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ADAM28 overexpression regulated via the PI3K/Akt pathway is associated with relapse in *de novo* adult B-cell acute lymphoblastic leukemia

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ABSTRACT

B-cell acute lymphoblastic leukemia (B-ALL) in adults is a very challenging disease. Relapse following remission after induction chemotherapy remains the major barrier to patient survival. ADAM28 is overexpressed in several human tumors and is related to cell proliferation and lymph node metastasis. To date, no information has been available on the prognostic role of ADAM28 in B-ALL. Fifty consecutive patients with *de novo* B-ALL and 22 healthy donors were enrolled in this study and were followed for 2.8 years. Our data suggested that ADAM28 expression in B-ALL patients was significantly increased ($P < 0.0001$). Patients experiencing disease relapse exhibited significantly increased ADAM28 expression, compared with those with favorable outcomes ($P = 0.0094$). Notably, ADAM28 overexpression was associated with lower probabilities of relapse-free survival (RFS) and event-free survival (EFS) ($P < 0.001$) and was a significant prognostic factor ($P < 0.001$). *In vitro*, the PI3K/Akt pathway inhibitor, as well as arsenic trioxide (ATO), down-regulated ADAM28 expression. Our results were the first to indicate that ADAM28 overexpression in B-ALL patients is correlated with relapse. ADAM28 overexpression is potentially regulated by the PI3K/Akt pathway. These data demonstrate that ADAM28 might serve as a novel biomarker for evaluating relapse in B-ALL and as a potential therapeutic target in B-ALL patients.

1. Introduction

B-cell acute lymphoblastic leukemia (B-ALL), the most common type of ALL in adults, is characterized by clonal expansion of developmentally arrested malignant B-cell precursors [1]. Complete remission (CR) rates by morphology have exceeded 85% in contemporary treatment series [2,3], but relapse is frequent, and survival has been in the 30–40% range in the best series [1–6]. Despite advances in the treatment of this disease, relapse following remission induction chemotherapy remains the major barrier to survival [7]. Treatment of relapsed B-ALL in adults remains a major challenge. It is generally accepted that bone marrow relapse in B-ALL, results from residual leukemic cells that have survived therapy. The current risk assignment uses age, white blood cell (WBC) count at diagnosis, karyotype, central nervous system (CNS) involvement and response to initial therapy to stratify patients for standard risk before chemotherapy [8–10]. Notably, many relapses occur in standard risk patients, who initially present with favorable prognostic features [2]; therefore, there is a clear need to improve the identification of patients at increased risk of relapse, particularly those currently stratified as standard risk, for whom more intensive treatments are already available.

ADAM28 is one of the metalloproteinase-type ADAMs (A disintegrin and metalloproteinases), and it is expressed in two alternative forms: a prototype membrane-anchored form (ADAM28m) and a short secreted form (ADAM28s) [11,12]. ADAM28 is expressed in human lymphocytes and the spleen, and it is involved in various biological events, including cell adhesion, cell fusion, membrane protein shedding, and proteolysis [11–14]. Overexpression of ADAM28 has been detected in many solid cancer types, including breast carcinoma, non-small cell lung carcinoma,

Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; ATO, arsenic trioxide; ADAMA, disintegrin and metalloproteinases; RFS, relapse-free survival; EFS, event-free survival; OS, overall survival; CR, complete remission; CNS, central nervous system; ATRA, all-trans retinoic acid; MRD, minimal residual disease; BM, bone marrow; ELISA, enzyme-linked immunosorbent assay; RT-PCR, real-time quantitative polymerase chain reaction; ROC, receiver operating characteristics.

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chondrosarcoma and bladder transitional cell carcinoma, and it is related to cell proliferation, lymph node metastasis and prognosis [15–19]. In chronic lymphocytic leukemia of B cells (B-CLL), ADAM28 expression has been shown to be significantly higher than that in normal $CD19^+$ B cells [20]. Based on these collective findings, ADAM28 is likely to play an important role in cancer cell proliferation, invasion and metastasis [11]. To date, there has been no information available on the prognostic and relapse relevance of increased ADAM28 levels in adult B-ALL patients.

In this study, we consecutively enrolled 50 B-ALL patients, assessed their ADAM28 expression levels and prospectively recorded their prognostic outcomes. Our results demonstrated that B-ALL patients with ADAM28 overexpression suffered shorter event-free survival (EFS) and relapse-free survival (RFS) than patients with average ADAM28 expression levels, and ADAM28 expression levels were shown for the first time to be a potential prognostic biomarker in B-ALL. We further demonstrated that ADAM28 overexpression is regulated via the PI3K/Akt pathway and could be modulated by selective signal transduction inhibitors in primary B-ALL samples.

2. Materials and methods

2.1. Clinical samples and treatment regimen

Newly diagnosed patients with B-ALL (between October 2012 and October 2013, $n = 50$) were prospectively enrolled in our study after providing written informed consent, in accordance with the Declaration of Helsinki. The bone marrow samples of 6 patients were evaluated with *in vitro* experiments. As normal controls bone marrow ($n = 22$) samples from healthy donors were analyzed. Diagnosis, staging, and response evaluation were based on the standard criteria used in general practice [6,8,21]. The choice of initial therapy and clinical management, as well as the scheduling of laboratory tests and follow-up (from October 2012 to July 2015), was undertaken by the treating physician. All of the patients were prospectively contacted for outcomes.

The induction chemotherapy regimen included daunorubicin, cyclophosphamide, vincristine, prednisone (VDCP), and L-asparaginase. Consolidation chemotherapy regimen included hyper-CVAD (B) (methotrexate and cytosine arabinoside), high-dose methotrexate with/without L-asparaginase, and the VDCP regimen, which were given in turn. After 3 to 4 courses of consolidation therapy, patients entered the HSCT program. Prophylaxis for central nervous system leukemia was given to every enrolled patient, which consisted of intrathecal chemotherapy with methotrexate, cytosine arabinoside, and dexamethasone for at least 4 doses during induction and consolidation chemotherapy [22].

2.2. Methods

2.2.1. Flow cytometry

Immunophenotype and ADAM28 expression levels in the bone marrow (BM) of B-ALL patients were studied by 7-color flow cytometry. Distinct cell populations (clusters) were identified based on any combination of forward and orthogonal light scatter properties and on fluorescence intensity with various antibody combinations. Expression levels of ADAM28 were compared with the degree of fluorescence of the same specimen stained with the isotypic control antibody [23]. Immunophenotypic abnormalities within leukemic blast populations were determined on the basis of deviations from normal patterns of B-lymphoid development.

2.2.2. Isolation and culture of leukemic cells

BM samples from adult acute leukemia patients without any treatment were obtained at diagnosis by aspiration from posterior iliac crest. Blast cells were obtained by density-gradient centrifugation. All leukemic cells were propagated in RPMI 1640 medium [24]. To block the PI3K/Akt pathway, blast cell samples were incubated with a PI3K inhibitor, Ly294002, for 24 h [25]. To observe the effects of arsenic trioxide (ATO) and all-trans retinoic acid (ATRA) on the expression of ADAM28, blast cell samples were treated with different doses of ATO(0.5 μmol/l, 1 μmol/l, 2 μmol/l, 4 μmol/l, and 8 μmol/l, respectively), and ATRA (0.001 μmol/l, 0.01 μmol/l, 0.1 μmol/l, 1 μmol/l, and 10 μmol/l, respectively) for 24, 48, 72 and 96 h [26,27].

2.2.3. ELISA

Each serum sample from the B-ALL patients was analyzed for the concentration of ADAM28 in duplicate using commercially available enzyme-linked immunosorbent assay (ELISA) kits (E99202Hu, Uscn Life Science Inc., Richmond Avenue, Houston, TX, USA) [16,18]. The protein concentrations were measured by standard protocols according to the suggestions of the manufacturer. The ELISA reader was a model Multiskan EX (Thermo Scientific, Vantaa, Finland).

2.2.4. RNA extraction and RT-PCR

ADAM28 mRNA expression levels in BM were measured by real-time quantitative polymerase chain reaction. Total RNA was extracted from the leukemic cells by Isogen (Nippon Gene Co., Ltd., Toyama, Japan), and evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) according to the manufacturer's protocol. Two micrograms of RNA was reverse transcribed with SuperScript II (Life Technologies Inc., Rockville, MD) using a random oligonucleotide hexamer (Takara Bio Inc., Shiga, Japan) at 42 °C for 50 min, followed by heating at 70 °C for 15 min. cDNA was stored at −20 °C until PCR analysis [19,20]. Primers specific for ADAM28 isoforms (ADAM28m forward primer sequence 5′-GCTGTGATGCTAAGACATGT-3′, reverse primer sequence 5′-TGAACAGCCTTTACCATCTG-3′; ADAM28s forward primer sequence 5′-GCTGTGATGCTAAGACATGT-3′, reverse primer sequence 5′-GTTTATGATCTTAGTAGGGTTGCC-3′ [17,19]) were designed based on sequences available in the GenBank. PCR was performed on an ABI Prism 7900 Real Time PCR instrument (Applied Biosystems, Foster City, CA, USA) with SYBR Green I as a double-stranded DNA-specific binding dye. All of the reactions used triplicate samples on a 384 plate, amplifying mRNAs for ADAM28m and ADAM28s and the housekeeping gene, GAPDH [20].

2.2.5. Western blot

ADAM28 expression levels in isolated and cultured leukemic cells were measured by western blot. After extracting protein from B-ALL samples, the blots were incubated with antibodies against the phosphorylated form of Akt (Thr308), the nonphosphorylated form of Akt or ADAM28 in mice, and antibody binding was visualized with an enhanced chemiluminescence detection system. The membranes were then stripped using 0.2 M NaOH solution for 30 min at room temperature and were reprobed for GAPDH. Signals were detected by a PhosphorImager (Storm 860, version 4.0; Molecular Dynamics, Sunnyvale, CA) and quantified by Scion Image software (Scion, Frederick, MD) [26], the protein signals of ADAM28 and pAkt were normalized against the protein signal of the GAPDH. Western blot results are expressed in terms of this ratio (relative expression). The western blots were performed in triplicate [25].

2.2.6. Cell migration assay

Leukemic cell migration was measured using modified Boyden chambers (Costar, USA), which had filters with an 8 μm pore size. Briefly, the cells were suspended in 500 μl of complete medium. A total of 2×10^5 leukemic cells were placed into the upper chamber, and complete medium in a volume of 600 μl, containing 10 ng/ml of stromal cell-derived factor-1a (SDF-1a, Sigma, USA), was placed

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