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Mitochondrial pro-apoptotic ARTS protein is lost in the majority of acute lymphoblastic leukemia patients

Ronit Elhasid[1,6], Dvora Sahar[2,6], Ayellet Merling[3], Yifat Zivony[3], Asaf Rotem[3], Miriam Ben-Arush[1], Shai Izraeli[4], Dani Bercovich[5] and Sarit Larisch*[,3]

[1]*Pediatric-Hemato-Oncology Department. Rambam Medical Center, Bat-Galim, Haifa 31096, Israel;* [2]*Molecular Hematology Lab, Rambam Medical Center, Bat-Galim, Haifa 31096, Israel;* [3]*Apoptosis and Carcinogenesis Research Lab, Pathology Department, Rambam Medical Center, Bat-Galim, Haifa 31096, Israel;;* [4] *Pediatric Hemato Oncology and the Cancer Research Center, Sheba Medical Center, Sackler Faculty of Medicine, Tel-Aviv University, Tel Hashomer 52621, Israel;* [5]*Migal-Galilee Technology Center, Human Molecular Genetics, PO Box 831, Kiryat-Shmona 11016, Israel*

Acquired resistance towards apoptosis is the hallmark of most if not all types of cancer. We have previously identified and characterized ARTS, a broadly expressed protein localized to mitochondria. ARTS was initially shown to mediate TGF-β induced apoptosis. Recently, we have found that high levels of ARTS induce apoptosis without additional pro-apoptotic stimuli. Further, ARTS promotes apoptosis in response to a wide variety of pro-apoptotic stimuli. Here, we report that the expression of ARTS is lost in all lymphoblasts of more than 70% of childhood acute lymphoblastic leukemia (ALL) patients. The loss of ARTS is specific, as the related non-apoptotic protein H5, bearing 83% identity to ARTS, is unaffected. During remission, ARTS expression is detected again in almost all patients. Two leukemic cell lines, ALL-1 and HL-60 lacking ARTS, were resistant to apoptotic induction by ara-C. Transfection of ARTS into these cells restored their ability to undergo apoptosis in response to this chemotherapeutic agent. We found that methylation process contributes to the loss of ARTS expression. We conclude that the loss of ARTS may provide a selective advantage for cells to escape apoptosis thereby contributing to their transformation to malignant lymphoblasts. We therefore propose that ARTS can function as a tumor suppressor protein in childhood ALL.
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Introduction

Apoptotic cell death serves as a major mechanism for the precise regulation of cell numbers, and as a defense against unwanted and potentially dangerous cells (Thompson, 1995; Hengartner, 2000; Meier *et al*., 2000). The regulation of apoptosis is fundamental to hematopoietic homeostasis. Stem cell renewal is continuously counterbalanced by apoptosis in functionally inactive or terminally differentiated cells (Vaux and Korsmeyer, 1999). Surplus cell production and apoptosis are therefore normal features of hematopoiesis (Domen, 2000). Aberrant apoptosis has been associated with development hematological malignancies (McKenna and Cotter, 1997; Rinkenberger and Korsmeyer, 1997; Hetts, 1998; Hanahan and Weinberg, 2000). We have previously described a human protein, termed ARTS, which was originally found to mediate TGF-β-induced apoptosis (Larisch *et al*., 2000). More recently, we have found that ARTS is also important for cell killing by a variety of other pro-apoptotic factors, such as arabinofuranosylcytosine (ara-C), etoposide and staurosporine (Gottfried *et al*., 2004). ARTS is a member of the septin family of proteins, which have been implicated in cytokinesis, exocytosis and scaffolds for assembly of signaling complexes (Field and Kellogg, 1999; Kartmann and Roth, 2001). Unlike other septins which are not involved in apoptosis and localize mainly to actin bundles (Field and Kellogg, 1999; Kartmann and Roth, 2001; Kinoshita *et al*., 2002), ARTS is localized to mitochondria and translocates to the nucleus upon pro-apoptotic stimuli (Larisch *et al*., 2000).

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children. It accounts for one-fourth of all childhood cancers and approximately 75% of all cases of childhood leukemia (Pui, 1995). Acquired resistance towards apoptosis is the hallmark of most, probably all types of cancer (Hetts, 1998; Hanahan and Weinberg, 2000; Green and Evan, 2002). In this study, we present evidence showing that ARTS is lost in the majority of ALL patients and that its loss is correlated with malignant state of the disease. Leukemic cells lacking ARTS are resistant to apoptotic induction by ara-C. Yet, transfection of ARTS into these cells restored their ability to undergo apoptosis in response to this chemotherapeutic agent. Methylation is one of the mechanisms responsible for the loss of ARTS expression in leukemia.

*Correspondence: S Larisch; E-mail: s_larisch@rambam.health.gov.il
[6]These authors contributed equally to this paper.
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We propose that the mitochondrial pro-apoptotic protein ARTS can function as a tumor suppressor in leukemia and may serve an important role in the pathogenesis of childhood ALL. These data provide a link between mitochondria, apoptosis and tumor suppression that has previously not been established.

Results

To investigate the involvement of ARTS in leukemia, we tested its expression both at the protein and at the RNA level in blood and bone marrow samples from children with ALL. In all, 24 samples from ALL patients were analysed by immunostaining with a specific antibody for ARTS (Sigma). In parallel, we assessed ARTS protein levels in bone marrow or blood samples from 26 healthy controls. Interestingly, major changes in ARTS protein expression were found exclusively in lymphocytes rather than in any other hematopoietic cell lineage. While all lymphocytes from healthy controls had very high expression of ARTS (Figure 1a, e), we found that 18 out of 24 ALL patients (75%) did not show any detectable levels of ARTS in their lymphoblasts (Figure 1c–f). Importantly, staining of bone marrow from ALL patients revealed that the loss of ARTS was complete in all malignant lymphoblasts. Staining of ARTS was still seen in the few remaining normal cells serving as internal control for specificity of staining (Figure 1f). To determine whether the absence of ARTS in lymphoblasts is the result of their failure to differentiate, we examined whether ARTS is expressed in CD34-positive precursor cells. Hematopoietic stem cells (CD34-positive) are the primary precursors of all blood cell types. CD34-positive cells were isolated from 11 normal donors using immunomagnetic beads with antibodies to CD34 (Miltenyi Biotec). All these CD34-positive cells expressed high levels of ARTS protein (Figure 1b). Therefore, it appears that ARTS protein is normally expressed throughout lymphocyte differentiation in healthy controls. These results suggest that the absence of ARTS in leukemic blasts is not simply the result of incomplete cellular differentiation, but is rather correlated with their malignant state.

Next, we examined whether the loss of ARTS protein expression in ALL patients was due to transcriptional or post-transcriptional changes. As major changes in ARTS protein expression were exhibited specifically in lymphocytes, we examined ARTS RNA levels in isolated lymphocytes from 32 ALL patients and 36 healthy donors. RNA levels were determined using semi-quantitative RT–PCR with primers specific for ARTS mRNA (see Patients and methods). We found that the vast majority (24 out of 32, 72.7%) of ALL patients had significantly reduced ARTS RNA levels (lower than 30%). In comparison, all healthy controls showed high ARTS RNA levels (higher than 30%) (Figure 2a). A cutoff of 30% was determined taking into consideration remaining normal lymphocytes containing ARTS, which are usually present within the examined

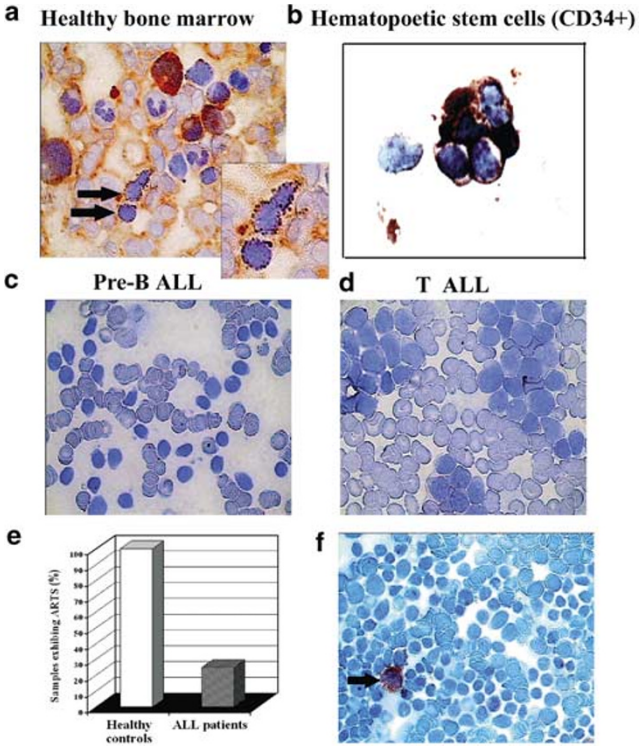

Figure 1 Immunohistochemistry of ARTS in cells of healthy controls and ALL patients. Immunohistochemistry staining of ARTS (red) in (**a**) Normal bone marrow sample and in (**b**) Hematopoietic stem cells (CD34+) (**c**) pre-B ALL and (**d**). T ALL sample. ARTS is highly expressed in normal lymphocytes (arrows) and in the precursor stem cells from healthy controls (a and b respectively). Inset in (**a**) shows mitochondrial staining of ARTS in lymphocytes. ARTS protein is absent in lymphoblasts isolated from a child with pre B-ALL (**c**) and from a child with T-ALL (**d**). e Only 25% of 24 ALL patients exhibit ARTS protein in their lymphoblasts at disease state. In 75% (18 out of 24 patients), there was no detectable ARTS in their lymphoblasts. All tested 26 healthy controls (100%) showed expression of ARTS protein. (**f**) ARTS is completely lost in lymphoblasts from ALL patient. Only normal remaining lymphocyte contains ARTS protein (arrow) and serves as an internal control for specificity of staining

patient samples (Figure 1f). These data suggest that ARTS expression is affected at the transcriptional level. Furthermore, these results confirm our protein results showing ARTS loss in lymphoblasts of more than 70% of tested ALL samples.

We went on to investigate whether this loss of ARTS was specific, or whether it also affected the related H5 transcript. Both ARTS and H5 proteins are derived from the same locus and share 83% identity (see Supplementary Figure 1). Although, they differ in their cellular localization and function, unlike ARTS, H5 does not promote apoptosis (Larisch *et al*., 2000) (Figure 3a). To verify that ARTS loss is linked to its pro-apoptotic function, we examined the expression of H5 as compared to ARTS expression. We tested lymphocytes isolated from 21 ALL patients and 25 healthy controls for their H5 expression. All ALL patients showed normal levels of H5 transcripts when compared to healthy controls (Figure 3b, c). Likewise,

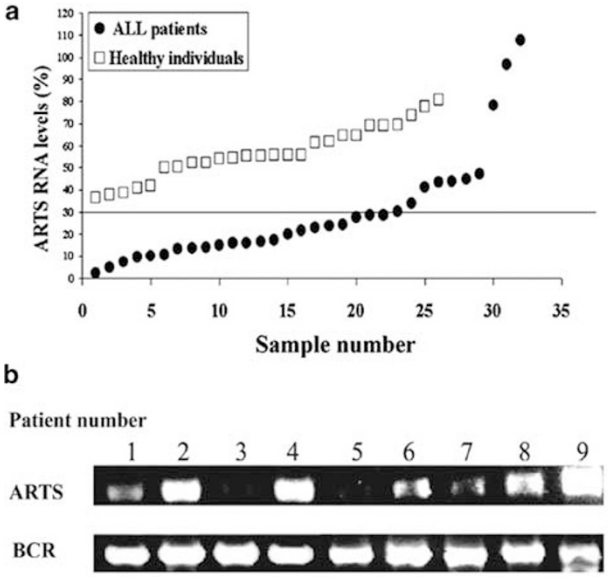

Figure 2 ARTS mRNA levels in healthy controls and ALL patients. ARTS mRNA levels in lymphocytes isolated from ALL patients and healthy controls. (**a**) The distribution of ARTS mRNA levels in healthy controls and in ALL patients. A cutoff of 30% was determined taking into consideration remaining normal lymphocytes containing ARTS, which are present within the examined samples. The majority of ALL patients exhibit ARTS levels below 30% (24 out of 32, 72.7%). Normal values of ARTS in healthy controls are above 30% in all tested samples. Samples are arranged from lowest to highest levels. (**b**) Representative PCR reaction demonstrating ARTS mRNA levels in some of the tested patients. BCR mRNA of each sample served as loading controls (see Patients and methods)

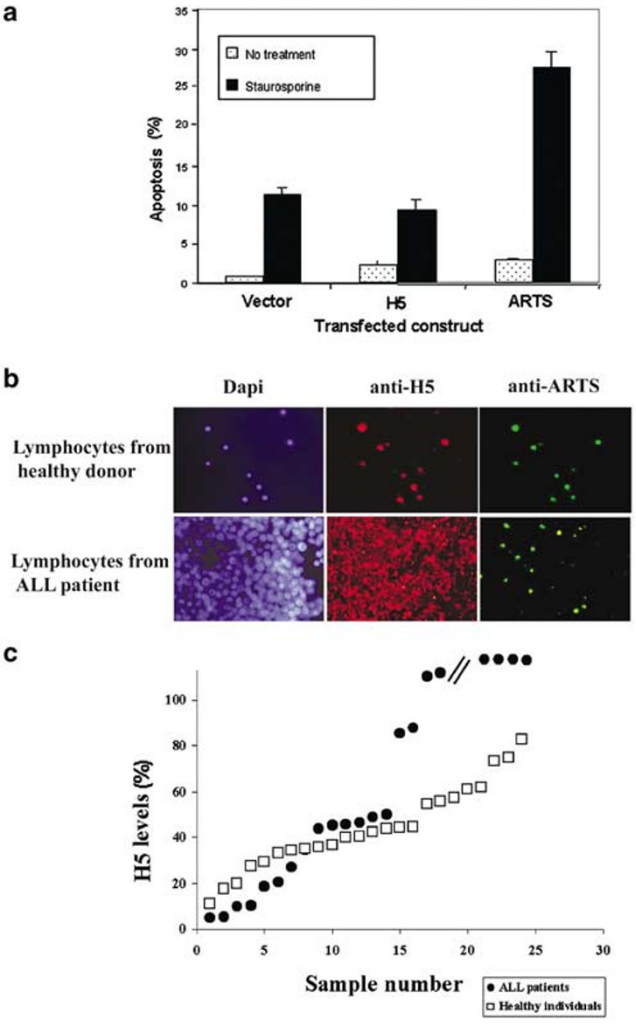

Figure 3 Levels of H5, a closely related septin, remain unaffected in ALL patients. ARTS is derived by differential splicing from the H5/Peanutl2 gene and shares most of its exons with H5 (see Supplementary Figure 1). However, unlike ARTS, H5 does not promote apoptosis. (**a**) COS-7 cells transfected with control vector, H5 and ARTS expression vectors. Only transfection of ARTS promotes apoptosis. (**b**) Immunofluorescence staining showing lymphocytes isolated from healthy donor (upper panel) and from ALL patient (lower panel). Staining with Dapi, showing nuclei of cells (blue), with anti-H5 (red) and anti-ARTS (green). Whereas all lymphocytes in healthy control contain both ARTS and H5 (upper panel), the sample from ALL patient exhibits severe reduction in ARTS staining, showing specific staining of few remaining normal cells.(**c**) The distribution of H5 mRNA levels in lymphocytes isolated from 21 ALL patients and 25 healthy controls. Four ALL patients contained H5 levels higher then 100%. Unlike ARTS, H5 levels remain unaffected in ALL patients. Interestingly, eight out of 21 (38%) ALL patients showed much higher levels of H5 in their lymphocytes compared to healthy controls

H5 protein levels seemed unaffected in ALL patients (Figure 3c). Interestingly, eight out of 21 (38%) ALL patients showed much higher levels of H5 in their lymphocytes compared to healthy controls (Figure 3c). Therefore, it appears that there is a specific down-regulation of ARTS at both the transcriptional and protein level in most ALL patients, which is probably linked to its apoptotic function.

During remission, when most cancer cells are eradicated and healthy cells repopulate the bone marrow of the patients, we would expect ARTS levels to return to normal. To test this idea, we examined nine bone marrow samples from children at remission that originally exhibited reduced ARTS levels. After treatment with chemotherapy, lymphocytes from eight out of nine patients showed a 2 to 30-fold increase in ARTS levels compared to ARTS levels at the disease state (Figure 4a–c). Therefore, loss of ARTS from lymphocytes in these patients was highly correlated with their malignant state.

To examine whether loss of ARTS in leukemic cells affects their ability to undergo apoptosis, we used HL-60 and ALL1 cells. Both cell lines do not contain any detectable levels of ARTS RNA or protein (ALL1 Figures 5a and 6a, HL-60 Figure 6a, b). We tested the ability of these cell lines to undergo apoptosis in response to the chemotherapeutic agent ara-C. We found that both these cell lines could not undergo apoptosis when treated with ara-C (Figures 5b and c). Similar results were seen with treatment of Etoposide (data not shown). However, this resistance could be reverted by forced expression of ARTS protein (Figure 5a). Transfection of ARTS restored sensitivity to ara-C treatment and led to strong apoptosis in both leukemic cell lines (Figure 5b, c). Thus, loss of ARTS

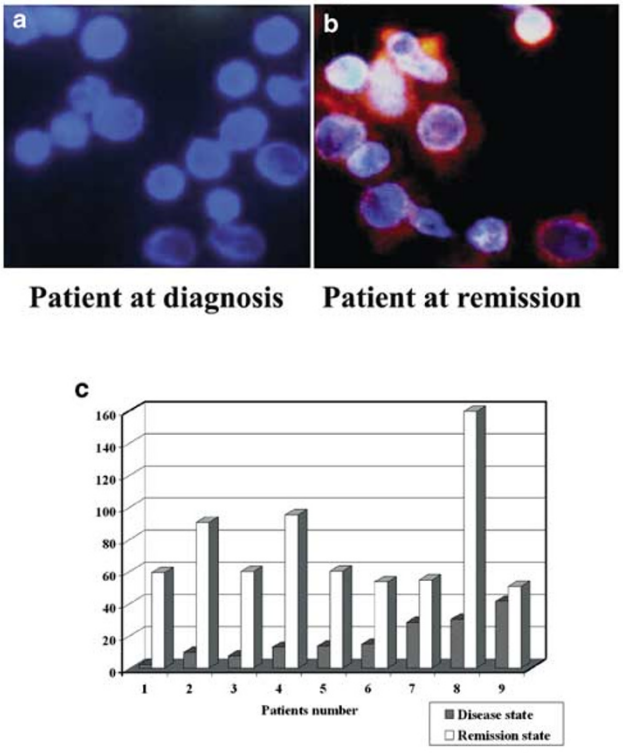

Figure 4 ARTS reappears at remission in patients showing severely reduced levels at disease state. (**a**) Immunofluorescence staining with Dapi (blue) and anti-ARTS (red) showing lymphoblasts isolated from ALL child at disease (left) and at remission state (right). Lymphoblasts from ALL patient at disease state do not contain ARTS protein. While at remission, lymphocytes from same patient show reappearance of ARTS (red). (**b**) ARTS mRNA levels in patients at disease and at remission state. Seven out of nine patients showed 2 to 30-fold increase in ARTS mRNA levels when at remission

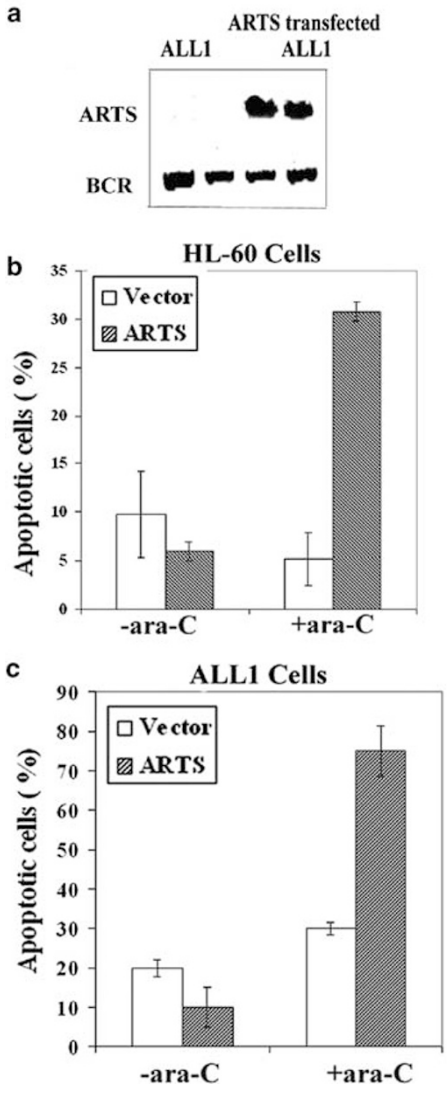

Figure 5 Apoptotic effect of ARTS reintroduction into leukemic cells lacking ARTS. (**a**) Western blot analysis of ALL1 cells before (left panel) and after (right panel) ARTS transfection. ALL1 cells lacking ARTS (left two lanes) display ARTS protein when transfected with ARTS expression vector (right two lanes). BCR protein in the same samples served as loading control. HL-60 and ALL1 cells lacking ARTS, do not undergo apoptosis in response to ara-C. Transfection of ARTS into these cells restores their ability to undergo apoptosis upon treatment with ara-C. (**b** and **c** respectively)

seems to provide a selective advantage of cells to escape apoptosis thereby contributing to their transformation to malignant lymphoblasts.

Methylation is one of the known mechanisms for inactivating tumor suppressor genes (Jain, 2003; Jones, 1999; Malfoy, 2000). To investigate whether methylation is responsible for the loss of ARTS in leukemic cells, we analysed HL-60 cells, which do not express ARTS RNA or protein (Figure 6a, b). Treatment of HL-60 cells with 5-azacytidine (5-aza), a methylation inhibitor, resulted in reappearance of ARTS protein (Figure 6b). The same phenomenon was seen when lymphoblasts from two ALL children were treated with 5-aza (Figure 6c–e, patients 4, and 31 in Supplementary Table 1). In contrast, lymphocytes from healthy donors did not increase ARTS RNA levels upon 5-aza treatment (Figure 6d, e). Finally, ALL1 cells did not show reappearance of ARTS upon treatment with 5-aza, suggesting that ARTS is lost using a different mechanism in these cells (data not shown). Thus, epigenetic silencing of ARTS by methylation is one mechanism of reducing ARTS expression in malignant leukemic blasts.

Discussion

In this study, we show that the expression of the pro-apoptotic ARTS protein is lost in lymphoblasts of the majority (>70%) of ALL patients during their disease state. ARTS reappeared in most patients during their remission stage. In comparison, the most common chromosomal aberration seen in childhood ALL, the translocation t(12;21), is found in 22–25% of patients (Pui, 1995; Shurtleff *et al*., 1995; Greaves, 2002; Rubnitz and Pui, 2003). Thus, loss of ARTS expression appears to be a very frequent molecular abnormality occurring in childhood ALL.

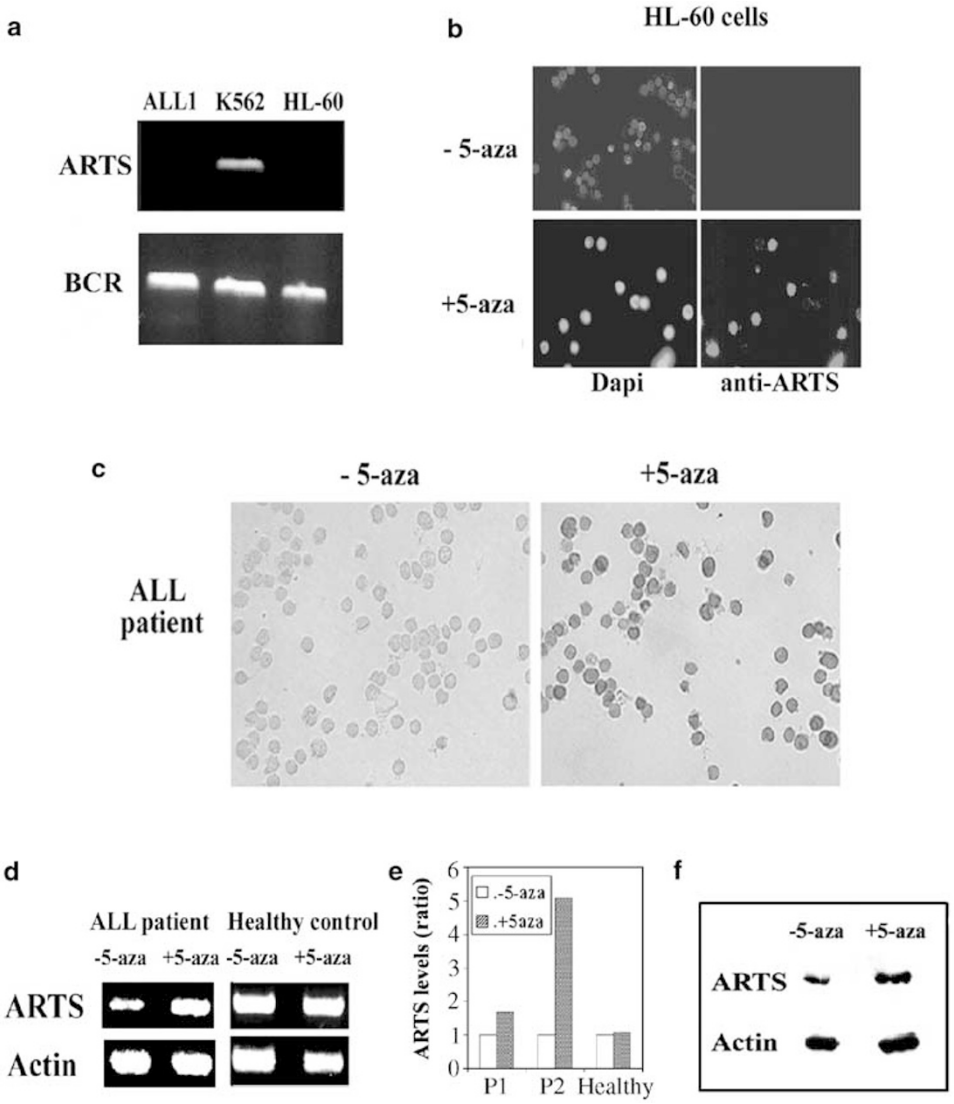

Figure 6 ARTS protein reappears in some ALL patients and cell lines following treatment with the methylation inhibitor 5-azacytidine. (**a**) RT-PCR analysis reveals that ALL1 and HL-60 leukemic cell lines do not contain ARTS mRNA, while K562 cells show ARTS RNA expression. BCR RNA products are shown as loading controls. (**b**) Immunofluorescence assay showing HL-60 cells lacking ARTS protein (upper panel). HL-60 cells regain ARTS expression upon treatment with 5-azacytidine (5-aza) a methylation inhibitor. (**c**) Immunohistochemistry staining of lymphoblasts isolated from ALL patient using, anti-ARTS antibodies (see Patients and methods). Lymphoblasts lacking ARTS (upper panel) show reappearance of ARTS upon treatment with 5-aza (lower panel). (**d**) Elevated levels of ARTS mRNA in lymphoblasts from ALL patient following 5-aza treatment. ARTS mRNA levels in treated lymphocytes from healthy control remain unchanged. (**e**) Diagrammatic presentation of mRNA ARTS levels in lymphoblasts from two ALL patients treated with 5-aza. Displayed are the ratios with and without 5-aza treatment, as compared to healthy control. (**f**) Western blot analysis of ARTS protein in lymphoblasts from one ALL patient reveals elevated levels of ARTS when treated with 5-aza

The loss of pro-apoptotic genes is one of the known mechanisms by which transformed cells obtain selective advantage through reduced susceptibility to apoptotic signals (Attardi and Jacks, 1999; Hanahan and Weinberg, 2000; Evan and Vousden, 2001; Igney and Krammer, 2002). We have shown that two leukemic cell lines lacking ARTS were resistant to the apoptotic effect of ara-C (Figure 5b, c). Transfection of ARTS into these cells increased ARTS protein levels (Figure 5a) and restored their ability to die upon ara-C treatment (Figure 5b, c). Moreover, levels of the related non-apoptotic H5 septin were unaffected in ALL patients, indicating specific selection against the pro-apoptotic function encoded by the ARTS gene, but not the septin function. Interestingly, two other septins, CDCrel-1 and MSF have been found as fusion proteins with MLL (ALL1, Htrx, HRX) in *de novo* leukemias in children and therapy-related acute leukemia patients (Megonigal *et al*., 1998; Osaka *et al*., 1999). Although the function of the MLL septin fusion proteins is yet unknown, evidence from mouse knockin models suggest that partners of MLL themselves are important for genera-

tion of leukemias (Corral *et al*., 1996). Moreover, MSF is located on chromosome 17q25 (Osaka *et al*., 1999) in the vicinity of ARTS, which maps to chromosomal location 17q23 (Larisch *et al*., 2000). This region is deleted in some ovarian and mammary tumors, and Kalikin *et al*. (2000) hypothesized that this region contains a tumor suppressor gene. We suggest that ARTS is this proposed tumor suppressor which contributes to the malignant transformation of hematopoietic cells, and possibly other cell types as well.

DNA methylation, has long been associated with transcriptional silencing of tumor suppressor genes (Jones, 1999; Malfoy, 2000; Rountree *et al*., 2001; Jain, 2003; Gaudet *et al*., 2003). Significantly, this includes Apaf-1, a key component of the apoptosome, which is commonly inactivated in metastatic melanoma. (Soengas *et al*., 2001) Agents reversing DNA methylation, such as 5-aza used in our assays, have been experimentally tested in clinical trials in humans with cancer (Karpf and Jones, 2002; Eden *et al*., 2003; Zagonel *et al*., 1993). The activity of these agents against cancer is presumably due to their reverting effect on silencing of tumor suppressor genes (Karpf and Jones, 2002; Rountree *et al*., 2001; Eden *et al*., 2003). Similarly, our data indicate that methylation is responsible for the loss of ARTS in two ALL patients and in HL-60 myeloid leukemia cells.

To the best of our knowledge, this is the first report of a mitochondrial pro-apoptotic protein that functions as a tumor suppressor protein.

We have preliminary evidence that ARTS expression is lost in other hematopoietic malignancies (e.g. in three AML patients as well as in HL-60 myeloid leukemia cell line). Moreover, ARTS maps to chromosomal location 17q23 (Larisch *et al*., 2000). This region is deleted in some ovarian and mammary tumors, and has been suggested to contain a tumor suppressor gene (Kalikin *et al*., 2000). We propose that the loss of ARTS contributes to the tumorigenesis process in leukemia and possibly other tumors as well. Since the loss of ARTS appears to be a very common molecular abnormality in childhood ALL, strategies aimed at restoring its function may provide promising new therapeutic opportunities.

Patients and methods

Patients and normal controls

This study involved 33 patients with ALL. Their median age at diagnosis was 6 years, with a range from 1 month to 16 years. There were 16 boys and 17 girls. In all, 28 patients had pre-B ALL while four patients had T-ALL and one patient had B-ALL. Follow-up was between 11 and 52 months (see Supplementary Table 1). In total, 36 healthy donors free of any known disease served as controls. This study was approved by the Institutional Review Board for human experimentation at the Rambam Medical Center, Haifa Israel (approval number 1183).

Cells

Measurements of ARTS and H5 RNA levels were done on RNA extracted from isolated lymphocytes. Lymphocytes were separated from patients or healthy donor blood samples on Ficoll–Histopaque (Sigma, St Louis, MO, USA). The leukemic cell lines, ALL1-acute lymphoblastic leukemia cells (Erikson *et al*., 1986), HL-60 and K562, acute myelocytic leukemia cells were grown in RPMI medium with 10% FCS, L-glutamate, antibiotics and sodium glutamate. COS-7 cells were maintained in DMEM.

RNA assays

Total RNA was extracted from isolated lymphocytes or cultured cells using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). cDNA was prepared using RT–PCR kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Resulting cDNA were analysed in two parallel PCR reactions; One reaction tested expression of exons 5 and 6 of ARTS, a second reaction tested bcr or actin expression, serving as house-keeping control genes for quantitation purposes. Specific primers used for ARTS exons; 5′CTCTTGGCCTGTTCCCTTG and 5′AGAGA GGATCATGCAAACTGT.

For bcr 5′GGAGCTGCAGATGCTGACCAAC and 5′GCATCCTCTTCAAGCTGAACTC. For actin-5′CA ACGGCTCCGGCATGTG and 5′CTCCTTAATGTC ACGCACGA.

For H5: 5′AAGGCTGTTTCTGTGTGTGAG and 5′ CAGCTTCAGTCCTGTCCTCA.

The reaction products were run on 2% agarose gel with 100 bp weight markers (New England BioLab, Beverly, MA, USA). Quantitative measurements of ARTS RNA levels were done using densitometry (Bio Profile, Vilber-Lourmat, sud Torcy, France, version 97.04) by comparing volume and ratio of control gene bands to those of the ARTS product band. Levels of ARTS and H5 mRNA were expressed as percentage of control measurements.

Apoptosis assays

Annexin V assay Leukemic cultured cells (6×10^6) were transfected with 15 μg ARTS plasmid or with 15 μg control vector using electroporation (900 μF, 250 mV), (Easyject Plus, EQIBIO, Ashford, UK). The cells were seeded in six-well plates (Falcon, Franklin Lakes, NJ, USA). After 48 h cells were treated with 100 μM ara-C (Cytosar) (Pharmacia, Uppsala, Sweden) for 4 h, or with 100 μM etoposide (Sigma, St Louis, MO, USA) for 8 h. To determine apoptosis levels, cells were separated using Annexin V-coated magnetic beads separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with Mini-Macs MS^+ separation columns. The bound apoptotic cells were counted in each sample, and apoptotic percentage was calculated from total cell number loaded on each column. Each graph represents average of four different experiments.

TUNEL assay Detection of apoptosis in COS7 cells was done using TUNEL assay according to the manufacturer's instructions. (In Situ Cell Death Detection Kit, TMR red, Roche, Indianapolis, IN, USA) Experiment was repeated three times.

Immunofluorescence assay Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA, USA), washed with 0.1% Triton X (Sigma, St Louis, MO, USA) in PBS followed by 0.5% Triton X in PBS for 5 min. Blocking was done in 5% goat serum (Biological Industries, Kibbutz Beit Haemek, Israel) in PBS for 1 h, then cells were incubated with either anti-ARTS (monoclonal anti-ARTS, Sigma, St Louis, MO, USA) or H5 antibodies (polyclonal anti-H5 peptide, costume made Sigma, Rehovot, Israel), followed by secondary antibodies conjugated with either fluorescein or rhodamine (Fluorescein-conjugated Gout Anti-Rabbit or rhodamine Red-X-conjugated Gout Anti-Rabbit, Jackson Immuno Research Laboratories Inc, West Grove, PA, USA); After washes, a drop of DAPI-containing mountain solution (Vector Laboratories, Inc., Burlingame, CA, USA) was added to each slide for staining of DNA in nuclei.

Immunohistochemistry assay Cells were fixed and washed with Triton X as indicated above. Slides were incubated with 0.3% hydroperoxide for 30 min to neutralize endogenous peroxide, followed by two 5 min washes with 0.1% Triton X in PBS. Cells were blocked with 5% BSA in PBS for 30 min followed by incubation with anti-ARTS antibody (polyclonal anti-ARTS, Sigma, St Louis, MO, USA), After three washes with 0.1% Triton X in PBS, slides were incubated with biotinylated secondary antibody and developed using HRP-conjugated substrate according to the manufacturer's recommendations (Histostain®-plus bulk kit-Zymed, San Francisco, CA, USA). Slides were developed using AEC substrate (Zymed, San Francisco, CA, USA). Hematoxylin solution was added to each slide for visualization of the nuclei.

Immunoblotting Protein was extracted from isolated lymphocytes using RIPA buffer (0.1% SDS, 1% NP-40, 1% Na-deoxychlorate, 150 mM NaCl, 10 mM Tris-PH 7.2, 2 mM EDTA and Complete Mini EDTA free protease inhibitors (Roche, Mannheim, Germany). SDS/polyacrylamide gel electrophoresis was performed and gels were blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Blocking was done with 3% blotting grade blocker nonfat dry milk (Bio-Rad, Hercules, CA, USA) in PBS for 1 h, then incubated with either anti-ARTS antibody (monoclonal anti-ARTS, Sigma, St Louis, MO, USA), or anti-Actin antibody (monoclonal mouse anti-Actin, (Ab-1), Oncogene, Cambridge, MA, USA) followed by incubation with secondary HRP-conjugated antibody (anti-mouse HRP-conjugated, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). Detection was done using Supersignal® west pico Chemiluminescent substrate-ECL (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations.

Treatment of cells with 5-azacytidine Lymphocytes were separated from blood samples on Ficoll–Histopaque (Sigma, St Louis, MO, USA) and freezed using 90% FCS and 10% DMSO. Cells were thawed in 10 ml saline (5% dextrose and 0.45% sodium chloride, (TEVA Medical, Ashdod, Israel) containing 10% BSA (Sigma, St Louis, MO, USA) for 2 h. Lymphocytes and HL-60 cells were treated with 7.5 μM of 5-Azacytidine (Sigma, St Louis, MO, USA) a methylation inhibitor, for 6 h. RNA was extracted from the cells using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH, USA).

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