A high risk of a high WBC count at diagnosis was found in 10 patients happened to develop RAS. Our results are in agreement with a report that high WBC (more than 10×10^9/L) was a risk factor for differentiation symptoms [21].

Mechanisms underlying RAS are known to involve two aspects - one is that several cytokines are secreted by differentiated promyelocytes

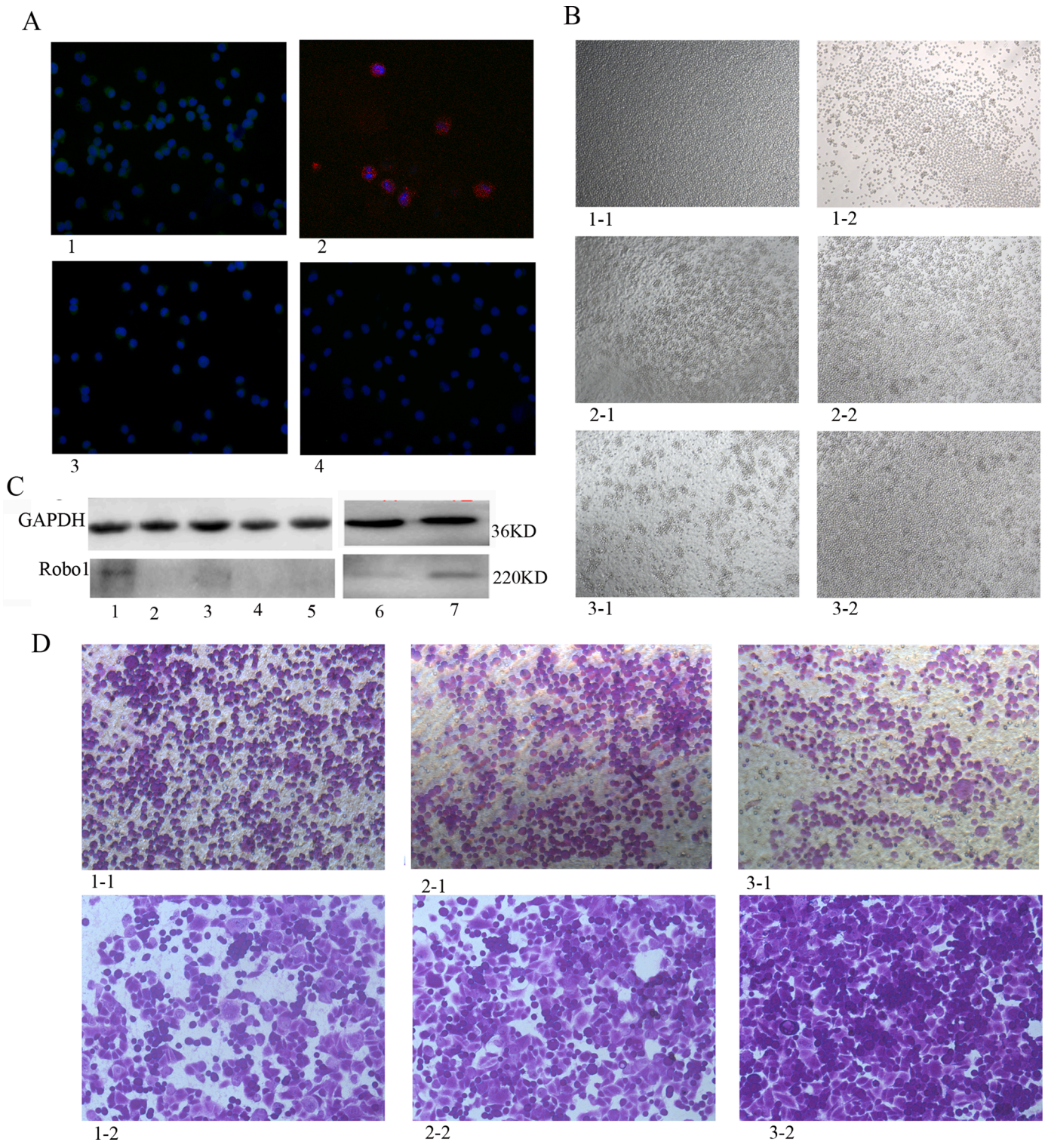

Fig. 1. (A) Results of Robo1 in differentiated cells by immunofluorescence. 1–2, from RAS+ patients; 3, from RAS- patients; and 4, from the control individual. (B) Transwell assay results of NB4 cells as rhIL-8 and Slit2-N were added to the lower layer. 1–1, 2–1, and 3–1 showed the NB4 cells in the upper layer after migration; 1–2, 2–2, and 3–2 showed the migrated NB4 cells in the lower layer; 1–2 showed the FBS in the lower layer, 2–2 showed the addition of Slit-N; 3–2 showed the addition of rhIL-8 and Slit2-N. The migration frequency increased as rhIL-8 and Slit2-N were added to the lower layer. (C) Robo1 protein estimation using western blotting: line 1, when RAS developed in a patient; line 2, before the development of RAS; line 3, after the development of RAS; line 4, in an APL patient without RAS; line 5, in a control; line 6, in a mild-RAS patient; line 7, in a severe-RAS patient. (D) Transwell assay results of co-culture of NB4 and A549 cells with rhIL-8 and Slit2-N. 1–1, 2–1, and 3–1 show the rest NB4 cells in the upper layer after migration; 1–2, 2–2, and 3–2 show the migrated NB4 cells and A549 cells in the lower layer; 2–2 shows the addition of rhIL-8; 3–2 shows the addition of rhIL-8 and Slit2-N. The migration frequency increased as rhIL-8 and Slit2-N were added to the lower layer.

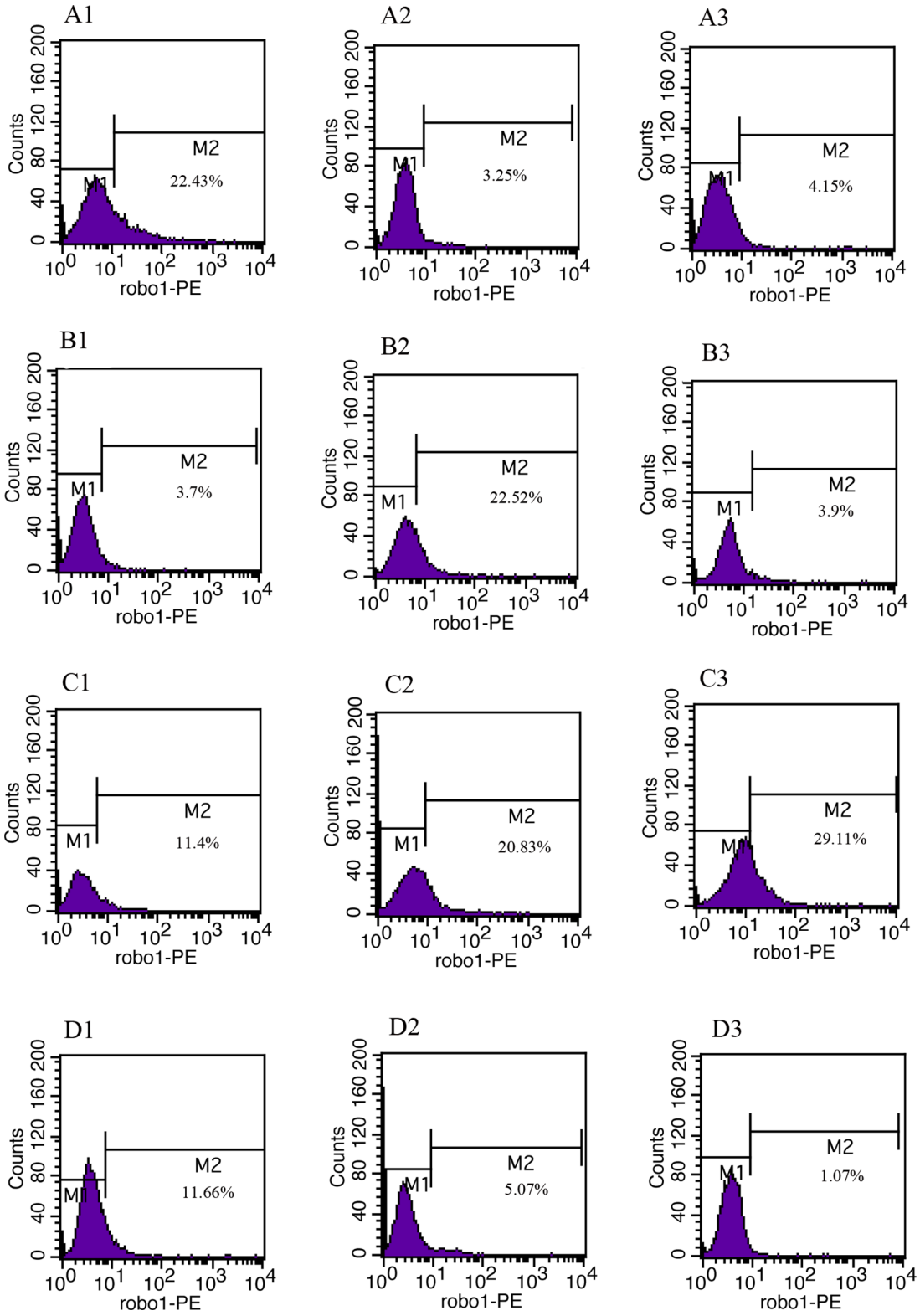

Fig. 2. The Robo1 expression in APL patients and control individuals. A1-A3, the Robo1 levels in different groups; A1, in APL patient with RAS; A2, in APL patient without RAS; A3, in control people. B1-B3, the expression of Robo1 in an APL patient during the development of RAS; B1, before RAS; B2, during RAS; B3, after RAS. C1-C3, the frequency of Robo1 in RAS+ patients; C1, in a mild-RAS patient; C2, in a moderate-RAS patient; C3, in a severe-RAS patient. D1-D3, The level of Robo1 in NB4 cells, the frequency in ATRA- and fMLP-treated NB4 cells, ATRA-treated NB4 cells and untreated NB4 cells.

induced by ATRA alone or combined with arsenite, and the other is that multiple differentiated cells are induced by the cytokines to infiltrate the organs, leading to RAS [1,18]. The use of corticosteroids contributes to cure RAS, but it was not sufficient to improve the outcomes [1]. Still, 5–9% of patient deaths are associated with RAS [18]. A study suggested that the mechanism underlying RAS is similar to inflammatory response and ARDS [22]. The infiltration of activated leukocytes through alveolar epithelium induced by cytokines secreted by lung cells is an important

Table 2
The differences in Slit2, Robo1, and cytokines between RAS+ and other groups.

Item	Method	RAS+(16 cases)	RAS -(46 cases)	Control group (20 cases)	P*
Robo1 (%)	FCM	17.06 ± 6.33	2.45 ± 0.77	3.29 ± 0.89	0.000
IL-8 (pg/ml)	ELISA	6.41 ± 2.40	1.02 ± 0.39	0.94 ± 0.22	0.000
Slit2 (pg/ml)	ELISA	1549.38 ± 540.33	45.06 ± 12.89	126.75 ± 55.07	0.000
IL-1β (pg/ml)	ELISA	4.17 ± 0.74	1.87 ± 0.64	1.20 ± 0.49	0.000

Note: FCM: Flow cytometry, ELISA: Enzyme-linked immunosorbent assay.

Table 3
The correlation of Slit2, Robo1, and cytokines with the development of RAS in 16 patients.

Item	Method	#Before RAS	*At RAS	#After RAS	*P**	*P#*
Robo1 (%)	FCM	2.25 ± 0.64	17.06 ± 6.33	2.63 ± 1.09	0.000	0.563
IL-8 (pg/ml)	ELISA	1.05 ± 0.13	6.41 ± 2.40	1.14 ± 0.19	0.000	0.371
Slit2 (pg/ml)	ELISA	35.31 ± 9.37	1549.38 ± 540.33	45.06 ± 12.34	0.000	0.157
IL-1β (pg/ml)	ELISA	2.17 ± 0.62	4.17 ± 0.74	2.57 ± 0.78	0.000	0.121

Note: FCM: Flow cytometry, ELISA: Enzyme-linked immunosorbent assay.

Table 4
Correlation between the expressions of Slit2, Robo1, and IL-8 and the severity of RAS.

Item	Method	RAS (16patients) Mild (+)	Moderate (++)	Severe (+++)	*P*	r
Robo1 (%)	FCM	11.25 ± 1.71	14.17 ± 2.48	23.83 ± 4.49	0.000	0.893
Slit2 (ug/ml)	ELISA	1.09 ± 1.87	1.34 ± 0.30	2.07 ± 0.45	0.001	0.808
IL-8 (pg/ml)	ELISA	3.15 ± 0.91	6.40 ± 1.05	6.60 ± 1.21	0.000	0.783
IL-1β (pg/ml)	ELISA	3.63 ± 0.57	4.32 ± 0.77	4.38 ± 0.33	0.246	0.363

Note: FCM: Flow cytometry, ELISA: Enzyme-linked immunosorbent assay.

step in the development of ARDS [23]. The study found that the expression of Slit2 and Robo1 upregulated during inflammation [9,24]. So Slit2 and Robo1 were shown a correlation with cells migration in inflammatory diseases and tumors.

In the present study, the expression of Robo1 in APL patients was low before treatment, even lower than that in healthy people. However, we found that the expression of Robo1 in differentiated cells upregulated in APL patients during the development of RAS and was negative in healthy people and APL patients without RAS. Furthermore, we found that the more serious the RAS, the higher expression of Robo1. The expression of Robo1 began to rise consistently with the development of RAS and was downregulated when RAS was alleviated. A study found that Robo1 was upregulated in response to inflammation in the brain [9]. The expression of Robo1 was low in HL-60 cells and increased when the cells were stimulated with fMLP [8]. Another study identified Robo1 as a factor that promoted inflammation [16]. A study proved that a change in the level of Robo1 may be related to the changes in the micro-environment due to the different stages during the development of a tumor, which is often accompanied by chronic inflammation [24]. Robo1 might be a useful factor for the diagnosis and treatment of RAS.

Therefore, upregulation of Robo1 expression in promyelocytes by certain factors may be related to the occurrence and development of RAS. The expression of Robo1 was not significantly upregulated in NB4 cells treated with ATRA. According to literature, the expression of Robo1 was upregulated in HL-60 cells treated with fMLP, suggesting that ATRA may not promote the expression of Robo1 in differentiated cells but fMLP and cytokines may. When RAS develops in APL patients, a variety of cytokines are secreted, and it may be accompanied by infection. Our study showed that the expression of Robo1 was higher in APL patients with RAS, which might be due to stimulation by the cytokines in RAS and underlying mechanisms are worth exploring.

We found that the expression of Slit2 protein in newly diagnosed APL patients was lower than that in healthy people but it was not significantly different. *SLIT2* gene methylation was observed in acute myelocytic leukemia (AML), chronic lymphocytic leukemia (CLL), and blood tumor cells [25]. The secretion of Slit2 correlated with the development of RAS, and it decreased when RAS was alleviated, while it did not decrease in the 3 patients who died of RAS. Certain studies showed that Slit2 and Robo1 were upregulated in animals during infection (skin and brain inflammation) [9,11].In ATRA-treated NB4 cells, no upregulation in Slit2 was detected, while a high level of Slit2 was found in A549 cells treated with ATRA. Studies have found that Slit2 protein is mainly secreted by the lung, heart, kidney, and brain tissues [8], and the concentration gradient of lung Slit2 protein gradually increases from alveoli to the trachea [12].

IL-8 and IL-1β levels in APL patients before treatment were found to be comparable to that in the control group and elevated as RAS developed. They also increased in ATRA-treated NB4 cells, especially IL-8. It has been found to induce leukocyte infiltration into the lungs [26]. A study showed that the activation of NF-KB in differentiated cells during the treatment with ATRA promotes the secretion of various cytokines [27]. Accordingly, we considered IL-8 as the main cytokine involved in and closely related to RAS.IL-8, Slit2, and Robo1 levels were elevated when RAS developed, and the increase in IL-8 was detected before that of Slit2 during RAS. A study found that cytokines promoted the secretion of Slit protein [14]. In our study, there was no significant increase in cytokines in patients with renal injury, but an increase in Slit2 protein was observed. The increase in Slit2 protein in RAS may be related to the organs damage by cytokines.

In our study, the migration of NB4 cells increased as Slit2-N was added to the lower layer and decreased as Slit2-N was added to the upper layer. It is possible that NB4 cells tended to have a high Slit2-N concentration. In the study by Wang et al., SLIT2-N induced the migration of HUVECs from the low-concentration area of S2-N to the high-concentration area [14]. In drosophila, the Slit protein acted as a chemokine to induce the migration of Robo-positive cells to Slit-enriched regions [15]. Certain studies have shown that the Slit2 protein has an inhibitory effect on chemokine-induced leukocyte migration [10,11]. We found that IL-8 induced cell migration in the lower layer, but the addition of Slit2-N did not reduce cell migration, suggesting that Slit2-N did not inhibit IL-8-induced cells migration.

Cytokines secreted in APL patients led to lung damage and stimulated the secretion of Slit2 protein. Il-8 induced the migration of inflammatory cells into the lungs, suggesting that the lungs are the preferred site. In the study by London, Slit2 was shown to mainly maintain endothelial integrity, stabilize vascular intima, and reduce permeability through endothelial Robo4, rather than inhibiting chemotaxis of Robo1-positive cells in ARDS, sepsis, and other inflammatory reactions [17]. The expression of Slit2 and Robo1 increased in atherosclerosis, asthma, and skin allergy [11,12].

In the present study, of the 9 APL patients that received 400 ml plasma daily for 10 days, only 1 high-risk patient developed RAS. It is considered that plasma infusion may play a certain role in reducing the concentration of Slit2 protein. Therefore, the upregulation of Robo1 and Slit2 in our study might be correlated with the development of RAS, and

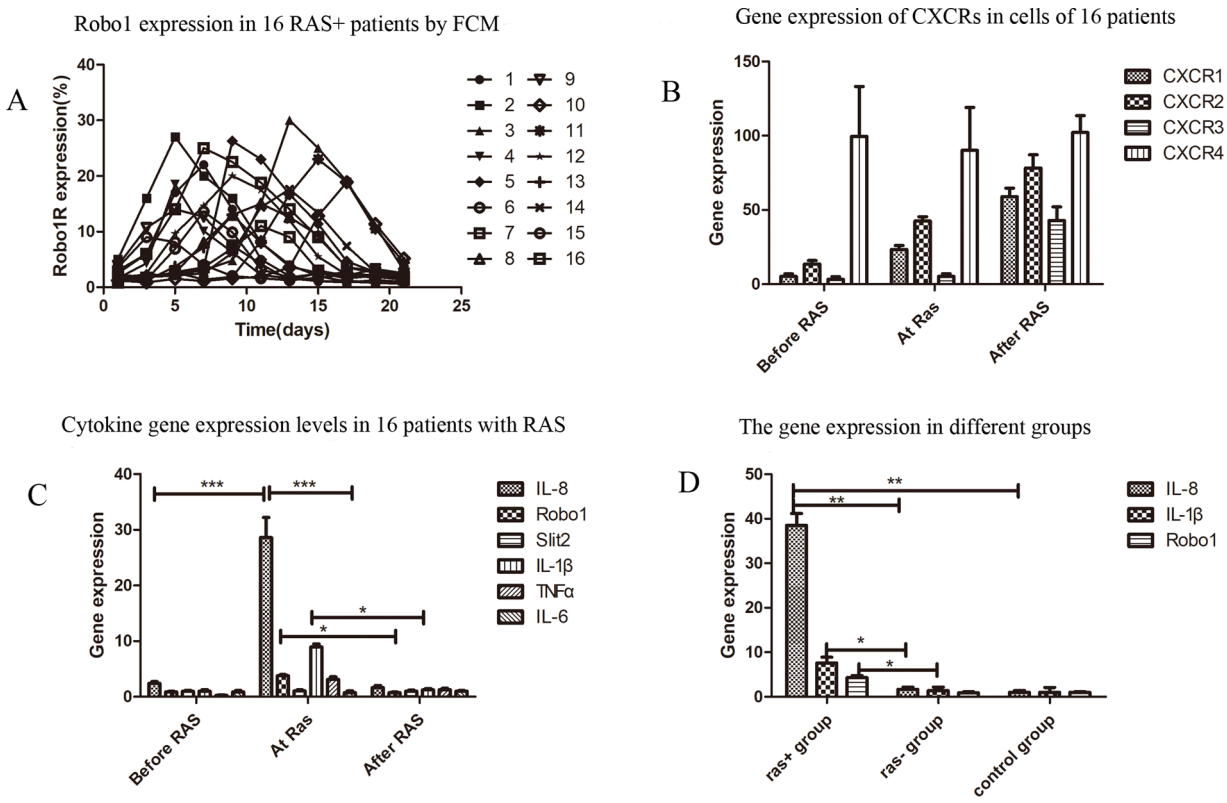

Fig. 3. (A) Robo1 expression measured using FCM in 16 APL patients with RAS from day 1 to day 21, showing the development of RAS from 9% to 30%. (B-C) The gene expression of CXCR1, CXCR2, CXCR3, CXCR4, Robo1, Slit2, and cytokines in differentiated cells of the 16 APL patients with RAS. CXCR1, CXCR2, CXCR3, and CXCR4 showed no relation to RAS. The expression of IL-8, IL-1β, and Robo1 upregulated when RAS developed. (D) Robo1, IL-8, and IL-1β in different groups, results showed they were higher in the RAS+ group than the RAS- and control groups.

might contribute to the migration of differentiated cells.

Conclusion

In the present study, the expression of Robo1 in differentiated cells was significantly upregulated in APL patients with RAS and NB4 cells treated with ATRA and fMLP. We think that the increase of Robo1 might be related to RAS. The expression of Slit2 protein upregulated in the serum of APL patients during the development of RAS, while it did not significantly increase in NB4 cells suggesting that Slit2 was probably mainly released not by differentiated cells. IL-8 was upregulated in the serum of APL patients during RAS and supernatant of NB4 cells treated with ATRA and might be secreted by the differentiated cells. In the present study, Slit2 and IL-8 were involved in the cell migration. Conclusion, we think that Robo1/Slit2 and IL-8 might be closely related to the occurrence and development of RAS. However, the exact underlying mechanism needs to be further elucidated.

Declarations

Ethics approval and consent to participate

CRediT authorship contribution statement

Haiyan Yang: Writing – review & editing, Visualization. **Shengsheng Zhou:** Writing – review & editing. **Dong Lan:** Investigation, Writing – review & editing. **Yehong Bin:** Investigation, Writing – review & editing. **Wenguang Bao:** Methodology, Writing – review & editing. **Man Wang:** Methodology, Writing – review & editing. **Fengxiang Huang:** Methodology, Writing – review & editing. **Zhigang Peng:** Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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