RESEARCH ARTICLE

Prognostic Significance of CD44v6/v7 in Acute Promyelocytic Leukemia

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Abstract

CD44v, especially splice variants containing exon v6, has been shown to be related closely to development of different tumors. High levels of CD44v6/v7 have been reported to be associated with invasiveness and metastasis of many malignancies. The objective of this study was to detect expression of CD44v6-containing variants in patients with acute promyelocytic leukemia (APL) and evaluate the potential of CD44v6/v7 for risk stratification. Reverse transcription polymerase chain reaction (RT-PCR) followed by PCR product purification, ligation into T vectors and positive clone sequencing were used to detect CD44 v6-containing variant isoforms in 23 APL patients. Real-time quantitative PCR of the CD44v6/v7 gene was performed in patients with APL and in NB4 cells that were treated with all-trans retinoic acid (ATRA) or arsenic trioxide (As_2O_3). Sequencing results identified four isoforms (CD44v6/v7, CD44v6/v8/v10, CD44v6/v8/v9/v10, and CD44v6/v7/v8/v9/v10) in bone marrow mononuclear cells of 23 patients with APL. The level of CD44v6/v7 in high-risk cases was significantly higher than those with low-risk. Higher levels of CD44v6/v7 were found in three patients with central nervous system relapse than in other patients inthe same risk group. Furthermore, in contrast to ATRA, only As_2O_3 could significantly down-regulate CD44v6/v7 expression in NB4 cells. Our data suggest that CD44v6/v7 expression may be a prognostic indicator for APL.

Keywords: CD44v - acute promyelocytic leukemia - extramedullary relapse - prognosis - As_2O_3

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Introduction

CD44 is a transmembrane glycoprotein, a member of a family of cell adhesion molecules, and a mediator of interaction between bone marrow stromal cells (BMSCs) and leukemia cells. CD44 can be divided into standard and variant isoforms: CD44 standard (CD44s) and CD44 variant (CD44v). The CD44v gene has ten variant exons (v1–v10) which can be selectively spliced, leading to production of different CD44 variant isoforms (Ponta et al., 2003). Of these CD44 variants, the v6-containing variant isoforms have been reported to correlate with metastasis and infiltration in multiple cancers (Herold et al., 1996; Naor et al., 2008; Yu et al., 2010; Zhang et al., 2012).

The higher frequency of CD44 v6-containing variant isoforms were found in mononuclear cells (MNCs) from patients with chronic myelogenous leukemia (CML) and lymphoma than in MNCs from healthy donors (Akisik et al., 2002). CD44v6/v7 was demonstrated to be the principal isoforms able to bind osteopontin (OPN), leading to the initiation of a signal transduction cascade that may result in phosphorylation of some kinases such as phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and protein kinase C (PKC), and consequently in suppression of apoptosis. In addition, this process can activate matrix degradative enzymes such as urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs), resulting in invasion and metastasis of cancers (Katarigi et al., 1999; Chakraborty et al., 2006; Pamela, et al., 2009; Zhang et al., 2010) .

Acute promyelocytic leukemia (APL) is a special subtype of acute myeloid leukemia (AML). Based on the white blood cell count at initial treatment, the patients with APL can be classified as high-risk and low-risk (Avvisati et al., 2011). The expression of v6-containing variant isoforms in APL cells and the role of CD44v6/v7 for risk stratification in APL have not been reported. In the present study, the expression of v6-containing variant isoforms in bone marrow MNCs was detected in 23 patients with APL. Sequencing results have identified four isoforms (CD44v6/v7, CD44v6/v8/v10, CD44v6/v8/v9/v10, and CD44v6/v7/v8/v9/v10). In further experiments, expression of CD44 v6/v7 mRNA was analyzed in bone marrow MNCs from patients with high-risk and low-risk APL in order to determine whether v6/v7 mRNA expression could be used for risk stratification in APL.

All-trans retinoic acid (ATRA) and arsenic trioxide (As_2O_3) are the classic drugs used for induction therapy of APL. As compared to ATRA treatment, As_2O_3 treatment leads to higher molecular remission rate and longer disease-free survival time (Zheng et al., 2007). This

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may be attributed to the elimination of leukemia stem cells (LSCs) by As_2O_3 (XW et al., 2010). However, it remains unclear whether As_2O_3 can block the interaction between LSCs and the surrounding microenvironment. In the present study, the effects of ATRA and As_2O_3 on CD44v6/v7 expression in NB4 cells were investigated in order to determine whether the different regulation effects on adhesion molecules result in the different therapeutic efficacy between ATRA and As_2O_3.

Materials and Methods

Subjects

A total of 23 patients with newly diagnosed APL (12 males, 11 females; median age 35 years [range: 17–51]) were recruited from the Department of Hematology, Affiliated Union Hospital of Fujian Medical University. The diagnosis of APL was based on World Health Organization (WHO) 2008 criteria. The clinical characteristics of these patients are shown in Table 1 and include age, genders, peripheral white blood cell (WBC) count and proportion of leukemia cells in all bone marrow nucleate cells. Bone marrow was collected and MNCs were isolated using standard methods (Kortlepel et al., 1993). No collections were performed without the consent of the subjects involved.

Cell culture

The human APL cell line NB4 was kindly provided by the Dr. P. Z. Zheng. NB4 cells were maintained in continuous culture in RPMI 1640 (Invitrogen, Carlsbad, CA, USA), supplemented with heat-inactivated fetal bovine serum (FBS, 10%), penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (0.25 mM) at 37 °C in 5% CO_2 humidified air. The viability of cells was determined by Trypan Blue dye exclusion. Cells were maintained in log phase with viability greater than 95%.

Table 1. Patient Data

Patient no.	Age/sex	WBC($\times 10^9$/L)	%Blasts
1	17/F	94.2	90
2	47/M	7.1	86
3	47/F	1.0	61
4	38/M	7.6	31
5	29/M	25.5	62
6	26/M	0.5	47
7	37/M	3.1	42
8	32/F	23.6	72
9	47/F	94.19	62
10	20/M	1.4	45
11	19/F	19.26	48
12	32/F	11.4	56
13	23/M	16.3	43
14	51/F	11.0	52
15	32/F	210	56
16	52/F	2.8	42
17	23/M	16.7	47
18	51/F	3.46	40
19	33/F	3.68	44
20	38/M	43.16	37
21	50/M	3.39	40
22	17/M	3.4	41
23	29/M	1.2	33

WBC, white blood cells; %Blasts, proportion of leukemia cells in bone marrow all nucleate cells

Sequencing of v6-containing variant isoforms in bone marrow MNCs from APL patients

Total RNA was extracted with TRIZOL (Invitrogen) and used for reverse transcription into cDNA. In the presence of DNA polymerase (Promega, Madison, WI, USA), PCR was performed using previously described procedures (LJ et al., 2000): pre-denaturation at 95°C for 5 min; 35 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 30 s and extension at 72°C for 60 s followed by a final extension at 72°C for 10 min. The primers in the PCR were as follows: β-actin: 5'AGTGTGACGTGGACATCCGCAAAG3' (forward), 5'ATCCACATCTG CTGGAAGG TGGAC 3' (reverse); CD44S-v6: 5'TCCAGGCAACTCCTAGTA 3' (forward), 5'-AGTCCACTTGGCTTTCTGTC-3' (reverse); CD44v6-S: 5'-GACGAAGACAGTCCCTGGATCA-3' (forward), 5' CAGCTGTCCCTGTTGTCG3' (reverse). Primers were synthesized by Guangzhou Yingwei Chuangjin Co., Ltd, China. The PCR products were collected and purified by Gel DNA Extraction Kits (Shanghai Sangon, Co., Ltd., China). The purified products were then ligated into T vectors (Promega), which were then used to transfect competent Escherichia coli JM-109. A total of 50 bacterial clones were selected per patient. Then PCR identification and positive clones sequencing were performed.

CD44v6/v7 expression in bone marrow MNCs from APL patients

The QuantiTect SYBR Green PCR Kit and a real-time PCR instrument (ABI PRISM7500, Applied Biosystems, Foster City, CA, USA) were employed to measure the expression of CD44v6/v7 mRNA in bone marrow MNCs. The reaction mixture (25 μl) consisted of 12.5 μl of 2×QuantiTect SYBR Green PCR Master Mix (ABI), 0.2 μmol/L each primer, 2.5 μl of cDNA, and RNase-free water. The primers were as follows: the primers for β-actin (mentioned above); CD44v6/v7: 5'TCCAGGCAACTCCTAGTA 3' (forward), 5'CCATCCTTCTTCCTG 3' (reverse). The PCR conditions were as follows: pre-denaturation at 95°C for 15 min followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. Each reaction and each experiment were done in triplicate, and β-actin served as an internal reference. Data analysis was performed using the $2^{-\triangle CT}$ method.

Effects of ATRA and As_2O_3 on CD44v6/v7 mRNA expression in NB4 cells.

NB4 cells (1×10^5/ml) were treated with 1 μmol/L ATRA (Sigma, St.Louis, MO, USA) and 1 μmol/L As_2O_3 which was a gift from Dr.M.J.Huang, respectively, for 72 h and harvested. QuantiTect SYBR Green PCR Kit was employed for the detection of expression of CD44v6/v7 mRNA. The reaction mixture and condition were the same as mentioned above.

Statistical analysis

Data obtained from the three independent experiments

Table 2. Analysis of V6-containing CD44v Molecules in Bone Marrow Mononuclear Cells from 23 Patients with Acute Promyelocytic Leukemia

Type no.	v6-containing exons	Size(bp)
1	v6	414
2	v6 v7	545
3	v6 v8 v10	718
4	v6 v8v9 v10	807
5	v6 v7 v8 v9 v10	938

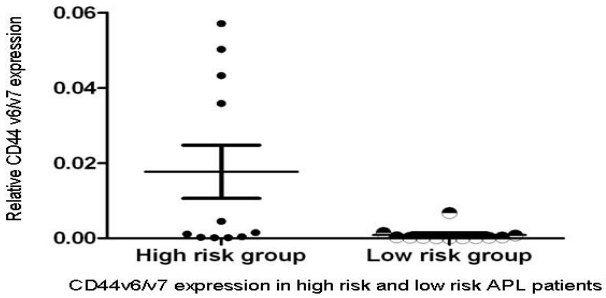

Figure 1. Relative CD44v6/v7 Expression in APL Patients with High Risk and Low Risk. Calculation of CD44v6/v7 expression was done using the $2^{-\triangle CT}$ method ($\triangle CT=CT_{target\ gene} - CT_{internal\ reference}$) and β-actin served as an internal reference

were expressed as mean ± standard deviation (SD). Statistical analysis was performed by either Student's unpaired two-tailed t-test or one-way analysis of variance (ANOVA) using SPSS 17.0 software. Values of $P<0.05$ were considered statistically significant.

Results

Sequencing of v6-containing variant isoforms from bone marrow MNCs of APL patients

Different sizes of v6-containing variant isoforms were identified. The PCR products were ligated into T vectors and positive clones were selected for sequencing. Four types of v6-containing variant isoforms (CD44v6/v7, CD44v6–v10, CD44v6/v8/v10, and CD44v6/v8/v9/v10) were detected and their respective sizes are shown in Table 2.

CD44v6/v7 mRNA expression in APL patients

At the time of initial treatment, APL could be classified as high-risk APL (WBC count $>10.0\times10^9$/L) and low-risk APL (WBC count $\leq10.0\times10^9$/L). As shown in Figure 1, CD44v6/v7 expression was higher in MNCs from APL patients with high- risk than from the patients with low-risk (0.01771 vs 0.0008607, $P<0.01$). Of note, CD44v6/v7 expression was higher in all the 3 patients (2 with high-risk, 1 with low-risk) with extramedullary relapse (central nervous system relapse) than in other patients at the same risk group. The CD44v6/v7 expression of the 2 patients with high-risk was 3.14- and 4.17-times the median level of CD44v6/v7 expression of the all high-risk patients, respectively. On the other hand, the expression of the 1 patient with low-risk was 7.8-times the median level of CD44v6/v7 expression for all patients with low- risk APL.

Effects of ATRA and As_2O_3 on CD44v6/v7 mRNA expression in NB4 cells.

NB4 cells were treated with 1 μmol/L ATRA or As_2O_3 for 72 h, harvested, and assayed by real-time PCR to measure CD44v6/v7 mRNA. CD44v6/v7 expression was reduced to 86.55±16.43% of the pretreatment level after ATRA treatment and 33.82±14.80% of the pretreatment level after As_2O_3 treatment. As_2O_3 treatment led to a significantly greater reduction in CD44v6/v7 expression ($P<0.01$).

Discussion

The CD44v gene has 10 variant exons, which may produce different CD44v variant isoforms with distinct functions after selective splicing. In the present study, four types of v6-containing variant isoforms (v6/v7, v6/v8/v10, v6/v8/v9/v10 and v6–v10) were found in the MNCs of 23 patients with APL .CD44v6/v7 expression has been positively associated with the invasion and metastasis of some solid cancers (such as gastric cancer and colon cancer) (Chun et al., 2000; Cristiana et al., 2010). Transfection of non-invasive rat pancreatic cancer cells with v6/v7-containing vectors was shown to confer the ability to metastasize (Wolfgang et al., 1993). This ability may attribute to the binding of CD44v6/v7 encoded proteins to OPN, which then activates downstream signaling pathways (such as the PI3K/Akt pathway), resulting in the activation of promoting cell proliferation and inhibiting cell apoptosis. In addition, it may activate some matrix degrading enzymes, leading to the invasiveness and metastasis of cancer cells (Marroquin et al., 2004). The bone marrow of AML patients has significantly higher OPN level than that of healthy controls, and high OPN expression is a predictor of poor prognosis in AML patients (Powell et al., 2009).

APL is a subtype of AML and is characterized by PML/RARα fusion protein (Brown et al., 1994). After regular induction and sequential therapies, the remission rate and disease-free survival time are usually higher in patients with APL than patients with AML of other subtypes (Hu et al., 1999; Fenaux et al., 2000; Tallman et al., 2002). However, extramedullary relapse, especially central nervous system relapse, is still an important factor affecting the long term disease-free survival of APL patients (Colovic et al., 2002). Although detection of PML/RARα fusion protein is a sensitive indicator of early hematological relapse, the diagnosis of extramedullary relapse usually relies on definite symptoms, signs, and related laboratory findings. However, the monitoring mentioned above usually could not be performed promptly. To date, no sensitive strategy has been developed for monitoring extramedullary relapse of APL. In the present study, CD44v6/v7 expression was markedly higher in APL patients with high-risk than in patients with low-risk, and higher in all three patients with extramedullary relapse (central nervous system metastasis) than in other patients at the same risk group. This is consistent with the finding that CD44v6/v7-transfected benign pancreatic tumor cells acquire invasive ability. Thus, we speculate that leukemia cells from APL patients with high CD44v6/v7 expression are more susceptible to release from the bone marrow, and more invasive than cells with low CD44v6/v7 expression.

More studies with a larger sample size will be required to confirm whether CD44v6/v7 expression in MNCs from APL patients receiving initial treatment can be used as a predictor of extramedullary metastasis.

Currently, the induction therapy in APL patients receiving initial treatment is usually ATRA or As_2O_3. Both of them may achieve similar rates of hematologic remission, but As_2O_3 achieves a higher molecular remission rate and disease-free survival rate than ATRA. This may well be because As_2O_3 can specifically degrade PML/RARα protein and impair the self-renewal of leukemia initiating cells (XW et al., 2010). Our previous studies showed As_2O_3 but not ATRA could downregulate CD44v6 expression in NB4 cells. And the present study indicateed the inhibitory effect of As_2O_3 on CDv6/v7 expression was more evident than that of ATRA. Thus, we postulate that As_2O_3 may inhibit interaction between leukemia cells and OPN via downregulating CD44v6/v7 expression, which then interferes with relapse.

In summary, on the basis of current findings, future studies with a larger clinic sample size are planned to further assess the roles of CD44v6/v7 expression as a prognostic indicator of risk and extramedullary recurrence of APL. In addition, we hope that As_2O_3 and CD44v6/v7 monoclonal antibody can be used to reduce the extramedullary relapse of APL.

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