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 ($\text{As}_2\text{O}_3$). 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 in the same risk group. Furthermore, in contrast to ATRA, only $\text{As}_2\text{O}_3$ 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 - $\text{As}_2\text{O}_3$

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 ($\text{As}_2\text{O}_3$) are the classic drugs used for induction therapy of APL. As compared to ATRA treatment, $\text{As}_2\text{O}_3$ treatment leads to higher molecular remission rate and longer disease-free survival time (Zheng et al., 2007).
may be attributed to the elimination of leukemia stem cells (LSCs) by As<sub>2</sub>O<sub>3</sub> (XW et al., 2010). However, it remains unclear whether As<sub>2</sub>O<sub>3</sub> can block the interaction between LSCs and the surrounding microenvironment. In the present study, the effects of ATRA and As<sub>2</sub>O<sub>3</sub> 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<sub>2</sub>O<sub>3</sub>.

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 log phase with viability greater than 95%. The human APL cell line NB4 was kindly provided by the Dr. P. Z. Zheng. NB4 cells were maintained in log phase with viability greater than 95%. The human APL cell line NB4 was kindly provided by the Dr. P. Z. Zheng. NB4 cells were maintained in log phase with viability greater than 95%

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: 5'-GACGAAGACAGTCCCTGGATCA-3' (forward), 5'-AGTGTGACGTGGACCATCCGAAAG-3' (reverse); CD44S-6: 5'-GCCAGCAATCTCCTAGTA-3' (forward), 5'-AGTCCAATTGCTGTTCGTGCT-3' (reverse); CD44v6-S: 5'-GACGAGAACACGTGGACTGATCA-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.

Table 1. Patient Data

| Patient no. | Age/sex | WBC(x10<sup>9</sup>/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
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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 promotion of 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 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 promotion of 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).

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 x 10^9/L) and low-risk APL (WBC count ≤ 10.0 x 10^9/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 As₂O₃ on CD44v6/v7 mRNA expression in NB4 cells.

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 v8/v9 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^−(ΔCT) method (ΔCT=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 x 10^9/L) and low-risk APL (WBC count ≤ 10.0 x 10^9/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 As₂O₃ on CD44v6/v7 mRNA expression in NB4 cells.
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$_2$O$_3$. Both of them may achieve similar rates of hematologic remission, but As$_2$O$_3$ achieves a higher molecular remission rate and disease-free survival rate than ATRA. This may well be because As$_2$O$_3$ can specifically degrade PML/RARα protein and impair the self-renewal of leukemia initiating cells (XW et al., 2010). Our previous studies showed As$_2$O$_3$ but not ATRA could downregulate CD44v6 expression in NB4 cells. And the present study indicated the inhibitory effect of As$_2$O$_3$ on CDv6/v7 expression was more evident than that of ATRA. Thus, we postulate that As$_2$O$_3$ 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$_2$O$_3$ and CD44v6/v7 monoclonal antibody can be used to reduce the extramedullary relapse of APL.

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References

Akisik E, Bavbek S, Dalay N (2002). CD44 variant exons in leukemia and lymphoma. Pathology Oncology Research, 8, 36-40.

Bendall LJ, Bradstock KF, Gottlieb DJ (2000). Expression of CD44 variant exons in acute myeloid leukemia is more common and more complex than that observed in normal blood, bone marrow or CD34+ cells. Leukemia, 14, 1239-46.

Brown D, Kogan S, Lagasse E, et al (1994). A PML/RARalpha transgene initiates murine acute promyelocytic leukemia. Proc Natl Acad Sci U S A, 91, 2551-6.

Chakraborty G, Jan S, Behera R, et al (2006). The multifaceted roles of osteopontin in cell signaling, tumor progression and angiogenesis. Curr Mol Med, 6, 819-30.

Chun SY, Bae OS, Kim JB (2000). The significance of CD44 variants expression in colorectal cancer and its regional lymph nodes. J Korean Med Sci, 6, 696-700.

Colovic N, Bogdanovic A, Miljic P, Jankovic G, Colovic M (2002). Central nervous system relapse in acute promyelocytic leukemia. Am J Hematol, 71, 60-1.

Cristiana B, Carla O, Xiaogang W, et al (2010). De novo expression of CD44 variants in sporadic and hereditary gastric cancer. Lab Invest, 90, 1604-14.

Fenaux P, Chevret S, Guerci A, et al (2000). Long-term follow-up confirms the benefit of all-trans retinoic acid in acute promyelocytic leukemia. Leukemia, 14, 1371-7.

Herold Mende C, Seiter S, Born AJ, et al (1996). Expression of CD44 splice variants in squamous epithelia and squamous cell carcinomas of the head and neck. J Pathol, 179, 662-731.

Hu J, Shen ZX, Sun G, et al (1999). Long-term survival and prognostic study in acute promyelocytic leukemia treated with all-trans-retinoic acid, chemotherapy, and As$_2$O$_3$; an experience of 120 patients at a single institution. Int J Hematol, 70, 248-60.

Katarigi YU, Steemans J, Fujii H, et al (1999). CD44 Variants but not CD44s cooperate with b1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Research, 59, 219-26.

Kortlepel K, Bendall LJ, Gottlieb DJ (1993). Human acute myeloid leukemia cells express adhesion proteins and bind to bone marrow fibroblast monolayers and extracellular matrix proteins. Leukemia, 7, 1174-9.

Marroquin CE, Downey L, Guo H, Kuo PC (2004). Osteopontin increases CD44 expression and cell adhesion in RAW 264.7 murine leukemia cells. Immunology Letters, 15, 109-12.

Naor D, Wallach-Dayan SB, Zalhaka MA, Sionov RV (2008). Involvement of CD44, a molecule with a thousand faces, in cancer dissemination. Seminars in Cancer Biology, 18, 260-7.

Pamela K, Rachid M, Thorsten J, et al (2009). CD44 variant isoforms promote metastasis formation by a tumor cell-matrix cross-talk that supports adhesion and apoptosis resistance. Molecular Cancer Research, 7, 168-79.

Ponta H, Sherman L, Herrlich PA (2003). CD44: from adhesion molecules to signaling regulators. Nature Reviews Molecular Cell Biology, 4, 33-45.

Powell JA, Thomas D, Barry EF, et al (2009). Expression profiling of a hematopoietic cell survival transcriptome implicates osteopontin as a functional prognostic factor in AML. Blood, 114, 4859-70.

Sanz MA, Lo Coco F, Martin G, et al (2000). Definition of relapse risk and role of nonanthracycline drugs for consolidation in patients with acute promyelocytic leukemia: a joint study of the PETHEMA and GIMEMA cooperative groups. Blood, 96, 1247-53.

Tallman MS, Andersen JW, Schiffer CA, et al (2002). All-trans retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup protocol. Blood, 100, 4298-302.

Wolfgang R, Martin H, Reinhard SA, et al (1993). The two major CD44 proteins expressed on a metastatic rat tumor cell line are derived from different splice variants: each one individually sufficient to confer metastatic behavior. Cancer Research, 53, 1262-8.

Yu PZhou L, Ke W, et al (2010). Clinical significance of pAKT and CD44v6 overexpression with breast cancer. J Cancer Res Clin Oncol, 136, 1283-92.

Zhang AM, Fan Y, Yao Q, et al (2010). Identification of a cancer stem-like population in the Lewis lung cancer cell line. Asian Pac J Cancer Prev, 13, 761-6.

Zhang LS, Ma HW, Geyrner HJ, et al (2010). Inhibition of cell proliferation by CD44: Akt is inactivated and EGR-1 is down-regulated. Cell Prolif, 43, 385-95.

Zhang XW, Yan XJ, Zhou ZR, et al (2010). Arsenic trioxide is a specific inhibitor of osteopontin expression in murine leukemia cells. Leukemia, 24, 1555-63.

Zheng X, Seshire A, Ruster B, et al (2007). Arsenic but not all-trans retinoic acid overcomes the aberrant stem cell capacity of PML/RARalpha-positive leukemic stem cells. Haematologica, 92, 323-31.