Correlation of Notch1/Hes1 Genes Expression Levels in Egyptian Paediatric Patients with Newly Diagnosed and Persistent Primary Immune(Idiopathic) Thrombocytopenic Purpura

Rania Mohsen Gawdat¹ · Amira Ahmed Hammam¹ · Dina Ahmed Ezzat²

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Abstract Notch signalling is involved in the development of several autoimmune diseases, one of such diseases is ITP. The aim of this study was to investigate and compare the expression levels of Notch1 receptor and its target Hes1 gene in Egyptian paediatric ITP patients. Real-time quantitative reverse transcriptase polymerase chain reaction was used to analyse the expression levels of Notch1 and Hes1 in 42 children with primary ITP (22 newly diagnosed and 20 persistent) cases. Twenty age and sex matched non-ITP controls were included. The expression levels of Notch1 were higher in newly diagnosed and persistent cases than controls with high statistical significant difference (P value < 0.001 , $P < 0.001$) respectively, similarly as regards the expression levels of HES1 (P value < 0.001 , $P < 0.007$) respectively. A significant positive correlation was found between Notch1 and Hes1 expression levels in newly diagnosed cases ($r = 0.587$, P value = 0.004). There was an association between levels of both genes in most of ITP patients but Hes1 was markedly elevated than Notch1 in few cases. High expression levels of Notch1/Hes1 indicated the important role of Notch signalling in both newly diagnosed and persistent ITP. High expression levels of Hes1 than Notch1 may shed light on its value as a therapeutic target for future research in ITP.

Keywords Idiopathic thrombocytopenic purpura · Notch1 receptor · Transcriptional factors · Gene expression · Real time-PCR

Introduction

In mammals, Notch signalling is comprised of 4 trans-membrane receptors, Notch 1–4 and five ligands, Jagged 1, 2 and Delta-like-1, 3, 4 [1]. It requires cell–cell interaction which is initiated when Notch receptors engage cognate ligands, resulting in Notch receptors cleavage by the enzymatic action of γ -secretase, followed by translocation of Notch intracellular domain (Notch-IC) to the nucleus [2, 3]. Within the nucleus, it interacts with a number of cytoplasmic and nuclear proteins, resulting in activation and regulation of numerous target genes such as hairy enhancer of split (Hes) family of basic helix-loop helix (bHLH) transcriptional repressors [4] which is formed of seven members (Hes1–7), where Hes1 is the classic and the most described gene regulated by Notch1 in multiple cell types [4, 5].

Notch signalling plays an important role in the immune system [6]. It regulates multiple steps of T and B cell development, T cell activation, regulatory T-cell function and T-helper cell differentiation [7–11]. Furthermore, it helps in the development and differentiation of many other hematopoietic and immune cells [12]. Deregulation of Notch signalling has been linked to multiple human diseases especially T-acute lymphocytic leukemia [13, 14] and recently, it has been related to the development of many autoimmune disorders as ITP [15].

The goal of this study is to detect Notch1/Hes1 genes expression among newly diagnosed and persistent Egyptian paediatric ITP patients, to focus on the correlation of

✉ Amira Ahmed Hammam
amirahammam@gmail.com

¹ Department of Clinical Pathology, Faculty of Medicine, Beni Suef University, Beni Suef, Egypt

² Department of Pediatrics, Faculty of Medicine, Beni Suef University, Beni Suef, Egypt

Notch1 expression levels to Hes1 in both ITP stages and to emphasize the importance of Hes 1 gene in ITP for the future design of novel therapeutic approaches targeting Hes1 gene.

Patients and Methods

The present study was conducted on Forty two paediatric patients (median of age: 6.3, with range 8 months–17 years), which is the most common age presenting with ITP. The patients were 22 girls and 20 boys who were presented to the paediatric hematology department, Faculty of medicine, Beni-Suef University hospital from the period of September 2012–September 2013. Exclusion criteria included infants below 6 months of age, recent manifestation of active infection, splenomegaly and secondary causes of ITP.

The patients were diagnosed and classified by duration according to the definitions confirmed by Vicenza Consensus conference [16, 17] into newly diagnosed ITP patients (within 3 months from diagnosis) and persistent ITP patients (between 3 and 12 months from diagnosis, including patients not achieving spontaneous remission or not maintaining complete response after stopping treatment during this period).

Accordingly, in our study there were 22 newly diagnosed ITP cases (10 boys and 12 girls; median of age: 5.8, with range 8 months–17 years) and 20 persistent ITP cases (12 boys and 8 girls; median of age was 8.5, with range 2–17 years).

In addition, Twenty age and sex-matched non-ITP controls (10 boys and 10 girls; median of age: 9.5, with range 6–14 years) with normal complete blood picture and normal platelet count were included in the study. They had been selected from the children that underwent a routine health examination in our outpatient department and from the hospitalized patients who had been admitted for elective surgery with no history of recent viral infection, atopy or medication at the time of taking blood samples.

Informed consent from the parents/guardians of both ITP and control groups of children were obtained at the time of their enrolment in the study, in accordance with Helsinki declaration of Bioethics [18]. A permission was taken from the ethics committee of Beni-Suef University hospital.

The diagnosis of primary ITP was made based on proper history taking, physical examination and laboratory assessment including: 1—complete blood count which revealed platelet count $\leq 100,000/\mu\text{L}$, normal HB concentration and normal TLC (the cut-offs used for HB and TLC as an exclusion criteria were according to different ranges

in different age groups [19]). 2—Bone marrow aspiration which revealed increased number of megakaryocytes than normal [20] with defective nuclear lobulation, cytoplasmic granulation and platelet budding. 3—Platelet antibodies were detected by using platelet immunofluorescence test as described [21].

RNA Isolation and Real-time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RTQ-PCR)

Two milliliters of peripheral blood was collected in EDTA tubes from both patients and controls. Total RNA were isolated from the peripheral blood using a QIAamp RNA blood kit (Qiagen, Germany). Complementary DNA (cDNA) was synthesized using (dt) 15-mer primer by superscript III Reverse transcriptase and stored at -20°C till use.

RTQ-PCR was conducted using the Light-Cycler system ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA) in accordance to the manufacturer instructions. The real-time PCR amplifications were performed in a reaction mixture of 6 μL of cDNA, 10 μL of 2 $\times$ SYBER Green Mastermix kit (Promega, Madison, WI, USA), and 1 μL of each of the forward and reverse specific primers of Notch1, GAPDH and Hes1 genes to reach a final volume of 20 μL in separate real-time PCR reaction assay. GAPDH was used as an internal control (reference gene).

The reaction protocol used involved heating for 10 min at 95°C , followed by 40 amplification cycles (15 s at 94°C and 1 min at 60°C). The PCR products were analysed by melt curve analysis.

The relative concentrations of the PCR products derived from both target genes were calculated using Light Cycler System software. The results were normalized relative to GAPDH. All experiments were conducted in triplicate and the triplicate results were averaged for each sample.

The Sequence of Primers Used for Detection of Notch1, Hes1 and GAPDH Genes [22]

Notch1

Forward: 5'-TCAGCGGGATCCACTGTGAG-3'
Reverse: 5'-ACACAGGCAGGTGAACGAGTTG-3'.

Hes1

Forward: 5'-TGATTTTGGATGCTCTGAAGAAAGATA-3'
Reverse: 5'-GCTGCAGGTTCCGGAGGT-3'.

GAPDH

Forward: 5'-GCACCGTCAAGGCTGAGAAC-3'

Reverse: 5'-TGGTGAAGACGCCAGTGGA-3'.

The expression levels of Notch1 and Hes1 genes in tested samples were expressed in the form of CT (cycle threshold) level then Normalized copy number (Relative quantitation) was calculated using the $\Delta\Delta$ CT equation.

Statistical Methods

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. χ^2 test was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann–Whitney test (non-parametric *t* test). Comparison between three groups was done using Kruskal–Wallis test (non-parametric ANOVA) followed by pair-wise comparison based on Kruskal–wallis test. Spearman-rho method was used to test correlation between numerical variables. A *P* value < 0.05 was considered significant.

Results

Clinical examination revealed that all patients have purpura and ecchymosis at the time of presentation, two patients had bleeding per gums, five had bleeding per rectum and seven had epistaxis).

The expression of Notch1 and Hes1 genes m RNA were determined in the blood samples of 42 pediatric primary ITP patients to be compared with 20 non-ITP controls.

The mean expression levels of Notch1 in all ITP patients was 165.89 ± 369.94 (range 0.016–1544.37) with a median level: 6.45, while its mean was 0.34 ± 0.17 (range 0.03–0.53) and median level: 0.39 in controls. The expression levels of Notch1 were significantly higher in cases than in controls (*P* value < 0.001).

The mean expression levels of Hes1 in all ITP patients was 312.93 ± 761.86 (range 0.01–3432.49) with a median level: 2.7, while its mean was 0.20 ± 0.15 (range 0.01–0.42) and median level: 0.25 in controls, showing higher statistical significant difference when comparing Hes1 levels in ITP to controls (*P* value < 0.001).

The 42 patients were then divided into 2 groups: 22 of them were newly diagnosed (1st group) and 20 were persistent cases (2nd group). Each group was compared to the 3rd control group (Table 1).

Although the expression levels of Notch1 in newly diagnosed patients were higher than those in persistent cases, but there was no statistically significant difference when comparing its expression levels between both groups (*P* value < 0.125), and similarly, as regards Hes1 levels (*P* value = 1).

There was an association between Notch1 and Hes1 expression levels in both newly diagnosed and persistent ITP cases, but the results showed higher values of Hes1 expression levels than Notch1 expression levels in few cases: 11/22 (50 %) of newly diagnosed ITP and 7/20 (35 %) of persistent ITP cases (Table 2).

When comparing the median of Notch1 or Hes1 expression levels between the three studied groups, the median of the levels of each of them was the highest in the newly diagnosed ITP than persistent ITP cases and controls with a high statistical significant difference (*P* value < 0.001, *P* value < 0.001) respectively (Fig. 1).

The median of platelet count at diagnosis of patients was $64.5 \times 10^9/L$, range 5–99 $\times 10^9/L$) which was significantly lower than that of the controls (median $278 \times 10^9/L$, range 200–348 $\times 10^9/L$), *P* value < 0.001.

Correlations

A statistical significant positive correlation was found between Notch 1/Hes1 expression levels in newly diagnosed cases (*r* = 0.587, *P* value < 0.004), while such correlation was not present in persistent ITP.

Neither Notch1 nor Hes1 had any correlations with either the clinical data or laboratory findings of ITP patients, except of a negative correlation between Hes1 and platelets' count in ITP patients with no statistical significance (*r* = 0.28, *P* value = 0.06).

Discussion

The choice of candidate genes for ITP study has largely focused on immune-related genes for which there is an approve of involvement in ITP or other autoimmune diseases [23].

In the immune cell development, Notch1 signal was essential for T-cell lineage commitment and is increased in activated but not naïve T cells [1, 24]. Meanwhile, Hes1 regulates the fate of various cell lineages in developing organs, including T-cells [25].

In the present study, the expression levels of Notch1 and Hes1 were analysed in 22 newly diagnosed and 20 persistent pediatric ITP patients with 20 matched age and sex non-ITP controls. Notch1 and Hes1 genes were expressed in all ITP cases and controls. Statistical analysis showed

Table 1 Comparison between Notch1/HES1 expression levels in the new diagnosed and persistent ITP cases (1st and 2nd groups) with non-ITP controls (3rd group)

	1st group	3rd group	P value	2nd group	3rd group	P value
Expression levels of Notch1	186.18 ± 386.44* (11.53, 0.208–1544.37)**	0.34 ± 0.17* (0.39, 0.03–0.53)**	<0.0001***	143.5 ± 359.54* (6.04, 0.16–1229.03)**	0.34 ± 0.17* (0.39, 0.03–0.53)**	<0.0001***
Expression levels of HES1	466.30 ± 991.07* (12.39, 0.27–3432.49)**	0.01 ± 0.17* (0.25, 0.02–0.42)**	<0.0001***	144.22 ± 331.25* (1.27, 0.15–1355.67)**	0.01 ± 0.17* (0.25, 0.02–0.42)**	<0.007***

* Mean ± SD

** Median, Range

*** High statistically significant

Table 2 Newly diagnosed and persistent ITP cases with higher Hes1 expression levels than Notch1

Newly diagnosed ITP	Hes1 levels	Notch1 levels	Persistent ITP	Hes1 levels	NOTCH1 levels
Case no.			Case no.		
6	48.51	38.46	4	1.27	0.016
9	2395.80	335.83	7	486.99	15.33
10	2.40	1.0	9	110.31	5.95
11	71.05	6.5	12	503.93	5.53
12	2545.18	24.39	13	399.32	4.5
14	131.70	10.81	18	14.96	3.7
15	3432.49	16.84	19	1355.67	6.04
17	9.07	3.62			
18	6.51	0.56			
21	15.72	0.76			
22	0.93	0.20

Fig. 1 A comparison between the three studied groups, as regards the median of the expression levels of Notch1 and Hes1, being higher in the newly diagnosed ITP than in the persistent ITP cases and non-ITP controls with high statistical difference (P value < 0.001, P < 0.001) respectively

significant high levels of both genes in all ITP (newly diagnosed and persistent) cases than controls.

Ma et al. [22], who were the first to explore the expression of Notch1 and Hes1 in ITP, by QRT-PCR, but

in adult patients, reported that high Notch1/Hes1 expression levels were significantly higher in patients than controls which showed an activation of Notch Signaling in ITP patients which was in agreement with our findings.

We didn't find a statistical significant difference between newly diagnosed and persistent ITP patients as regards Notch1 and Hes1 expression levels although both are higher in the former group than the later one but this may be explained by the small number of samples used in our study. Our results were in consistent with Ma et al. [22], as no significant differences of Notch1/Hes1 levels were found between their studied groups (untreated and recurrent ITP).

In the present study, we noticed an association between expression levels of Notch1 and Hes1 in both newly diagnosed and persistent ITP cases, with a significant positive correlation between Notch1/Hes1 expression levels in newly diagnosed cases ($r = 0.587$, P value < 0.004), but such correlation was not in consistent with Ma et al. results [22], which may be due the small number of samples (22 samples) used in their study. Such Positive correlation should be confirmed with more future studies in both of paediatric and adult ITP as it may be of value in

using Notch1 and Hes1 as therapeutic target genes in ITP [6, 26].

According to our findings, 11/22(50 %) of newly diagnosed ITP and 7/20(35 %) of persistent ITP cases showed very high Hes1 expression levels associated with very low Notch1 expression levels, which may be related to the multiple signal inputs from different pathways such as Sonic Hedgehog, c-Jun-N-terminal kinase and tumor necrosis factor α pathways that control Hes1 expression in different cell types [27–31].

In addition, previous studies revealed that Hes1 may act independently of Notch signalling and showed that its expression could not be affected by genetic or pharmacological blocking of Notch as in Ewing's sarcoma [32, 33].

In our study, neither Notch1 nor Hes1 had any correlations with either the clinical data or laboratory findings of ITP patients, except of a negative correlation between Hes1 and platelets in ITP patients with no statistical significance ($r = -0.28$, P value = 0.06) which agreed with Ma et al. [22].

High expression levels of Notch1/Hes1 presented in our study were similar to those reported in other autoimmune diseases [34–37].

On the contrary to our results [38], lower levels of Notch1 were detected in active SLE than controls, but the difference in Notch1 levels between ITP and SLE was explained by the nature of ITP disease and the difference in the biological samples used in both studies [22]. Later on, it was revealed that these decreased levels were due to Notch1 transcriptional repression in SLE that occurred through epigenetic and CAMP responsive element modulator α (CREM α) [39] and this was not present in ITP.

In conclusion, our study showed that overexpression of Notch1/Hes1 genes may play an important role in pediatric ITP and increased Notch1 activity is reflected by increase in Hes1 expression levels. Also, High Hes1 levels than Notch1 may shed light on the importance of further researches in ITP to evaluate the relationship between Hes1 and other signaling pathways rather than Notch that may help to understand ITP biology and encourages designing novel therapeutic approaches targeting Hes1 gene.

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