Role of Long non Coding RNAs, NEAT1 and Lnc-DC Expression in Pediatric Immune Thrombocytopenic Purpura

Soha Mohamed Hamdy[1], Marwa Saad Ali[1], Rehab Galal Abd El-Hmid[2], Noha Khalifa Abdelghaffar [3], Omayma Owees Abdelaleem*[4]

Abstract

Background: Pediatric immune thrombocytopenic purpura (ITP) is an autoimmune disease; whose etiology is unknown. lncRNAs are regulators of numerous actions, which participate in the development of autoimmune diseases. We evaluated the expression ofNEAT1 and Lnc-RNA in dendritic cell (Lnc-DC) in pediatric ITP.

Methods: Sixty ITP patients and 60 healthy subjects were enrolled in the present study; Real-time PCR was performed to assess the expression levels of NEAT1 and Lnc-DC in sera of children with ITP as well as healthy children.

Results: Both lncRNAs, NEAT1 and Lnc-DC were significantly upregulated in ITP patients in comparison to controls (p <0.0001 and P= 0.001 respectively). Furthermore, significant upregulation of the expression levels of NEAT1 and Lnc-DC were observed in the non-chronic compared with chronic ITP patients. Also, there was significant negative correlation between each of NEAT1 and Lnc-DC and platelet counts before treatment (r= -0.38; P= 0.003 and r= -0.461; P< 0.0001, respectively).

Conclusions: serum lncRNAs, NEAT1 and Lnc-DC could be used as potential biomarkers in differentiating childhood ITP patients and healthy controls in addition to differentiating non-chronic from chronic ITP which may provide a theoretical basis for the mechanism and treatment of immune thrombocytopenia.

Keywords: Lnc-DC, NEAT1, Pediatric, ITP.

Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disorder with an estimated incidence of 4–5 cases per 100,000 children per year (1). Platelet counts $<100 \times 10^9/$ L with isolated thrombocytopenia is a hallmark of ITP (2). At variance with adults, in children most cases resolve during the first 12 months from diagnosis, but in a minority of them (around 20-30 % of cases) a chronic disease occurs requiring treatment to avoid risk of bleeding. Most non-chronic forms often occur with an "acute onset" (1). Identifying predictive biomarkers to differentiate chronic from self-limited non-chronic disease could help to differentiate the pathogenesis of the disease and to guide treatment strategies (3).

For unknown reasons, platelet surface proteins become antigenic and stimulate the immune system to induce cytotoxic T lymphocytes and auto-antibodies resulting in reduce platelet production as well as immune-mediated damage of platelets (3,4).

Long non-coding RNAs (lncRNAs) are RNAs of >200 nucleotides that are not

1: Departments of Chemistry, Faculty of Science, Fayoum University, Fayoum, Egypt.
2: Departments of Pediatrics, Faculty of Medicine, Fayoum University, Egypt.
3: Departments of Clinical pathology, Faculty of Medicine, Fayoum University, Egypt.
4: Departments of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Fayoum University, Egypt.
*Corresponding author: Omayma Owees Abdelaleem; Tel: +01 049491030; E-mail: ooa00@fayoum.edu.eg.

translated into proteins. In recent times, numerous research verified that lncRNAs are implicated in many biological and physiological processes, such as activation and maturation of innate and acquired immune response (5). The lncRNAs have been concerned with gene expression modification through various mechanisms, like binding with translational and transcriptional factors as well as DNA epigenetic modifications (6,7), giving a possible explanation of their role in autoimmune diseases.

Nuclear paraspeckle assembly transcript 1 (NEAT1) is a lncRNA in animal cells which is essential for biogenesis and maintenance of nuclear bodies, chromatin remodeling, gene expression regulation, stress and immune responses, organogenesis, and the development of pathological diseases including cancer (8).

Lnc-DC is specifically expressed in DCs. Numerous researchers found that differentiation of monocytes into DCs and ability of DCs to stimulate T cell activation are affected by knockout of the lnc-DC which helps phosphorylation of tyrosine-705 of signal transducer and activator of transcription 3 (STAT3) via binding to STAT3 in the cytoplasm, affecting the downstream genes transcription and increasing the maturation and differentiation of DCs (9).

Our aim was to investigate the expression levels of NEAT1 and Lnc-DC in children with ITP as novel biomarkers and to assess their expression levels with different clinical and laboratory data.

Materials and Methods

The current study was done at the Pediatric Department of Fayoum University Hospital, El Fayoum, Egypt.

The current study included 60 children diagnosed with ITP who were attending pediatric ward at Fayoum University Hospital. They were 42 (70%) females and 18 (30%) males aged 5(1-13) years by median (IQR).

The diagnosis of ITP was either prompted by the presence of ecchymosis and or purpuric eruption or by a confirmed finding of incidental thrombocytopenia (less than 100,000/uL) in the absence of any apparent underlying disease. Furthermore, 60 healthy children of matching age and sex, who attended the Outpatient clinic were recruited as controls. They were 44 (73.3%) females and 16 (26.7%) males aged 6 (4-15) years by median (IQR).

The following patients were excluded from the study:1-Patients with thrombocytopenia with other autoimmune or immune deficiencydiseases.2-Patients with thrombocytopenia due to bone marrow aplasia, sepsis, or malignant cell infiltration. 3-Patients older than 18 years.

Blood sample collection and storage

3ml peripheral venous blood sample was withdrawn from each subject by using vacutainer system. Serum separator tubes were used to collect the samples, left to clot for 15 minutes, and then centrifuged at 4000 xg for 10 minutes. Sera were separated and stored at -80 °C until the time of analysis. This serum was used in long non-coding RNAs extraction and detection of fold change of NEAT1 and Lnc-DC using real time PCR.

Long non coding RNAs extraction

Purification of serum total RNA, together with long non-coding RNA was done using miRNeasy mini kit (Qiagen, Valencia, CA, USA). NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA) was used to assess RNA quantitation and purity

Reverse transcription (RT) of RNA into complementary DNA (cDNA)

RT2 First Strand Kit (Qiagen, Maryland, USA) was used to reverse transcription whole RNA in a final volume of 20 ul RT reactions.

Quantitative Real-time PCR (qPCR) for Detection of long-non coding RNAs NEAT1 and Lnc-DC

The reagents of RT2 SYBR Green ROX q PCR Master mix, RT2 IncRNAqPCR Assay (Qiagen, Maryland, USA), and cDNA synthesis reaction were used to form PCR

reaction mix fora 25μl per well reaction volume.

The real-time cycler (DNA-technology thermocycler, DT lite 4S1, Russian) was programmed according to the following steps: initial heat activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and combined annealing/extension at 60 °C for 60 s.

Expression levels of the lncRNAs, NEAT1 and Lnc-DC were evaluated with GAPDH as internal control (10) using primers for NEAT1, Lnc-DC and GAPDH, that were ready-made. Catalog no. of NEAT1 is 330701LPH 15809A and its refSeq Accession no. is NR_028272.1, Gene ID: 283131 Catalog no. of Lnc-DC is 330701 LPH23184A and its refSeq Accession no. was NR_030732.1, Gene ID: 645638 Catalog no. of GAPDH is 330701LPH31725A and its refSeq Accession no. was ENST00000496049.0. Fold change (FC) was calculated using $2^{-\Delta\Delta Ct}$ for each patient.

Values of FC that were more than 1 indicated that the long noncoding RNA was over expressed; while that of FC that were less than 1 indicated that it was under expressed. Control value was equaled 1, because $-\Delta\Delta Ct$ for control subjects equals zero and 2^0 equals one.

Statistical analysis of data

Software statistical computer package version 18 (SPSS Inc, USA) was used for organization, tabulation and analyzing the collected data statistically. For quantitative data, the mean, median, standard deviation (SD), and range were calculated. Kolmogorov-Smirnov test (KS) test was performed as a test of normality. Chi square ($\chi 2$) was performed for presentation of qualitative data as number and percentages. The relation of NEAT1 and Lnc-DC with study parameters was assessed using Spearman correlation. The receiver operating characteristic (ROC) curve was used to determine the discrimination value of NEAT1 and Lnc-DC for cases differentiation. Significance was set at $P \leq 0.05$.

Results

The demographic characteristics of all patients and the controls are summarized in Table 1. No significant difference was observed between both groups regarding age (P = 0.416) and sex (P = 0.658).

However, there were significant differences between the ITP patients and the controls regarding white blood cell counts, hemoglobin and platelets counts before and after treatment (P=0.023, P= 0.0001 and P= 0.0001, respectively).

The relative expression levels of NEAT1 and Lnc-DC were upregulated in ITP patients, (median fold change 12.91, range, 0-131.6; P <0.0001) and (median fold change 4.8, range, 0-295.57, P<0.0001) for NEAT1 and Lnc-DC, respectively (Fig. 1).

Moreover, NEAT1 was increased significantly in non-chronic ITP group compared to control group (P< 0.0001). Furthermore, significant upregulation of the expression level of NEAT1 was observed to be elevated in the non-chronic compared with chronic ITP patients (P= 0.036). However, no significant differences were observed between chronic ITP and control (P =0.243). Also, Lnc-DC serum expression level was significantly increased in non-chronic ITP group compared to control group (P< 0.0001) and significant differences between the non-chronic and chronic ITP patients (P= 0.024) was observed. There were no significantly differences between chronic ITP patients and control (P =0.659) regarding the expression level of Lnc-DC (Table 2).

Table 3 illustrates that there were no statistically significant differences between NEAT1 or Lnc-DC and clinical characteristics such as sex, family history, bleeding, splenomegaly, and *H. pylori*. However, there was statistically significant upregulation of Lnc-DC among patients who had preceding febrile illness (PFI) than those without PFI, P=0.018.

As shown in Table 4, there was statistically significant positive correlation betweenNEAT1 and Lnc-DC (r= 0.849; P< 0.0001). Also, there was significant negative correlation between

NEAT1 and platelet counts before treatment (r= -0.38; P= 0.003). Similarly, significant negative correlation between Lnc-DC and platelet counts before treatment was observed (r= -0.461; P< 0.0001).

Receiver-operating characteristic (ROC) analysis revealed that serum NEAT1 and Lnc-DC levels differentiated chronic ITP patients from non-chronic ITP patients. For NEAT1, the optimal cut-off value was 0.84% with a sensitivity of 87%, a specificity of 42.9%, P< 0.352 and AUC 0.618(95% CI= 0.444-0.972). Also, the optimal cut-off value of Lnc-DC was 3.56% with a sensitivity of 65.2%, a specificity of 71.4%, P< 0.148 and AUC 0.682 (95% CI= 0.531-0.836) (Fig. 2).

Table 1. The baseline demographic and laboratory data of ITP patients and control. Data are expressed as median (IQR), mean ± SD, or n (%).

	Cases (N=60)	Control (N=60)	P-value
Age (years)	5(1-13)	6(4-15)	0.416
Sex: **Male, n (%)** **Female, n (%)**	18(30.0) 42(70.0)	16(26.7) 44(73.3)	0.658
HB (g/ dl)	10.2 ±3.4	13.9 ±1.3	**<0.0001***
WBC ×10³ /µl	8.4±3.9	7.1±1.9	**0.023***
Platelets before treatment (×10³ /µl)	8.5(1-55)	228 (135-287)	**<0.0001***
Platelets after treatment (×10³ /µl)	82.5 (20-219)	228 (135-287)	**<0.0001***
Family history, n (%) Negative Positive	56(93.3) 4(6.7)		
Chronic, n (%) No Yes	46(76.7) 14(23.3)		
Bleeding, n (%) Yes No	16(26.7) 44(73.3)		
Splenomegaly, n (%) Negative Positive	58(96.7) 2(3.3)		
***H. pylori* Ag, n (%)** Negative Positive	56(93.3) 4(6.7)		
PFI, n (%) No Yes	22(36.7) 38(63.3)		

HB: Hemoglobin: White blood cell, *H. pylori: Helicobacter pylori*, PFI: preceding febrile illness. *Significant<0.05

Table 2. Comparison between ITP acute cases, ITP chronic cases and controls regarding expression levels of NEAT1 and Lnc-DC.

	Acute cases (N=46)	Chronic cases (N=14)	P-value
NEAT1	13.36 (0-131.6)	6.77(0.02-65.80)	**0.036[a]**
Lnc-DC	4.99 (0-295.57)	2.81(0.04-28.64)	**0.024 [a]**

Fold change levels represent noncoding RNA expression relative to controls that were calculated as $2^{-\Delta\Delta CT}$. Data are expressed as median and Intra quartile range. *Significant <0.05, a: comparison between acute and chronic cases, b: comparison between chronic cases and controls, c: comparison between acute cases and controls.

Table 3. Association between NEAT1 and Lnc-DC and clinical characteristics.

		NEAT1	P-value	Lnc-DC	P-value
Sex	Female	10.13 (0.02-131.6)	0.846	4.72(0.04-295.57)	0.698
	Male	20.81 (0-114.56)		4.99(0-70.52)	
Family history	negative	12.91 (0-131.6)	0.742	4.25(0-295.57)	0.067
	positive	24.35 (5.06-43.63)		57.34(10.13-104.56)	
Bleeding	bleeding	14.24(0.02-131.6)	0.815	4.52(0.04-26.94)	0.242
	No bleeding	12.91(0-120.77)		4.8(0-295.57)	
Splenomegaly	negative	12.47(0-131.6)	0.092	4.72(0-295.57)	0.191
	positive	65.4(65-65.8)		28.8(28.6-29)	
***Helicobacter Pylori* Ag**	negative	17.08(0-131.6)	0.077	4.93(0-295.57)	0.058
	positive	2.88(1.36-4.41)		1.55(0.34-2.77)	
PFI	no	6.77(0.002-65.88)	0.307	2.81(0.04-28.64)	0.018*
	yes	20.81(0-131.6)		5.83(0-295.57)	

H. pylori: Helicobacter pylori, PFI: preceding febrile illness
*Significant <0.05

Table 4. Correlations between NEAT1, Lnc-DC and other variables among study group.

	NEAT1		Lnc-DC	
	r	P-value	r	P-value
Lnc-DC	**0.849**	**<0.0001***		
Age (years)	-0.079	0.547	-0.068	0.606
HB (g/dl)	0.013	0.924	0.050	0.704
WBC×10³ /µl	0.132	0.314	0.128	0.331
Platelets before	**-0.380**	**0.003***	**-0.461**	**<0.0001***
Platelets after	0.035	0.789	-0.039	0.766
lymphocyte count	-0.007	0.958	0.016	0.906

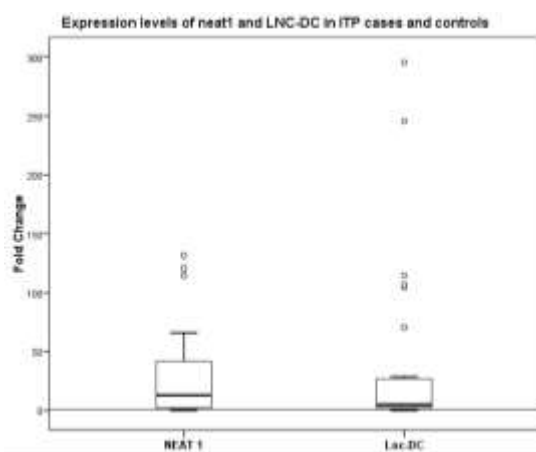HB: Hemoglobin, WBC: White blood cell. *Significant P< 0.05.

Fig. 1. Box plot representation indicative of the serum expression of NEAT1 and Lnc-DC (fold change) in pediatric Immune Thrombocytopenic Purpura compared with the levels in healthy controls. Data are presented as a box plot (median, upper, and lower quartiles). The horizontal line represents the expression levels of NEAT1 and Lnc-DC in healthy subjects.
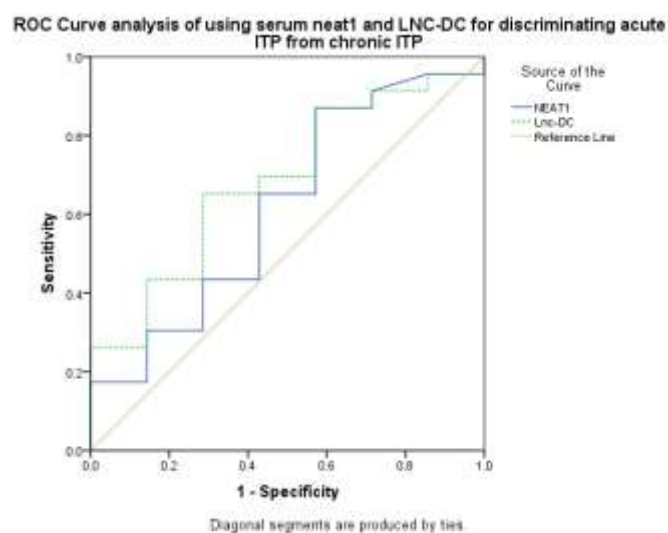

Fig. 2. ROC curve analysis of serum NEAT1 and Lnc-DC for differentiating children with chronic from nonchronic ITP ones.

Discussion

In the current study, of the 60 children with ITP, 76.7% were diagnosed with non-chronic ITP and 23.3% with chronic ITP and nearly 26.7% of children had bleeding manifestations. These results are in agreement with the reports of Alexandreos et al. who reported in their results that 39 (68%) children had newly diagnosed, 4 (7%) persistent, and 14 (25%) chronic ITP. Also, Tu¨lin et al. demonstrated that 71.6% of the patients in their study had non-chronic ITP and 28.4% had chronic ITP (3, 11).

Also, according to our results, there was significant difference between the ITP patients and the controls regarding white blood cell count which is in accordance with Sheema et al. and Jeon et al. results (12,13). However, significantly low Hb level and platelet count were noticed in the ITP group when compared to control group which is similar to the results of Badrawy et al. and Faki Osman (14,15). As known, one of the hallmarks of ITP is the decreased number of circulating platelets that results in bleeding which cause anemia and low Hb level which could explain our results (16).

In this present study, more than 63.3% of patients suffered from ITP after a preceding febrile period. Our results agree with those of Talaat et al. who reported in their results that 85% of the patients with ITP disease had history of preceding infection, also, Ayoub et al. was reported that ITP had been preceded with a febrile illness in 66.7% of the patients (17,18).

Also, according to our results, 6.7% of patients suffered from ITP after H. pylori infection which came in line with those of Ayoub et al. who reported that ITP had H. pylori infection in 10% of the patients and Sheema et al. who reported also that ITP patients had diagnosed after infection with H. pylori which detected in 34 (40%) positive and 51 (60%) negative (12,17).

Our study showed that the relative expression levels of lncRNAsNEAT1 and Lnc-DC were significantly over expressed in the serum of ITP patients when compared to control group and there were significant differences in NEAT1 and Lnc-DC between ITP patients and the controls groups. But no significant differences were noted in the expression levels of NEAT1 and Lnc-DC between chronic ITP patients and the control group.

Numerous research had proposed that the differential expression of lncRNAs might participate in the progression of ITP through affecting both the proinflammatory and inflammatory biomarkers (17, 19). However, to the best of our knowledge, the associations between NEAT1 and Lnc-DC with ITP in pediatrics have not been studied yet.

It was reported in previous study that NEAT1expression level was increased in RA patients and in differentiated Th17 cells (20). The authors in the abovementioned work suggested that NEAT1 helped CD4+ T cells differentiation into Th17 cells through inducing the STAT3 protein level accelerating the inflammatory response in RA. Of note, it was documented that Th17 cells and its downstream cytokine (IL-17) were obviously elevated in ITP patients (21, 22) we hypothesized that up regulation of NEAT1 may results in differentiation of Th17 cells which play vital role in pathogenesis of ITP, though further works are needed to prove these results.

Furthermore, Zhang et al. showed that NEAT1 expression level was positively correlated with disease activity in patients with SLE. The elevated NEAT1 expression could result in secretion of a various cytokines and chemokines including IL-6 and CXCL10(C-X-C motif chemokine ligand 10) in addition to activation of TLR4-mediated inflammatory process in those patients (23).Interestingly, numerous researches proved involvement of IL-6, CXCL10 as well as TLR4 in the pathogenesis of ITP (24,25).

Additionally, Liu et al., reported that upon activation of purified CD4+T cells the expression of NEAT1 that promotes Th2

differentiation is increased resulting in production of antibodies and numerous cytokines as IL-4, IL-5 IL-6 and IL-13 which arises in ITP (24).

We hypothesized that elevated level of NEAT1 in ITP patients are related to activation of purified CD4+T cells resulting in increasing the levels of cytokines.

The current study verified that there was considerable negative correlation between NEAT1 and platelet counts before treatment which is in line with Xu et al. who showed that NEAT1 expression was significantly negatively correlated with platelet count. However, Bian et al. suggested that the NEAT1 was related to platelet activity; they reported that NEAT1 knockdown decreases platelets activity (26,27).

In the existing study we documented that the relative expression level of Lnc-DC was significantly increased in the serum of ITP patients when compared to control group.

There were growing researches to identify the role of lnc-DC in autoimmune diseases. Shaker et al. observed up regulation of serum levels of lnc-DC in patients with multiple sclerosis (MS) and proved that serum lnc-DC could be used as a potential novel biomarker for diagnosis of MS (28). As well, Zhuang et al. showed significant positive correlation between levels of Lnc-DC and the immune response of HBV (29). Also, Chen et al. verified marked association between values of Lnc-DC and Primary Sjögren's Syndrome (pSS) patients with ITP (30).

Lnc-DC, that is located on chromosome 17, near the STAT3 gene, is one of the lncRNAs that absolutely present in dendritic cells (DCs) which is concerned with the differentiation of DCs and of T cells activation (31). Lnc-DC binds directly to STAT3 in the cytoplasm, helping phosphorylation of STAT3 on tyrosine-705, thus affecting the transcription of downstream genes and inducing the maturation and differentiation of DCs (9).

Expression of the STAT3 to increase Th17 cells which secrete numerous cytokines is regulated by Lnc-DC and this promotes the

inflammation and affects the incidence and progression of the disease (30).

The apoptotic platelets are presented to T lymphocytes with the aid of DCs which is important in pathogenesis of ITP (32). Furthermore, treatment with prednisone results in reduction of circulating DCs that is associated with elevated levels of platelets in the circulation (32) suggesting that DCs activate antigen-specific immune responses in ITP. Besides, therapy of corticosteroid could reduce the number of circulating DCs diminishing the effects of the autoantibody on platelets in patients with ITP patients (30).Additionally, Catani et al., verified that increased tendency of platelets to go through apoptosis as well as dysfunction of DC could play a role in ITP through stimulation of the immune system(32). In addition, Zhou et al. showed that there is activation of dendritic cells in ITP patients (33). The abovementioned data indicate that DCs functions are involved in the pathogenesis of ITP. Our results showed that there was statistically significant upregulation of Lnc-DC among patients who had preceding febrile illness (PFI) than those without PFI.

It was documented that DCs differentiating from monocytes represents one of the important lines of defense of the body against invading pathogens (34). Interestingly, lnc-DC was absolutely present in dendritic cells helping its differentiation. Also, lnc-DC helps phosphorylation of STAT3 which plays a vital role in viral and bacterial infection and pathogenesis (35). From the previous data we may explain the upregulation of Lnc-DC among patients who had PFI.

We concluded that serum lncRNAs, NEAT1 and Lnc-DC were up regulated in childhood ITP patients compared with healthy control which may provide a theoretical basis for the mechanism and treatment of immune thrombocytopenia. Furthermore, NEAT1 and Lnc-DC were increased significantly in non-chronic ITP group compared to control group. Furthermore, significant upregulation of the expression levels of NEAT1 and Lnc-DC were

observed in the non-chronic compared with chronic ITP patients.

Ethics declarations

The Ethical Committee of Faculty of Medicine (protocol code R286), Fayoum University approved the study which carried out in compliance with the Helsinki Declaration (2009). Informed consent from the legal guardians of each case was taken.

Conflicts of Interest
None.

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