ANTICANCER RESEARCH *25*: 2645-2648 (2005)

DHX32 Expression Suggests a Role in Lymphocyte Differentiation

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Abstract. *RNA helicases constitute a large group of enzymes involved in all aspects of RNA metabolism. Several RNA helicases are dysregulated in cancer, whereas several others are involved in differentiation. DHX32 has previously been identified as a novel RNA helicase with a unique structure and expression pattern. DHX32 message was down-regulated in acute lymphoblastic leukemia cell lines and patient samples. In this report, anti-DHX32 was used to study its expression in thymus. Immunohistochemistry and flow cytometry showed positive correlation of DHX32 expression with thymocyte maturation. These results suggest that DHX32 might play a role in normal lymphocyte differentiation.*

RNA helicases constitute a large group of conserved enzymes characterized by the presence of a centrally located "helicase domain" (1). Based on variations of the sequence of the motifs of the helicase domain, RNA helicases are further classified into families (2). The nomenclature of RNA helicases has recently been revised, and the two largest human RNA helicase gene families, the *DDX* and *DHX* families, have been published (3). With a few notable exceptions, the biochemical activities and biological roles of *DDX* and *DHX* genes are not very well characterized. However, two general features for RNA helicases can be recognized, namely involvement in differentiation (4) and dysregulation in cancer (5, 6). These features are compatible with a possible role of RNA helicases in regulating the expression of critical genes.

A link between RNA helicases and normal/ abnormal hematopoiesis has been suggested. *DDX6* (7) and *DDX10* (8) have been identified as fusion partners in chromosomal translocations in hematopoietic malignancies. The *Schlafen* gene family, whose members have an RNA helicase-like motif, have been shown to be involved in lymphocyte development and activation (9). These findings raise the possibility that other RNA helicases might be involved in hematopoiesis.

DHX32 has been have identified as a novel RNA helicase gene which is down regulated in Acute Lymphoblastic Leukemia (10). The gene designation was changed to *DHX32,* according to the new nomenclature of RNA helicases (3), to reflect its homology to the *DHX* family of helicases. Since blasts in precursor acute lymphoblastic leukemia can represent early stages of lymphocyte differentiation, the possibility that *DHX32* expression is correlated with normal lymphocyte differentiation was investigated.

Materials and Methods

Immunohistochemistry. Anonymous archival specimens from the Department of Pediatric Laboratory Medicine were used. Five-μm formalin-fixed, paraffin-embedded tissue sections were mounted on positively-charged microscope slides. Tissue sections where baked overnight at 60˚C, dewaxed in xylene and hydrated to distilled water through decreasing concentrations of alcohol. The immunohistochemical procedure for anti-*DHX32* and the preimmune control serum was performed on the NEXES™ auto-immuno stainer (Ventana Medical Systems, Tuscon, Arizona, USA) at a dilution of 1:400. Immunodetection was carried out using an ABC system employing the Ventana DAB (3-3'-diaminobenzidine) Detection System, (Cat#250-001). All tissue sections were treated with Ventana Protease I (cat#250-2018) for 16 minutes, and blocked for endogenous biotin with the Ventana Endogenous Biotin Blocking Kit (Cat#250-050) as part of the automated staining protocol. The slides were counterstained with haematoxylin.

Flow cytometry. The cell suspension was performed from leftover anonymous thymic tissue with a Beckton Dickinson Medimachine according to the manufacturer's instructions (BD BioScience, Oakville, Ontario, Canada). Surface staining for CD4 and CD8 was performed by using PerCP-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies with the appropriate isotype controls according to the supplier's instructions. This was followed by plasma membrane

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Key Words: RNA helicase, DHX32, lymphocyte, differentiation.

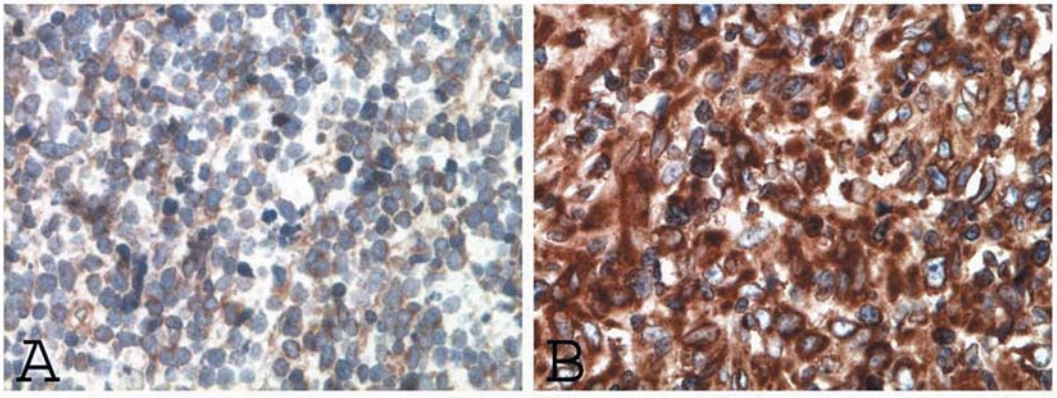

Figure 1. *DHX32 expression in lymphoma. Immunohistochemistry with anti-DHX32 Ab 1 in 400 dilution on protease-treated 5 micron sections of formalin-fixed and paraffin-embedded sections. A: a case of precursor T-lymphoblastic lymphoma showing very low expression. B: a case of large B-cell lymphoma showing strong expression.*

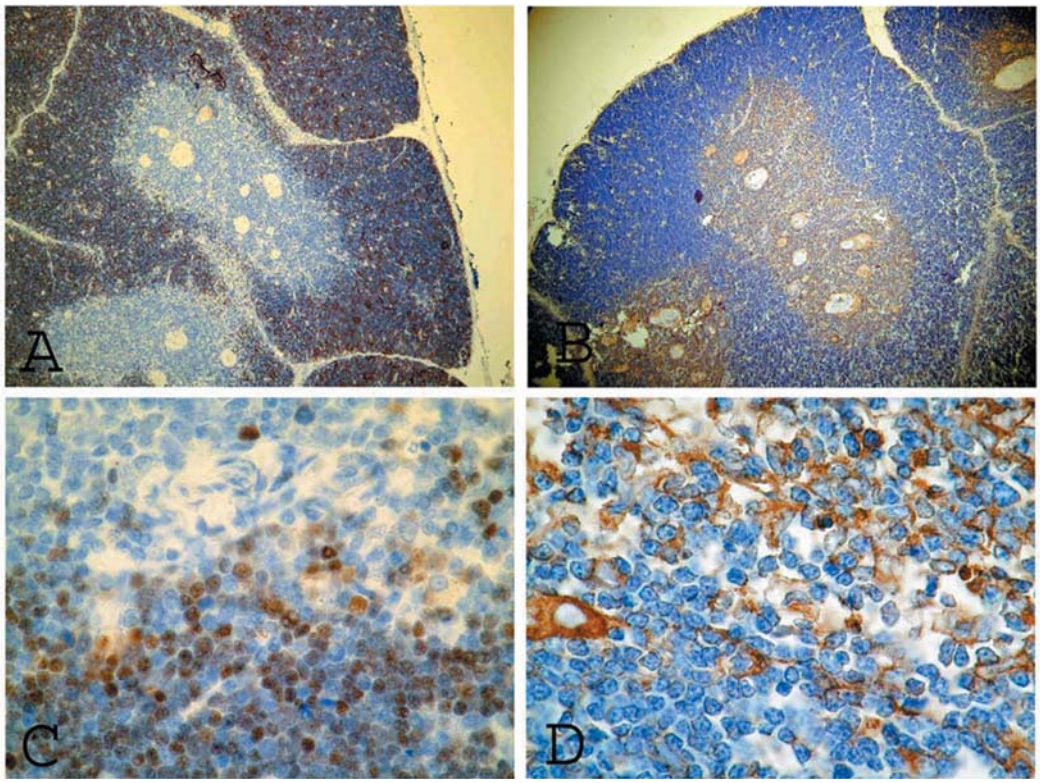

Figure 2. *DHX32 expression in normal human thymus. The architectural and immunological features of the thymus facilitate the correlation of expression with lymphocyte differentiation. Low and high power thymus sections stained with anti-TdT antibody (A and C, respectively). Low and high power sections stained with anti-DHX32 antibody (B and D, respectively). Immunohistochemistry showed that the outer cortex, which contains immature lymphocytes that express the nuclear enzyme TdT, had little DHX32p. On the other hand, more differentiated lymphocytes in the thymic medulla, negative for TdT, showed stronger staining with anti-DHX32 Ab.*

permeabilization using Intraprep permeabilization reagent (Immunotech, France), according to the manufacturer's instructions, and staining with anti-*DHX32* antibody (or pre-immune serum control) diluted 1:100 in PBS for 1 hour at room temperature. FITC-conjugated anti-rabbit antibody (Sigma, Oakville, Ontario, Canada) diluted 1:100 in PBS was incubated for 30 minutes. Samples were analyzed on FACSCALIBUR flow cytometer and data were analyzed and histograms generated by using the Cell Quest program.

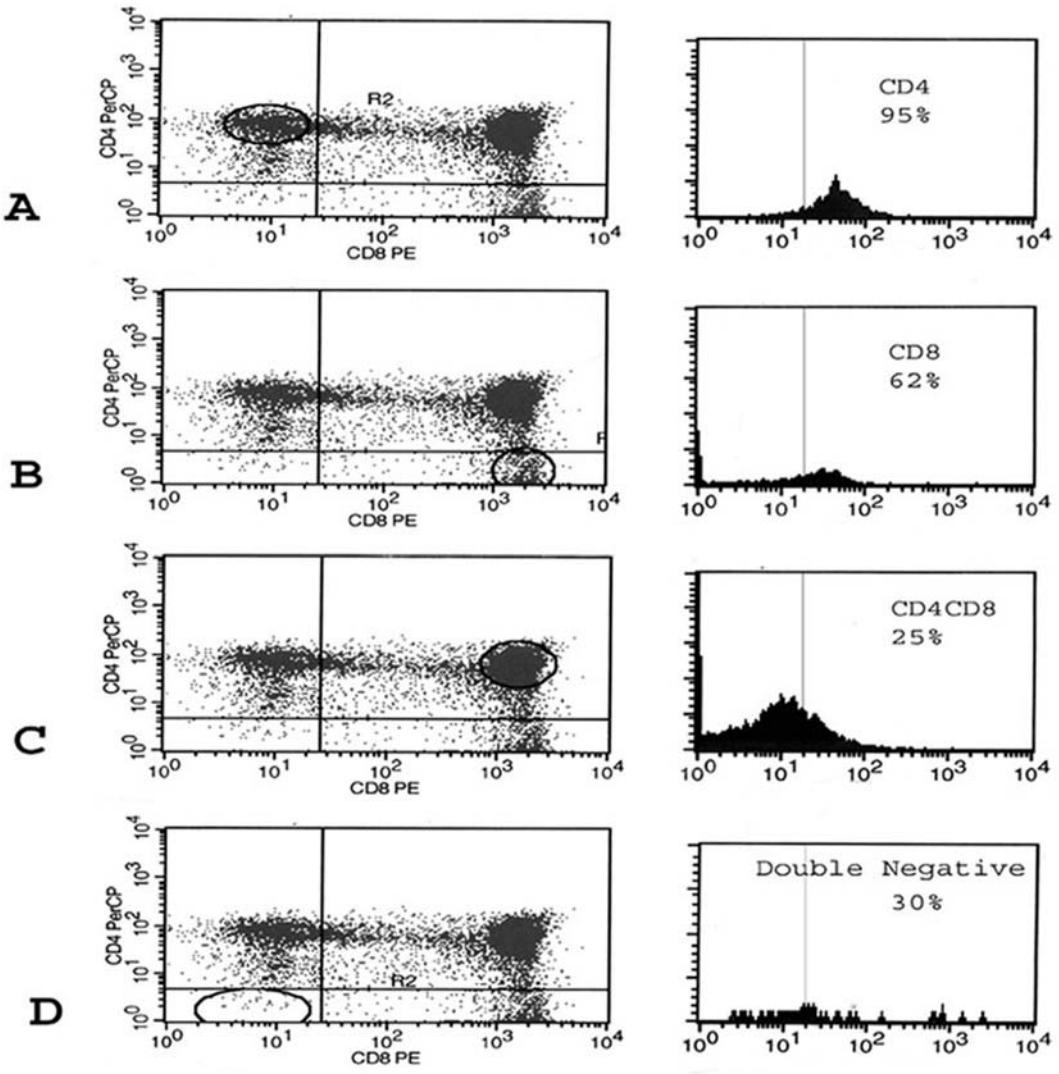

Figure 3. *Correlation of DHX32 expression with thymocyte maturation. Thymocytes were first stained with CD4 and CD8 antibodies to detect the presence of these antigens on the cell surface. Cells were then permeabilized and incubated with either pre-immune serum or anti-DHX32 antibody. Left panels show surface staining with CD4 (Y-axis) and CD8 (X-axis). Circles indicate the population used to produce the histogram on the right side, which is the intracellular staining with anti-DHX32 antibody. Pre-immune control was used to set the negative staining. The single positive CD4 and single positive CD8 lymphocytes showed 95% (A) and 62% (B) expression of DHX32 protein, respectively. The double positive and double negative cells showed much lower expression, 25% (C) and 30% (D), respectively.*

Results

Low expression of DHX32 protein in lymphoblastic lymphoma. It has previously been shown that the *DHX32* gene expression is down-regulated in precursor acute lymphoblastic leukemia cell lines and primary patient samples (10). To test *DHX32* expression at the protein level, anti-*DHX32* rabbit oligoclonal antibody directed against the C-terminus 15 amino acids was raised and optimized for use in formalin-fixed paraffin-embedded tissue sections. Low expression of *DHX32p* was noted in lymphoblastic lymphoma, whose cell of origin is a precursor lymphoblast similar to acute lymphoblastic leukemia. In comparison, strong expression was noted in large cell lymphoma, whose cell of origin is more mature lymphocytes (Figure 1).

Expression of DHX32 in the thymus is correlated with thymocyte differentiation. Immunohistochemistry showed that the outer cortex, which contains immature lymphocytes that express the nuclear enzyme TdT, had little *DHX32p*. On the other hand, more differentiated lymphocytes in the thymic medulla, which were negative for TdT, showed stronger staining with

anti-*DHX32* Ab (Figure 2). In order to directly correlate *DHX32p* expression with the stage of differentiation, three-color multiparametric flow cytometry analysis of the thymocyte cell suspension was performed. The most immature thymocytes are negative for both CD4 and CD8 surface antigens, *i.e.* "double negative". This stage is followed by "double positive" and finally mature "single positive" lymphocytes for either CD4 or CD8. The single positive CD4 and single positive CD8 lymphocytes showed 95% and 62% expression of the *DHX32* protein, respectively. The double positive and double negative cells showed much lower expression at 25% and 30%, respectively (Figure 3).

Discussion

DHX32 was originally identified as a novel putative RNA helicase gene whose expression was down-regulated in acute lymphoblastic leukemia (10). The cell of origin of precursor acute lymphoblastic leukemia is a lymphoblast at an early stage of differentiation. The architectural and immunological features of the thymus facilitate the correlation of expression with lymphocyte differentiation. In this study, we showed that there is increased expression of *DHX32* in mature normal thymocytes compared to less mature ones. This result suggests that *DHX32* might be involved in lymphocyte differentiation.

The requirements of tight regulation of gene expression during differentiation can be achieved at different levels. Through their modulation of RNA structure and interactions, and hence its availability for protein expression, RNA helicases are potential regulators of differentiation (4). There is evidence of the involvement of RNA helicases in differentiation. Examples include *DDX3* (11, 12) and *DDX25* in spermatogenesis (13). The *Drosophila* ortholog of *DDX4*, known as *vasa*, has been shown to be required for the formation of germ cells and oogenesis by a mechanism that involves regulating the translation of mRNAs essential for differentiation (14, 15). The role of *DHX32* in RNA processing is not yet known. The widespread expression of *DHX32* in other tissues does not rule out its potential involvement in lymphopoiesis. Developmentally-regulated expression of *DHX32* in lymphocytes could be explained by the involvement of lymphocyte-specific transcription factors in regulating its expression. This possibility is currently being tested through analysis of the promoter activity of the *DHX32* gene.

There are several examples of dysregulation of RNA helicases in cancer (5), mostly in the form of over-expression (6). However, the down-regulation of *DHX32* in acute lymphoblastic leukemia raises the question of whether RNA helicases could be targets for novel anti-cancer differentiating agents. Further characterization of the factors that regulate *DHX32* expression and its role in normal lymphopoiesis should shed more light on this possibility.

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Received January 18, 2005
Accepted April 14, 2005