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NOTCH1 Versus NOTCH3 in the Pathogenesis of T-Acute Lymphoblastic Leukemia

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ABSTRACT

Background: It has been established that Notch pathway plays a major role in the pathogenesis of T-ALL. The relative role of Notch1 versus Notch3 is still a controversial issue.

Objectives: The aim of this study was to evaluate the role of NOTCH1 and NOTCH3 in the pathogenesis of TALL. We also wanted to study the correlation of the expression of each of the two genes to various clinical and laboratory parameters.

Patients and Methods: We studied the expression of Notch1 and Notch3 in 46 T-cell acute lymphoblastic leukemia (T-ALL) patients (38 children/8 adults), in comparison with 12 cases of precursor B-ALL and 13 healthy adults, as control groups using Real Time PCR.

Results: The expression of both genes was increased in T-ALL, compared to precursor B-ALL and healthy subjects, and statistically higher for Notch3 (p=0.02) in children compared to adult T-ALL. Expression levels were higher in intermediate and late T-ALL group compared to early T-ALL for both genes (p=0.016 and 0.019) In T-ALL, a correlation was found between Notch1 and Notch3 (r=0.508/ p=0.0001). T-ALL and precursor B-ALL groups showed comparable Notch3/Notch1 relative expression ratios (p=0.312), however, it was significantly higher in comparison to healthy subjects, particularly for T-ALL (p=0.0001). The highest Notch3/Notch1 ratio was observed in T-ALL.

Conclusion: Our results confirm a pivotal role of Notch pathway in the pathogenesis of T-ALL. The higher Notch3/Notch1 ratio suggests that Notch3 dysregulation may play a more central role than Notch1 in T-ALL pathogenesis. This could open the field for Notch3 targeted therapy.

Key Words: *T-cell acute lymphoblastic leukemia – Notch1 – Notch3.*

INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is a heterogeneous disease with various subtypes that differ significantly in clinical outcome. Hence, there is a need to identify subtype-specific molecular markers, to improve our understanding of pathogenetic events and to facilitate risk assessment. T-cell ALL (T-ALL), which accounts for 10-15% of pediatric and 25% of adult ALL cases, includes several subtypes and these are thought to correlate with genetic aberrations at different stages of thymocyte differentiation **[1]**.

Members of the Notch protein family, have a role in cell-fate choice in several tissues, and control various steps of intrathymic T-cell development. Hence, dysregulated Notch signaling could be involved in the development of T-cell leukemia **[2]**.

Notch proteins are transmembrane receptors and are activated by ligand-mediated proteolysis, involving a series of mutually dependent cleavage events. This process releases the Notch intracellular domain (Notch-IC), which then translocates to the nucleus and forms a large transcriptional activation complex that includes proteins of the Mastermind family thus activating the transcription of target genes (e.g. The Hes family members) **[2]**.

Notch1 and Notch3 have been linked with distinct steps in the T-cell differentiation process that occurs inside the thymus **[3]**.

Regulation of the Notch-dependent T-cell developmental process appears to be affected in T-ALL. Notch3 was shown to be expressed in all the T-ALL patients examined, whereas its expression was dramatically reduced or absent in remission and in other types of ALL **[4]**. Notch1 was discovered as a partner gene in a t(7;9) chromosomal translocation resulting in <1% of T-ALLs **[5]**. Although the t(7;9) is rare, about 50% of human T-ALLs were noted to harbor activating point mutations in Notch1 that lead to aberrant activation of Notch signaling, placing the Notch1 pathway at the center of T-ALL pathogenesis **[6]**. However, other studies reported that Notch1 expression was not pathognomonic for T-ALL, because Notch1, but not Notch3 expression, was generally detected not only in normal peripheral blood T lymphocytes but also in non-T cell leukemias **[4]**. Some studies also reported that Notch1/Fbxw7 mutations are a good prognostic parameter in T-ALL with good overall survival and better outcome **[7,8]**. Alternatively, others reported the exact opposite **[9]**, while others reported that Notch1 mutations do not affect prognosis **[10]**.

Finally, Notch1 mutations have been reported to represent secondary events in both human and mouse T-ALL **[11,12]**.

Thus, the aim of this work was to study the expression of Notch1 and Notch3 and to verify the role of Notch1 versus Notch3 in the pathogenesis of T-ALL and to correlate the level of expression of both genes to other prognostic parameters.

PATIENTS AND METHODS

Patients and cell samples:

The study protocol was approved by the IRB of NCI and Faculty of Medicine, Cairo University. Under informed consent of the patients or their parents bone marrow (BM) or peripheral blood (PB) samples were taken from 46 newly diagnosed T-ALL patients (33 male and 13 female); 38 were children with a median age of 9 years (range: 18 months – <18.0 years), and 8 were adults with a median age of 27 years (range: 18-60 years). The study also included 12 cases of precursor B-ALL and 13 healthy adults as control groups. The choice of healthy adults as one of the control groups was based on ethical restrictions to use healthy children as control. Moreover, expression of the studied genes has not been reported to change with age in normal subjects.

Diagnosis of ALL was based on morphology and on cytochemical and immunophenotypic features. Immunophenotyping was performed on circulating leukemic blasts isolated using whole blood lysis technique, and cell-surface as well as intracytoplasmic antigens were detected by cytofluorimetric assay with a panel of monoclonal antibodies. The criteria for marker positivity and for the subclassification of T lineage ALL (early, intermediate, and late T-ALL) and non-T cell ALL (precursor B), were adopted as previously described (13). Mononuclear cells were isolated from BM and PB samples and stored at –80ºC until RNA extraction.

RNA extraction and cDNA preparation:

RNA was extracted from BM or PB samples of patients with newly diagnosed T-ALL, precursor B-ALL (both with >75% blast cells) and healthy subjects using the QIAamp RNA blood RNeasy Minikit (Qiagen, Hilden, Germany), according to manufacturers' instructions, further processed for RT-PCR, as previously described (4) and stored at –20ºC until use.

Evaluation of the tested genes:

Study of Notch1 and Notch3, genes' expression was carried out using Real-Time PCR (ABI Prism 7900 Sequence Detection System, Applied Biosystems) and TaqMan Universal PCR master mix (Applied Biosystems). Primer sequences are summarized in table (1). Gamma-secretase-resistant T-ALL cell lines with variable levels of expression of the studied genes were used as a reference for the methodology validation namely MOLT3, CCRF CEM, SKW3, JURKAT, HSB2, and LOUCY. The reaction was performed in 25µl mix containing 1X master mix, 1X assay on demand (AOD) mix, 900nM of each primer, 25ng cDNA and probe final concentration of 200nM. The data were analyzed using the relative standard method (relative fold change). For the tested genes, external standard curves were constructed using serial dilutions of known concentration templates (total thymocyte cD-

NA). The measured amount of the template from each gene was divided by the amount of cDNA from the housekeeping gene ß-actin measured in the same sample to normalize for possible variation in the amount and quality of cDNA between different samples **[14,15]**.

Statistical analysis:

SPSS package (version 15) was used for data management. Mean and standard deviation described quantitative data with median and range when appropriate (small number, no normal distribution). Parametric and non-parametric *t*-tests were used for comparing means of 2 independent groups and Kruskal Wallis ANOVA for comparing means for more than 2 independent groups. Parametric and non-parametric correlation analysis was done to elicit association between different genes and prognostic factors. *p*-value is significant at 0.05 level.

RESULTS

Clinical and laboratory characteristics of T-ALL patients' cohort:

Clinical data of T-ALL cohort showed 30% of cases with mediastinal mass, 13.5% with CNS involvement, 62.2% with hepatomegaly, 73% with splenomegaly and 66.7% with lymphadenopathy. Complete blood count showed 66.6% with haemoglobin level <10g/dl and 33.4% with a level ≥10g/dl, 16.2% with total leucocytic count (TLC) <10 x 10^9/L, 21.65% with TLC 10 - <50 x 10^9/L, 13.51% with TLC 50 - <100 x 10^9/L and 48.64% with TLC ≥100 x 10^9/L. BM or PB samples displayed a blast percentage with a range of 53-95% and 30-96% respectively. Immunophenotyping showed 20/46 cases to be early T-ALL (17 children and 3 adults), 20/46 intermediate T-ALL (17 children and 3 adults) and 6/46 late T-ALL (4 children and 2 adults).

Genes' expression of Notch family in T-ALL cases:

Forty six cases of newly diagnosed T-ALL were examined by Real-Time PCR compared to both precursor B-ALL and healthy subjects. Our results showed a significantly increased expression level of both Notch1 and Notch3 in T- ALL cases compared to the other 2 groups (Table 2). A moderate correlation was found between both genes (r=0.508, p=0.0001). Our study showed consistent results even when comparing between pediatric groups for both T-ALL and B-ALL cases (data not shown).

Genes' expression in T-ALL in relation to age:

In this work children showed increased gene expression of Notch1 and Notch3 compared to adults. However, the difference was found to be statistically significant only for Notch3 (Table 3).

Increased genes' expression in relation to T-cell differentiation:

Regarding the maturation stages of T-ALL our study examined the genes level in early, intermediate and late T-ALL. Due to the comparable level of gene's expression in both intermediate and late T-ALL, we considered them as one group as compared to early T-ALL. A statistically significant increase in gene expression in the intermediate and late T-ALL group was found for both genes compared to early T-ALL (Table 4).

Genes' expression in relation to other prognostic parameters in T-ALL:

In our study the level of Notch 1 and Notch3 genes' expression was compared in relation to prognostic parameters including age, hemoglobin level, TLC, mediastinal involvement, CNS involvement, hepatomegaly, splenomegaly, and lymphadenopathy. Significant results were found only for Notch1 expression in relation to hemoglobin level; Notch1 gene expression showed a higher level in the group with hemoglobin level <10g/dl compared to that with a level ≥10g/dl (p-value 0.049). Also a statistically significant moderate negative correlation was found between Notch1 expression and hemoglobin level (Fig. 1).

Notch3/Notch1 ratio among the studied groups:

Evaluation of Notch1 and Notch3 relative expression in the form of a ratio in T-ALL compared to both precursor B-ALL and normal control was done. Our results showed that Notch3/Notch1 ratio among T-ALL cases is comparable to that of precursor B-ALL cases (p-value 0.312), whereas both T-ALL and B-ALL cases showed a statistically significant higher ratio when compared to normal healthy subjects (p-value 0.0001 and 0.013 respectively) (Table 5).

Table (1): Reagents and primers for real time PCR: Applied biosystems.

Gene	Primer	Probe	Assay on demand*
Notch3	Fwd- ggatgagcttgggaaatcagc Rev- tccatttttgagcagggcc (cod. 4304971-80 nM) Rev- cacacctgtgggtagggctg (cod. 4304971-80nM)	ctgcggctgtgaaca (cod.4316033100uM)	
ßactin			Probe dye VIC-MGB 43263 15E-04 06005
Notch1			(cod. HS 00413187-M1 20x mix

* Assay on demand (AOD) sequence not provided.

Table (2): Genes' expression in T-ALL compared to control groups.

Gene	T-ALL (46 cases)	Precursor B-ALL (12 cases)	Healthy subjects (13 cases)	*p*1	*p*2	*p*3
Notch1	18.64±14.8* 0.34–62.53	7.25±9.53 0.15–34.63	1.0±0.6 0.28–2.24	0.019	0.0001	0.20
Notch3	3189.4±5089.02 0.204–21571.99	451.39±554.74 0.16–1877.60	1.0±0.98 0.16–3.86	0.044	0.0001	0.023

Mean±SD and range.
*p*1: *p*-value for T-ALL vs. B-ALL.
*p*2: *p*-value for T-ALL vs. Healthy subjects.
*p*3: *p*-value for B-ALL vs. Healthy subjects.

Table (3): Gene expression in T-ALL in relation to age.

Age group	No.	Notch1	Notch3
Pediatric	38	19.62±14.69*	3749.49±5430.78
Adult	8	13.96±15.39	528.97±895.96
p-value		0.21	0.02

* Mean±SD (Standard Deviation).

Table (4): Genes' expression in T-ALL in relation to immunophenotyping (IPT).

IPT	No.	Notch1	Notch3
Early	20	12.51±11.99* (0.349–33.98)	2077.30± 576.98 (0.20–11962.20)
Intermediate +late	26	22.95±15.25 (0.641–62.53)	3971.99±5867.37 (8.71–21571.99)
p-value		0.016	0.019

* Mean±SD

Table (5): Notch3/Notch1 ratio in T-ALL compared to control groups.

Group	Mean±SD	Range	Median
T-ALL	151.96±181.98	0.05–841.31	84.18
Precursor B-ALL	64.98±73.32	0.32–251.75	56.84
Normal Control	0.95±0.51	0.33–1.90	0.82

SD: Standard deviation.

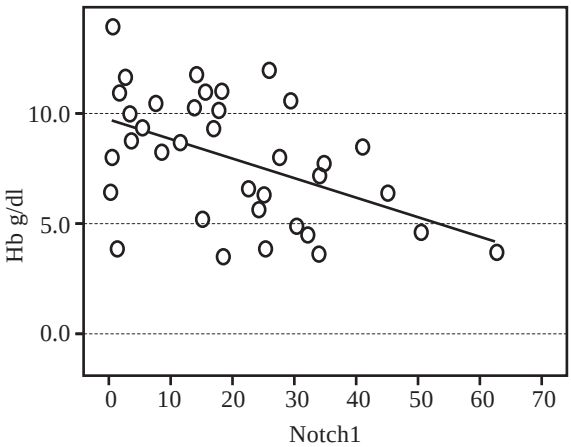

Fig. (1): Correlation between Hemoglobin level and Notch1 in 46 T-ALL cases: *r*-value:–0.476 *p*-value: 0.003.

DISCUSSION

Notch1 mutations are found in over 50% of T-ALL cases **[16,17]** and its role in the pathogenesis of T-ALL has been established. On the other hand, although no mutations were found in Notch3, its oncogenic role was supported in both mouse models and human T-ALL samples **[4]**. In this work we studied both genes to verify the role of Notch1 versus Notch3 in the pathogenesis of T-ALL.

Almost all studies concerning the role of Notch1 gene in T-ALL concentrated on studying the gene mutation rather than directly quantitating the gene expression as we did **[16-18]**. However, we propose that direct measurement of the gene expression would be more relevant as evidenced by the fact that Notch1 mutations were found only in 50% of cases **[16]**, whereas increased gene expression was reported in all T-ALL cases **[19]** as was encountered in the current study. The oncogenic role of Notch3 signaling was supported by mouse models in which enforced expression of Notch3-IC led to aggressive T cell leukemia reminiscent of human T-ALL **[4,20]**. Thus it could be postulated that the gene mutation is not the only factor affecting gene expression and that other mechanisms may influence Notch3 over-expression. Indeed, in the present work and, previously, some of us namely Bellavia et al. **[4]**, evidenced a significantly higher expression of Notch3 in all T-ALL cases examined with no evident mutations, compared to that of the controls. Notably, this expression level was significantly reduced or absent in cases that have undergone remission **[4]**.

In addition our study showed a significantly higher gene expression level for Notch3 in B-ALL cases compared to healthy controls and although there is a controversy regarding the role of activated Notch signaling in the development of B-cell malignancies **[19,22-24]**, the higher expression of this gene raises the possibility that Notch3 may be a contributing factor in the development of B-cell malignancies.

In our study children showed increased gene expression of Notch1 and Notch3 compared to adults; the difference was found to be statistically significant for Notch3. Up to our best knowledge, no other studies addressed this issue. We proposed separating our cohort in relation to age in view of the fact that in general ALL in children differs from adults in prognosis and response to therapy. The relevance of such findings to clinical outcome with children having better prognosis than adults needs further investigations.

With regards to the maturation stages of T-ALL, our results showed a statistically significant increase in gene expression in the intermediate and late T-ALL group, compared to early T-ALL for both genes. This suggests that the level of the genes is higher at both the double positive and the single positive stages in comparison with the double negative stage. Up to our best knowledge, no other studies addressed this issue using the same methodology on human subjects. However, other studies in murine models examining Notch1 mutations reported comparable results **[23,24-28]**.

In our study a correlation was found between both Notch1 and Notch3. A strong correlation between Notch3 and pTa was documented in both murine **[29]** and human T-ALL **[4]**; it was concluded that enforced expression of Notch3, which is ordinarily down-regulated as thymocytes mature, may sustain pre-TCR expression, causing dysregulated hyperplasia **[4]**. On the other hand, Chiaramonte and co-workers reported an increased level of Notch1 gene expression in T-ALL cases and identified pTa as a Notch1 pathway target gene **[19]**. Whether correlating with Notch3 or Notch1 all the previous studies show the strong interaction between the Notch pathway and pre-TCR signaling pathways documenting the role of both in T-cell ontogeny.

Our results showed a statistically significant negative correlation between Notch1 and Hb level. Up to our best knowledge no other studies addressed this issue. Notably Notch pathway is linked to early hematopoiesis during embryonic development **[30]**. Furthermore, it was reported that the Notch/RBPjk signaling pathway induces erythroid apoptosis in different hematopoietic tissues including yolk sac and bone marrow as well as in murine erythroleukemia cells **[31]**.

As one of the aims of our study was to evaluate the role of Notch1 versus that of Notch3 in the pathogenesis of T-ALL, we evaluated their relative expression in the form of a ratio in T-ALL compared to both precursor B-ALL and normal controls. Up to our best knowledge this issue was not addressed before. Our data suggest that dysregulation of the normal pathway of Notch genes occurs in ALL whether precursor B-ALL or T-ALL. The high Notch3/Notch1 ratio raises the possibility that Notch3 dysregulation and involvement in the pathogenesis of ALL may play a more central role than Notch1. Although, the relative ratio between Notch3 and Notch1 seems to be a character of

malignancy being comparable in precursor B-ALL and T-ALL yet the gene expression of both Notch3 and Notch1 is significantly higher in T-ALL cases. This could reflect the pivotal role of Notch pathway in the pathogenesis of T-ALL compared to its controversial role in B-ALL. Altogether, these data may question the hypothesis of some authors who reported that Notch3 could be a target of Notch1 **[32]** and has no real role alone in the pathogenesis of T-ALL, rather supporting the hypothesis of a prominent role of Notch3 in T-ALL development.

In conclusion, our study confirms a pivotal role of the Notch pathway in the pathogenesis of T-ALL. The higher Notch3/Notch1 ratio suggests that Notch3 dysregulation may play a more central role than Notch1, thus placing Notch3 as a major player in T-ALL pathogenesis. This could open the field for the new studies which identified monoclonal antibodies that specifically inhibit or induce activating proteolytic cleavages in Notch3 **[33]**. As was shown, intestinal epithelium cells express only Notch1 and Notch2 receptors, and Notch signaling plays an important role in homeostasis of intestinal progenitors **[34]**, thus one of the main drawbacks of using g-secretase inhibitors as a Notch signaling pathway inhibitor was the severe gut toxicity induced **[35]**. Consequently, it can be postulated that using monoclonal antibodies targeting only Notch3 could spare the gut these severe side effects. Thus proving Notch3 as a center stage player in the pathogenesis of T-ALL could revolutionize the ongoing targeted therapy into a new direction of targeting only a specific receptor instead of the whole pathway.

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