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ANKHD1, ankyrin repeat and KH domain containing 1, is overexpressed in acute leukemias and is associated with SHP2 in K562 cells

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Abstract

In the present study, increased levels of *ANKHD1* mRNA and protein expression in leukemia cell lines are reported, as compared with normal hematopoietic cells. Furthermore, a higher expression of *ANKHD1* mRNA was detected in primary acute leukemia samples than in normal hematopoietic cells ($P=0.002$). ANKHD1 was detected in the cytosolic and membrane fraction of cells and was co-immunoprecipitated with SHP2 in protein extracts of K562 and LNCaP cell lines. These findings suggest a role for ANKHD1 as a scaffolding protein that may be associated with the abnormal phenotype of leukemia cells.

Keywords: ANKHD1; MASK; Ankyrin repeat; SHP2 phosphatase; Acute leukemia

1. Introduction

Ankyrin-repeat-containing proteins regulate multiple cellular functions including transcriptional and cell-cycle regulation, ion channel, cell survival and cell signaling and participate in protein–protein interactions via their repeat motifs [1–4]. Multiple ankyrin repeat and single KH domain protein, MASK, was first identified in *Drosophila melanogaster* through a genetic screen designed to identify proteins that interact with the protein–tyrosine phosphatase Corskscrew (CSW), homolog to SH2-containing protein–tyrosine phosphatase (SHP2) in humans [5]. The phenotypic characterization of MASK in *Drosophila* suggests that it is a novel protein involved in receptor tyrosine kinase signaling (RTKs) and its activity is required for cell differentiation, cell survival and cell proliferation in *Drosophila* eyes [5]. In humans, an orthologous protein of *Drosophila* MASK, ANKHD1 (Ankyrin Repeat and KH Domain Containing 1), previously named hMASK, was first identified in LNCaP, a prostate cancer cell line, and described by Poulin et. al. [6]. However, protein expression patterns or protein interactions of ANKHD1 have not yet been described.

The nonreceptor protein–tyrosine phosphatase SHP2, encoded by the *PTPN11* gene, is a signal-enhancing component of growth factor, cytokine and extracellular matrix receptor signaling and plays an important role in regulating cell proliferation, differentiation and migration [7,8]. Recent studies implicate SHP2 in human disease, including Noonan syndrome [9], sporadic juvenile myelomonocytic leukemia, childhood myelodysplastic syndrome, B-cell precursor acute lymphoblastic leukemia (ALL), pediatric and adult acute myeloid leukemia (AML) [10–13] and some solid tumors [14]. More recently, SHP2 has been shown to be overexpressed in primary leukemia cells and in leukemia cell lines, and suppression of SHP2 expression induces apoptosis and growth inhibition in leukemia clonogenic cells [15].

Since MASK was first isolated through a genetic screen designed to identify proteins that interact with CSW/SHP2, the characterization of ANKHD1 expression in humans and

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its interaction with SHP2 would be of interest. The present study was aimed to evaluate the expression of *ANKHD1* mRNA and protein in normal tissues, normal hematopoietic cells, leukemia cell lines and primary acute leukemia samples. In addition, the association of ANKHD1 with SHP2 was studied in leukemia cell lines and the prostate cancer cell line, LNCaP.

2. Materials and Methods

2.1. Primary leukemia samples

A total of 38 adult cases (patient age range 18–83 years, median 47 years) diagnosed with acute leukemia at the Hematology and Hemotherapy Center of the State University of Campinas were studied, including 7 ALL, 1 biphenotypic acute leukemia and 30 AML (1 M0, 5 M1, 6 M2, 3 M3, 10 M4, 3 M5, 1 M6, 1 M7) based on the French-American-British (FAB) classification. Primary leukemia cell samples from bone marrow aspirate were obtained from patients before any treatment. The National Ethical Committee Board approved the study and informed-written consent was obtained from all patients. Normal hematopoietic cells ($n=11$), including bone marrows ($n=7$) and peripheral blood mononuclear cells (PBMNC) ($n=4$), were obtained from healthy donors. After removing erythrocytes by hemolysis, remaining cells were submitted to protein or RNA extraction.

2.2. Human leukemia cell lines

A panel of human leukemia cell lines was used which included KG-1, HEL, K562, NB4, HL-60, Jurkat, MOLT4, Raji, Daudi and Namalwa. A prostate cancer cell line, LNCaP, was used as a positive control. All cell lines were obtained from ATCC, Philadelphia, PA. Cells were cultured in RPMI containing 10% fetal calf serum and glutamine with addition of penicillin/streptomycin and amphotericin B and were maintained at 37 °C, 5% CO_2. For experiments, cells were seeded at a density of 3×10^5 cells/ml, cultured around 7 days and collected at an exponential phase of growth.

2.3. Protein analysis by immunoblotting

Equal amounts of protein were used for total extracts or for immunoprecipitation with specific antibodies followed by SDS-PAGE and Western blot analysis with the indicated antibodies and ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, UK) as described [16]. Antibody anti-ANKHD1 was raised in a rabbit against a synthetic peptide (CHPMHQQLSDPSTFSQ) comprising amino acids 2423-2437 from ANKHD1 exon 32, and was manufactured by Sigma-Aldrich (MO, USA). Dr. Francis Poullin (Department of Genome Sciences, Lawrence Berkeley National Laboratory, Berkeley, CA) kindly provided an additional antibody against ANKHD1 (RC2335) [6]. Monoclonal antibody against phosphotyrosine (SC-508) and polyclonal antibodies anti-SHP2 (sc-280), anti-actin (sc1616), anti-histone deacetylase (sc11419) and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal normal IgG (sc-2027) (Santa Cruz, CA, USA) was used as a control for immunoprecipitation.

2.4. Laser confocal analysis

Cells were washed with PBS and fixed with paraformaldehyde containing 4% sucrose. The primary antibody used was anti-ANKHD1. After washing, the slides were labeled with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes, Leiden, The Netherlands) for 2 h. Following this procedure, K562 and Jurkat cells were incubated with TRITC-conjugated phalloidin (Sigma-Aldrich, MO, USA) at room temperature and coverslips with ProLong Gold antifade reagent with DAPI (Molecular Probes, Leiden, The Netherlands) were applied; PBMNC were washed with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), incubated with 100 μg/mL DNase–RNase in 2× SSC for 20 min at 37 °C, washed with 2× SSC and incubated with Propidium Iodide (PI) (Molecular Probes), which binds to DNA, for 5 min at room temperature. Coverslips were applied with Vectashield (Vector Labs, CA, USA). Positive immunoreactivity was visualized by laser confocal scanning (Zeiss LM510). In the absence of primary antibodies, application of secondary antibodies (negative controls) failed to produce any significant staining.

2.5. Subcellular fractionation

Jurkat cells were washed twice and the pellet was resuspended in hypotonic buffer containing 10 mM HEPES (pH 7.9), 1.4 mM $MgCl_2$ and 10 mM KCl and allowed to swell on ice for 10 min. Cells were then lysed on ice by vigorous homogenization by 10 passages of the cell suspension through a 26.5-gauge needle. The extracts were centrifuged at $500\times g$ for 10 min at 4 °C. The supernatant was used as a cytosolic and membrane fraction. The pellet was washed twice with a hypotonic buffer containing 20 mM HEPES (pH 7.9), 25% Glycerol, 1.5 mM $MgCl_2$, 20 mM KCl and 0.2 mM EDTA and resuspended in hypertonic buffer containing 20 mM HEPES (pH 7.9), 25% Glycerol, 1.5 mM $MgCl_2$, 1 M KCl and 0.2 mM EDTA. The homogenate was incubated on ice for 30 min at 4 °C and centrifuged at $14.000\times g$ for 15 min at 4 °C. Supernatant was used as the nuclear fraction. Equal amounts of proteins were used for Western blot analysis.

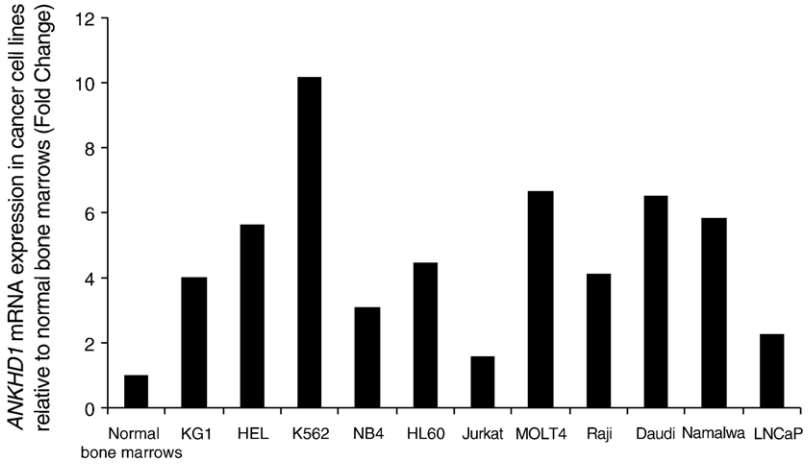

Fig. 1. Increased *ANKHD1* mRNA expression in leukemia cell lines. Real-time quantitative RT-PCR was performed on cDNA from a panel of human leukemia cell lines and the LNCaP cell line. Increased levels of *ANKHD1* mRNA were detected in all subtypes of leukemia cell lines, as compared with normal hematopoietic cells.

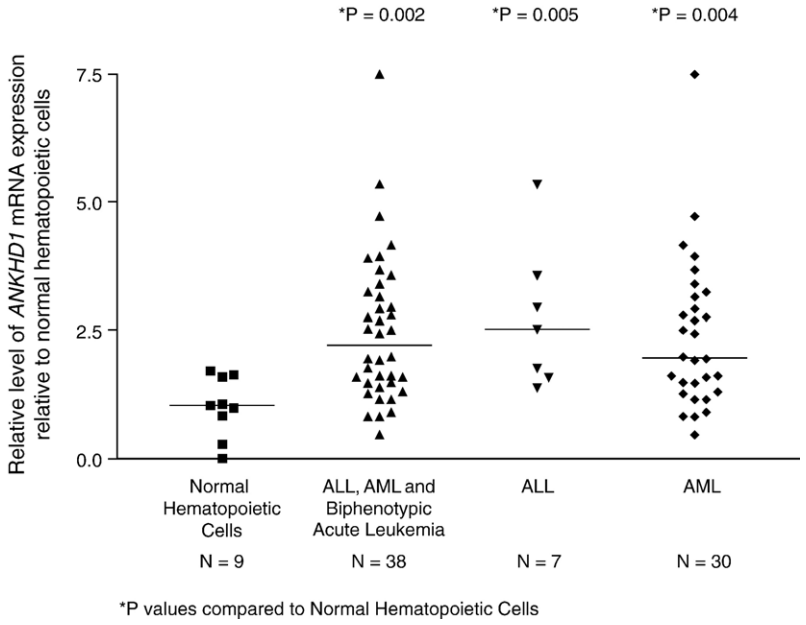

Fig. 2. Increased *ANKHD1* mRNA expression in primary leukemia cells. Real-time quantitative RT-PCR was performed on cDNA from fresh leukemia cell samples from patients at diagnosis or from normal hematopoietic cells. Horizontal lines represent medians. *ANKHD1* mRNA expression was significantly higher in all primary acute leukemias samples ($P=0.002$), acute myeloid leukemias (AML) ($P=0.004$) and acute lymphoblastic leukemias (ALL) ($P=0.005$) when compared with normal hematopoietic cells.

2.6. Real-time quantitative RT-PCR

Reverse transcription, primer sequences and real-time quantitative RT-PCR was performed as previously described [17]. Briefly, real-time detection of amplification was performed in an ABI 5700 Sequence Detector System (Applied Biosystems) using SybrGreen PCR Master Mix (Applied Biosystems). Four replicas were run on the same plate for each sample. β-Actin expression was used as an endogenous control and a pool of 3 normal bone

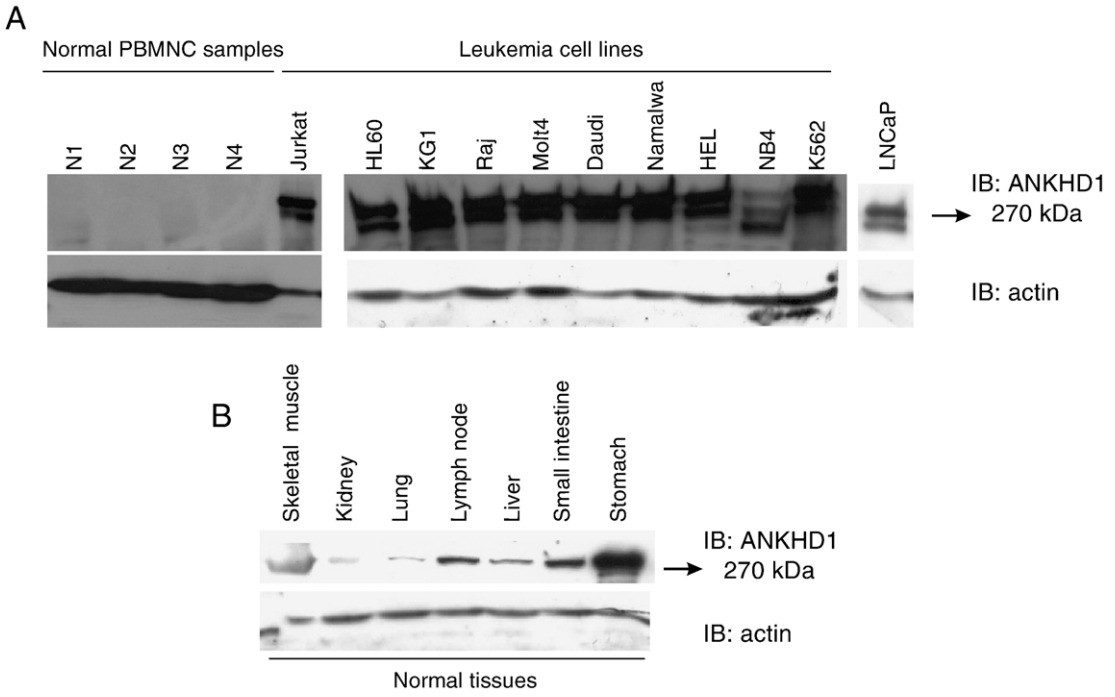

Fig. 3. Analysis of the ANKHD1 protein expression. Western blot analysis of peripheral blood mononuclear cells (PBMNC) from 4 healthy donors (N1 to N4), human cancer cell lines (A) and various human normal tissues (B) for the expression of the ANKHD1 protein. Total tissue or cell extracts were blotted with antibodies against ANKHD1 (270 kDa) or actin (42 kDa), as a control for equal sample loading, and developed with the ECL Western Blotting Analysis System.

marrows was used as the Calibrator. The relative quantification value of *ANKHD1* gene expression was calculated using the $2^{-\Delta\Delta CT}$ [18].

2.7. Statistical analysis

The relative level of *ANKHD1* mRNA expression was compared between normal hematopoietic cells, all primary acute leukemia samples, primary AML samples and primary ALL samples using a Mann–Whitney *U* test. A *P* value of ≤ 0.05 was considered to indicate statistical significance.

3. Results

3.1. ANKHD1 mRNA is highly expressed in leukemia cells

Real-time RT-PCR demonstrated increased levels of *ANKHD1* mRNA in all leukemia cell lines studied; KG-1, HEL, K562, NB4, HL-60 Jurkat, MOLT4, Raji, Daudi and Namalwa (up to 10-fold increase), as compared with normal bone marrow cells (Fig. 1).

ANKHD1 mRNA expression was significantly higher in primary acute leukemia samples when compared with normal hematopoietic cells (medians: 2.21 *versus* 1.05, $P=0.002$), and in ALL (medians: 2.53 *versus* 1.05, $P=0.005$) and AML (medians: 1.97 *versus* 1.05, $P=0.004$) when compared with normal hematopoietic cells. No significant difference in *ANKHD1* mRNA expression was observed in the comparison of AML versus ALL (Fig. 2). Among the AML samples, *ANKHD1* mRNA expression tended to be higher in the subtypes AML M1 and AML M2; however, there was no statistical significance among the subtypes of AML; neither among the subtypes of ALL.

3.2. ANKHD1 protein is highly expressed in leukemia cells

Immunoblot analysis detected a very low expression of ANKHD1 in PBMNC of normal donors. Conversely, a high expression of the protein was detected in the human leukemia cell lines (Fig. 3A); the expression of ANKHD1 was characterized with 2 bands of approximately 270-kDa in the immunoblot of leukemia cells, and the expression of ANKHD1 in LNCaP was used as a positive control, as previously described [6].

A broad expression of ANKHD1 was observed in all normal human tissues here studied, with a high expression in stomach, small intestine and lymph node and a low expression in liver, spleen, lung, kidney and skeletal muscle (Fig. 3B). Only 1 band of approximately 270 kDa, corresponding to ANKHD1, was detected in normal human tissues.

Immunoblotting analysis of leukemia cell lines with anti-phosphotyrosine antibody revealed that ANKHD1 was not phosphorylated at tyrosine (data not shown).

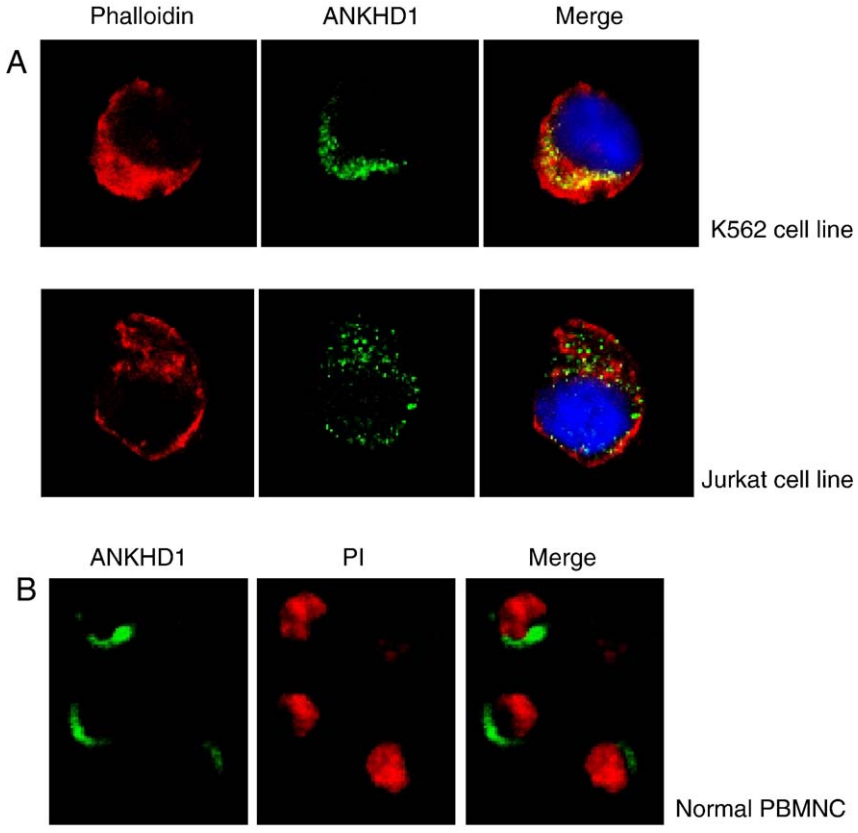

Fig. 4. Laser confocal analysis. K562 and Jurkat cells (A) were fixed and permeabilized and ANKHD1 was visualized by Alexa 488-conjugated anti-ANKHD1 antibody (green fluorescence). Phalloidin was used to visualize the actin (red fluorescence) and DAPI to mark the nuclei (blue fluorescence), as detailed in Materials and methods. Positive immunoreactivity was visualized by laser confocal scanning (Zeiss LM510). In the red, green and blue overlay (merge) the yellow signal indicates regions of red/green overlap. Normal peripheral blood mononuclear cells (PBMC) (B) were fixed, permeabilized and ANKHD1 was visualized by Alexa 488 conjugated anti-ANKHD1 antibody (green fluorescence). Propidium iodide (PI) was used to visualize the nuclei (red fluorescence). Positive immunoreactivity was visualized by laser confocal scanning (Zeiss LM510).

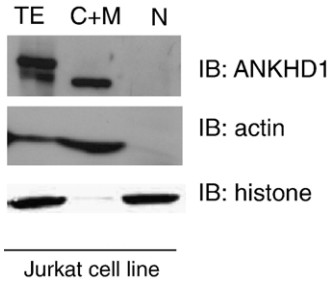

Fig. 5. Subcellular localization of ANKHD1 in the Jurkat cell line. Jurkat cells were lysed in hypotonic buffer and lysates were used to prepare total cellular protein extract (TE), cytosolic and membrane (C+M) and nucleus (N) fractions. An aliquot of each fraction (100 μg total proteins) was subjected to immunoblot analysis for ANKHD1. Anti-actin and anti-histone were used as the controls of subcellular fractions.

3.3. Subcellular localization of ANKHD1

Laser confocal analysis of K562 and Jurkat cell lines (Fig. 4A) and PBMNC (Fig. 4B) showed that ANKHD1 is located in the cytoplasm of leukemia and normal hematopoietic cells. This cytoplasmatic localization of ANKHD1 was further examined by immunoblotting performed with anti-ANKHD1 antibody and tissue fractions of Jurkat cell line (Fig. 5).

3.4. Association of ANKHD1 and SHP2 in cancer cell lines

Immunoprecipitation analysis was carried out using anti-ANKHD1 antibody. ANKHD1 was co-immunoprecipitated with SHP2 in protein extracts of K562 and LNCaP cell lines (Fig. 6, upper panels). To double-check this interaction, we performed the reverse assay; SHP2 was co-immunoprecipitated with ANKHD1 in the protein extracts of K562 and LNCaP cell lines (Fig. 6, middle panels), indicating that ANKHD1 protein associates with SHP2 in vivo. As a negative control for all assays, cell lysates were immunoprecipitated with rabbit polyclonal IgG and immunoblotted with anti-SHP2 or anti-ANKHD1, no band was observed; total extracts were used as a positive control for the immunoblot (Fig. 6, bottom panels).

ANKHD1 was not found to be associated with SHP2 in KG1, HL60, Daudi and Jurkat cell lines, even though all leukemia cell line studies presented a high level of SHP2 protein expression (Fig. 7).

4. Discussion

We, herein, report increased levels of *ANKHD1* mRNA and protein expression in leukemia cell lines, as compared with normal hematopoietic cells, and a higher expression of *ANKHD1* mRNA in primary acute leukemia samples, as compared with normal hematopoietic cells. Furthermore, Western blot analysis demonstrated a broad expression of ANKHD1 in normal human tissues. ANKHD1 is an orthologous protein of *Drosophila* MASK, suggesting that ANKHD1 belongs to a protein family conserved through evolution. In *Drosophila*, *Mask* was identified through a genetic screen to identify potential targets of CSW, the *Drosophila* homologue of *SHP2* [5]. In this study, the association of ANKHD1 with SHP2 was identified by immunoprecipitation in K562 and LNCaP cell lines. We also demonstrated that ANKHD1 is located in the cytoplasm of the cells, similar to the subcellular localization of SHP2 in leukemia cells [15], indicating that these proteins share subcellular compartments. Moreover, the expression pattern of ANKHD1 was found to be similar to that of SHP2, which is overexpressed in acute leukemia cell lines and primary leukemia cells [15].

In *Drosophila*, Mask was characterized as a novel protein involved in RTKs [5]. Our findings that ANKHD1 is not phosphorylated in tyrosine suggest that ANKHD1 may function as an adaptor protein, since SHP2 binds directly to some RTKs or to one or more scaffolding adapter proteins [8,19]. Protein–protein interactions are crucial for all biological processes. The generation of accurate cellular protein interaction networks is an ongoing process, in which the data produced by the study of new proteins contributes in a complementary manner.

SHP2 is a widely expressed protein–tyrosine phosphatase that seems to play a positive role in the activation of MAP kinase in response to growth factors [20,21]. Rongzhen Xu and colleagues [15] demonstrated that overexpression and constitutive activation of SHP2 protein is a common phenotype in various types of human leukemia and are closely associated with

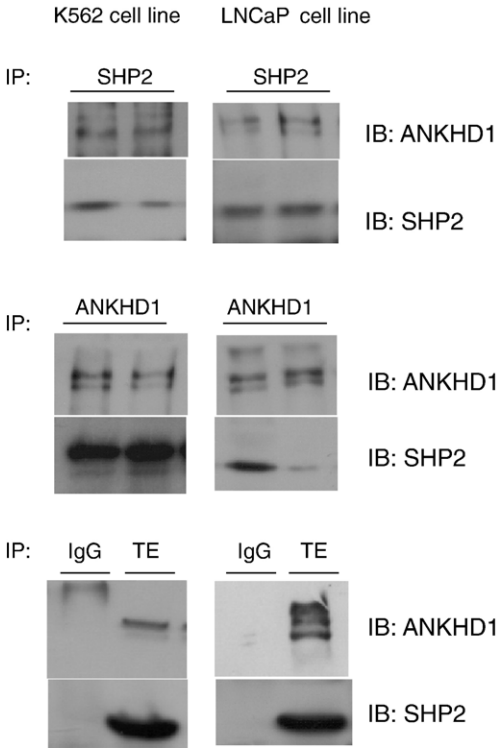

Fig. 6. ANKHD1 is a substrate of SHP2 protein. Lysates from K562 or LNCaP cells containing equal amounts of protein were immunoprecipitated (IP) in duplicate with anti-SHP2 antibodies (upper panel), anti-ANKHD1 antibodies (middle panel) or normal rabbit IgG (bottom panel) and immunoblotted (IB) with anti-ANKD1 antibodies or anti-SHP2 antibodies. K562 and LNCaP total cell extract (TE) were used as a positive control for the immunoblotting with anti-ANKHD1 or anti-SHP2 (bottom panel). Western blots were developed with the ECL Western Blotting Analysis System.

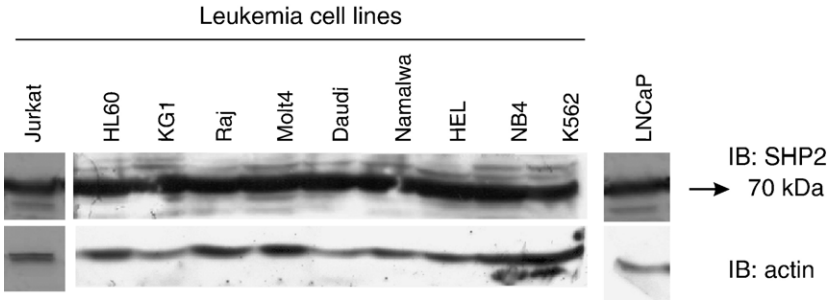

Fig. 7. High SHP2 protein expression in human cancer cell lines. Western blot analysis of human cancer cell lines for the expression of the SHP2 protein. Cell extracts were blotted with antibodies against SHP2 (70 kDa) or actin (42 kDa), as a control for equal sample loading, and developed with the ECL Western Blotting Analysis System.

the proliferative capacity of leukemia blasts. Therefore, overexpression of SHP2 might not be sufficient for the development of leukemia, and other factors may be required for leukemogenesis [15]. In this context, the finding that ANKHD1 is associated with SHP2 in K562 and LNCaP cells, and that *ANKHD1* mRNA is overexpressed in primary leukemia cells compared to normal hematopoietic cells suggests that ANKHD1 may be an adaptor protein and that its association with SHP2 may be required for leukemogenesis and the development of other cancers. However, the finding that ANKHD1 is not associated with SHP2 in KG1, HL60, Daudi and Jurkat cell lines, even though all leukemia cell line studies presented a high level of SHP2 protein expression, gives rise to the hypothesis that the association between ANKHD1 and SHP2 may be tissue specific and may play a different role in different cells. Another possible explanation for the different pattern of association may be the presence of variants of ANKHD1 and/or SHP2 within these cell lines. Besides, *PTPN11* mutations can also lead to SHP2 variants with altered susceptibility to activation and modified substrate selectively [22].

The previous findings that ANKHD1 interacts with GRINL1A [23], which has been recently identified as a cancer/testis antigen expressed in bone marrow and peripheral blood from AML patients, but not in normal donor samples [24], as well as the high expression of ANKHD1 in acute leukemia, described in our study, corroborate our hypothesis that ANKHD1 protein may be involved in leukemogenesis. The identification of new disease-specific targets for AML immunotherapy expands treatment options and increases our chances of successfully treating this heterogeneous disease and lowering the unacceptably high mortality rate.

Ankyrin repeat proteins carry out a wide variety of biological activities and have been detected in organisms ranging from viruses to humans [4]. Two new variants of ANKHD1, Vpr-binding ankyrin repeat proteins (VAPR-L and VAPR-S), may possess an antiapoptotic effect and protect cells during normal cell proliferation in HeLa and NT2 cells [25]. Like other ankyrin repeat proteins, ANKHD1, through its association with SHP2, may have a role in regulating cell proliferation; however, the exact role of ANKHD1 in acute leukemias is not yet fully understood and requires further studies.

ANKHD1 has a ubiquitous expression in normal human tissues and has a varied higher expression in acute leukemias. The presence of multiple ankyrin repeats suggests a role for ANKHD1 as a scaffolding protein, bringing together many signaling molecules. Our findings are the first to describe the expression of ANKHD1 in different human tissues and to characterize the association of ANKHD1 with SHP2. The identification of proteins that interact with ANKHD1 and that might direct ANKHD1 to various signal transduction pathways is essential to clarify the role of ANKHD1 in the signaling pathways in general. The higher expression of ANKHD1 in acute leukemia may be associated with the abnormal phenotype of the leukemia cell and may be a molecular target for a rational therapy for leukemia in the near future.

Acknowledgments

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