RESEARCH PAPER INTERNATIONAL JOURNAL OF PURE MEDICAL RESEARCH

Medicine

KEYWORDS: ITP, ABCB1, Glucocorticoids,polymorphisms

EFFECT OF MDR1 GENE POLYMORPHISMS ON RESPONSE TO ORAL PREDNISOLONE IN EGYPTIAN IMMUNE THROMBOCYTOPENIC PATIENTS

Volume-3, Issue-4, April - 2018

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Article History
Received: 26.01.2018
Accepted: 04.03.2018
Published: 10.04.2018

ABSTRACT.

Immune thrombytopenia(ITP) is an autoimmune disorder characterized by heterogeneous bleeding manifestations as well as marked-inter individual variation in response to glucocorticoid (GCs); the 1st line ITP treatment. This may be due to Pgp protein; the product of ATP binding cassette B1(ABCB1) gene.IT IS involved in effluxing drugs as steroids outside the intracellular space thus decreasing its intracellular concentration. The study investigated relation between single nucleotide polymorphism (SNPs) in ABCB1gene and variation of response to oral prednisolone treatment in 100 Egyptian ITP patients. Clinical response was measured during 1st 28 days and compared with C3435T and G2677T SNPs determined by PCR REPLF technique. No significant difference in C3435T between ITP and control. Carriers of mutant allele of G2677T were significantly higher in ITP compared with control (p value :02) suggesting possible role in disease predisposition. But No relation was between age of onset, initial platelet count, steroid response or steroid dependence and any of both polymorphisms

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder involving autoantibody and cell-mediated increased platelet destruction and/or decreased platelet production (Kashiwagi ,2013, Rodeghiero et al., 2009). It is characterized by clinically heterogeneous bleeding manifestations ranging from petechiae, ecchymoses, mucosal bleeding as vaginal, GIT bleeding, etc. to rare intracranial Hge (Neylon et al., 2003). Great possibility that there are genetic factors playing a pivotal role in several aspects of ITP as disease susceptibility, and inter individual variability of GCs response (D'Orazio et al., 2013) .Despite the precise mechanisms of which are not completely understood, glucocorticoids (GCs) have been the mainstay and first-line therapy used worldwide in the treatment of ITP for decades (Pang et al., 2010) Eighty to ninety percent of patients respond adequately. However, still minority of patients are unresponsive. On the other hand some patients necessitate keeping them on high doses, repeated courses of corticosteroid administration to maintain a platelet count or to avoid bleeding, a group defined as steroid dependent patient bringing more steroid toxicity (Rodeghiero et al., 2009). This inter-individual variation in steroid response may be due to group of transport proteins one of them is Pgp –efflux protein encoded by ABCB1 gene..Functional P-gp is highly expressed also in several hematological cells as natural killer (NK) cells, CD4and CD8lymphocytes which play an important role I ITP pathogenesis (Klimecki, 1994).This protein is involved in pharmacokinetics; absorption, distribution, and disposition of drugs by pumping effluxing them from the intracellular space back into the intestinal lumen decreasing intracellular drug concentration(Goh et al., 2002).Glucocorticoids are substrates of Pgp.ABCB-1 expression in lymphocytes has been reported to be negatively correlated with the response to prednisone, Many studies have reported that certain single-nucleotide polymorphisms (SNPs) in the MDR-1 gene are associated with altered drug disposition(Ieiri et a., 2004).Recently, more than 50 single nucleotide polymorphism (SNPs) were identified for ABCB1 among them, three SNPs of ABCB1 (C1236T, C3435T and G2677T/A) which were frequenty studied . Frequency of these polymorphisms as well as effect vary widely among different ethnic populations, indicating ethnicity has a major impact on genetic distribution and effect (Ameyaw et al., 2001). our study was done on C3435T and G2677T/A in Egyptian ITP, relation to predisposition , response to oral GCs, and disease course. We found C3435T had no difference thus no role in disease predisposition. G2677T is higher in ITP group suggesting role in ITP predisposion . however both had no effect on response to oral prednisolone, steroid deendene, age of ITP or initial platelet count .

Patients and method:

This was a prospective study conducted at hematology clinic and inpatient department of EL- minia and El Kasr El Einy university hospital at the period between Dec 2015 to Dec 2017
Subjects:

This study has included 2 groups:

1 Patient group: 100 ITP patients after providing written informed consent.
2 Control group: 100 age and sex matched healthy volunteers

I. Inclusion criteria

Patients were diagnosed as ITP according to The American Society of Hematology (ASH) 2011 evidence-based practice guideline for immune thrombocytopenia (Neunert et al., 2011) as follow:

- Isolated thrombocytopenia (platelet count <100 x 109/L.)
- Normal red cell indices, white blood cell count, and platelet morphology
- No other causes of thrombocytopenia

ii. Exclusion criteria

1- Patients aged younger than 18 years old
2- Pregnant females
3- Patient with thrombocytopenia due to other cause:

- HCV, HIV, chronic liver disease,
- Others autoimmune disease, SLE, anti-phospholipid syndrome
- Drug induced thrombocytopenia as quinine and heparin induced thrombocytopenia
- Lymphoproliferative disease

Methodology:

All groups s were exposed to confirm bleeding, exclude other causes of thrombocytopenia

A-Thorough history taking
B- Complete physical examination:
C- Laboratory work up

1- Routine Complete blood count
2- Work up to exclude secondary causes of thromboytopenia

a. Peripheral blood smear: Size and morphology of platelets, RBCs, and WBCs
b. ANA, anti-ds DNA
c. Anti-phospholipid Ab. , Anti-cardiolipin IgG, IgMAb
d. HCV Abs., HIV Abs by ELISA
e. H pylori Ag in stool
D- Imaging:

1- Abdominal US: for assessment of liver, spleen, and abdominal Lns.

E- Treatment:
All patients have received glucocorticoids in 1mg/ kg per day .After the 28-day continuous GCs treatment period, Patients were assessed for response. And According to response to GCs , patients were grouped into 3 groups (Rodeghiero et al., 2009):

1- The GCs responsive: A platelet count more than 30 000/dL or greater than 2-fold increase in platelet count from baseline measured on 2 occasions 7 days apart and the absence of bleeding. The quality of response is further divided into

- Partial response: platelet count > 30000 /L and at least 2-fold increase the baseline with cessation bleeding

- Complete response: A platelet count >100 000/L measured on 2 occasions 7 days apart and the absence of bleeding

2- The GC non response group platelet count less than 30000 /L or a less than 2-fold increase in platelet count from baseline or the presence of bleeding. Platelet count must be measured on 2 occasions more than a day apart

3- The GC dependent group; The need for ongoing or repeated doses administration of corticosteroids for at least 2 months to maintain a platelet count at or above 30 *109/L and/or to avoid bleeding .

F- Molecular studies
ABCB1 gene genotyping was done to all individuals included in this study for detection of 2 SNPS (C3435T and G2677T/A) using polymerase chain reaction restriction fragment length polymorphism (PCR-RELF) Technique .

For (G2677T) SNP:
It determines the presence of the T allele at position 2677:

- Upstream primer: 5~-TGCAGGCTATAGGTTCCA GG-3~
- Downstream primer: 5~-TTTAGTTTGACTCACCTTCCCG-3~

For (C3435T) SNP:
It determine the presence of the T allele at position 3435

- Upstream primer: 5~-GATCTGTGAACTCTTGTTTTC-3~
- Downstream primer: 5~-CTTGTTTTCAGCTGCTTGATGGCAA-3~

Statistical analysis
Statistical analysis was carried out using the SPSS program" Quantitative variables are presented as the mean ± standard deviation. Allelic and genotypic frequencies were estimated by genotype count. All statistical tests were two-tailed, and $p<0.05$ was chosen as the level of significance. The $\chi 2$ test was employed to perform univariate analysis of the association of each SNP with disease and categorical clinical features. The Kruskall- Wallis test was used to investigate the relationship between genotypes and quantitative clinical parameters .The association between genotypes and clinical characteristics was analyzed by logistic regression and expressed as odds ratio (OR) with 95% confidence interval (95% CI)

Results:
Our patient characteristics were shown in table 1. As regard C3435T, table 2 shows the frequency distribution of wild CC genotypes well as wild non mutant allele C was significantly higher compared to CT genotypes (CC versus CT) and T mutant allele© versus T) (p value:<0.001) in patient group as compared to control group. As regard G2677T, table 3 shows that T allele is significantly higher in patient group compared with control group than wild G allele but no significant difference at level of genotypes. There was also no statistically significant correlation between any of polymorphisms and age of onset of ITP or initial platelet count

Table 1: patient characteristics

ITP group		Descriptive statistics
Age	Range Mean ± SD	(19-70 y) 34.3±12.3
Sex	Male: n (%) Female: n (%)	35(35%) 65(65%)
BMI	Range Mean ± SD	19-32 14.3±12.3
Severity of bleeding N(%) Ÿ Minor (purpura) 4% Ÿ Mild (echymosis) 44 % Ÿ Moderate (mucosal) 52 % (vaginal, gum, epistaxis) Ÿ Severe (internal, intracranial) 0%		
Routine initial investigations :		
CBC: HB	Range Mean ± SD	10-13gm/dl 11± 1.7
TLC	Range Mean ± SD	4000-30000/dl 3000± 1800
Platelets	Range Mean ± SD	4000-30000/dl 13±8.1
(0ther invstigations to exclude 2ry thrombocytopenia)		
HCV ab , HIVab : ANA, Anticardiolipin IgG , IgM H Pylori Ag in stool Bone marrow aspirate Abdominal US	Negative Negative Negative Negative Hypercellular, giant megakaryocytes , no infiltration Normal liver, no splenomegaly	

Table 2: Distribution of C3435T polymorphism in studied population

	Control	ITP	P value (2)	OR / (95% CI)	P value (3)
C3435T					
CC CT TT	12(12.9%) 70(75.3%) 11(11.8%)	42(43.8%) 42(43.8%) 12(12.5%)	<0.001*	Ref. 0.17 / (0.08-0.36) 0.31 / (0.11-0.88)	<0.001* 0.028*

(1) Wild Mutant	82(88.2%) 11(11.8%)	84(87.5%) 12(12.5%)	0.888	Ref. 1.1 / (0.4-2.5)	0.888
C T	94(50.5%) 92(49.5%)	126(65.6%) 66(34.4%)	0.003*	Ref. 0.54 / (0.35-0.81)	0.003*

Table 3:Distribution of G2677T polymorphisms in studied population

	Control	ITP	P value (2)	OR / (95% CI)	P value (3)
G2677T					
GG GT TT	23(23%) 40(40%) 37(37%)	16(16%) 32(32%) 52(52%)	0.097	Ref. 1.2 / (0.5-2.5) 2 / (0.9-4.3)	0.729 0.071
(1) Wild Mutant	63(63%) 37(37%)	48(48%) 52(52%)	0.033*	Ref. 1.8 / (1.04-3.2)	0.034*
G T	86(43%) 114(57%)	64(32%) 136(68%)	0.023*	Ref. 1.6 / (1.1-2.4)	0.023*

	Response		P value (3)
	Complete	Partial	
G2677T 0/ C3435T 0 G2677T 0/ C3435T 1 G2677T 1/ C3435T 0 G2677T 1/ C3435T 1	24(35.8%) 8(11.9%) 32(47.8%) 3(4.5%)	11(42.3%) 1(3.8%) 14(53.8%) 0(0%)	0.574

There was no significant difference in genotype and allele frequency and distribution of any of both polymorphisms separately or combined as shown in table 4 found between early and late responders, partial and complete

Table 4: synergistic effect of both polymorphisms on time, quality of response and steroid

	Time of response		P value (3)
	Early	Late	
G2677T 0/ C3435T 0 G2677T 0/ C3435T 1 G2677T 1/ C3435T 0 G2677T 1/ C3435T 1	24(36.4%) 8(12.1%) 32(48.5%) 2(3%)	11(40.7%) 1(3.7%) 14(51.9%) 1(3.7%)	0.685

	Dependence		P value (3)
	Negative	Positive	
G2677T 0/ C3435T 0 G2677T 0/ C3435T 1 G2677T 1/ C3435T 0 G2677T 1/ C3435T 1	11(34.4%) 5(15.6%) 15(46.9%) 1(3.1%)	25(39.7%) 4(6.3%) 32(50.8%) 2(3.2%)	0.531

0,) wild + heterozygous; (1), mutant. (3) Chi square test or Fisher exact test if expected frequency <:

Discussion

The study examined each of C3435T and G2677T polymorphisms of ABCB1 in Egyptian ITP and control patients, relation with response to steroid therapy. The study was done on 100 adult Egyptian ITP patients, 100 healthy controls. At the baseline, frequency distribution of each of the SNPs was examined and compared in ITP and control. Concerning C3435T distribution, there was significantly higher distribution of wild C allele compared to mutant T allele (C versus T) (P value; 0.003) in patient as compared with control group. This result suggests no role of this polymorphism in predisposition to ITP. It is also consistent with (El-beblawy et al 2015) done in Egypt on pediatric ITP patients and revealed that frequency of the C3435T gene in all patients with ITP and control was not significant (P value .090). Again results are further supported by result obtained by El-beblawy et al 2015 stating that children with wild CC genotype of this polymorphism significantly have the eldest age of ITP diagnosis(P value:.02). M. Xuan et al 2014 is another study done on adult ITP in China (Xuan et al 2014)and Akin et al., 2011 in Turkey on pediatric ITP revealed the same results (Akin et al., 2011) . As regard other polymorphism G2677T, we found mutant T allele was significantly higher as compared with wild G allele in patients as compared with control group. This may give impression to possibility of relation to disease predisposition. This polymorphism was never studied previously in Egyptian population. Outside Egypt, also it didn't have the same chance to be studied extensively in ITP. ONLY one study M. Xuan et al 2014 done on ITP in association with C3435T in China and found there was no statistical difference found between ITP and normal Also, There was no significant differences at age of diagnosis, initial platelet count between different genotypes or alleles of either C3435T or G2677T polymorphism. This comparison was also done in El-beblawy et al 2015 and there was a significant difference in age at diagnosis of C3435T gene with the CC genotype had the eldest age and lowest initial platelets count (P ¼ .029 and P ¼ .004). The newly diagnosed ITP patients were given prednisolone orally 1 ml/kg (max. 60mg).Of our total 100 ITP cases 97% were responders and 3 % only were resistant. These results were consistent with that found in El-beblawy et al 2015 which was done on Egyptian ITP patients.;9% were resistant 91% were responsive (El-beblawy et al 2015). In Xuan et al., 2014 study done on 471 ITP patients, resistant cases was considerable (24%) and 76% were responsive (Xuan et al., 2014). After one month of oral steroid ttt , gradual withdawal over period of 6 months. 70% of total responders were steroid dependent or persistent ITP group and the other 30% were steroid non-dependent remission group. Results differ from that found in El-beblawy et al 2015. The steroid dependent cases were (19%) and (81%) were steroid non-dependent remission group reflecting different disease course between adults and pediatric (El-beblawy et al 2015) As regard C3435T, there was no statistical significant difference or special distribution in genotypes or alleles between groups of partial or complete response, early or late responders, dependent or non-dependent group.This suggests no role of this polymorphism in determination course of disease, responseto steroid ttt rapidity or pattern. Xuan et al 2014 patients received the same steroid oral regimen. They found no statistically significant difference in frequency or genotype distribution ofC3435T polymorphism between the GCs-responsive and non-responsive group (Xuan et al 2014). Our results also were consistent with El-beblawy et al 2015. In addition, they received different ttt modalities high dose dexamesazone , high dose methylprednisolone),and other ttt modalities as IVIG, anti D Ig, and TPO .The response was assessed as a whole from regarding time, best reached platelet count, and post-treatment platelet count. There was no significant difference in response to different modalities of treatment , time of treatment, and post-treatment platelet count in newly diagnosed patients with ITP between different genotypes and alleles of C34353T polymorphism .Furthermore, there was no significant difference (P value 0.89) between genotypes of the gene as regards the progression of the disease whether had remission or had persistent course of the disease (El-beblawy et al 2015).Few number of newly diagnosed cases has made number of children receiving each ttt modality might be few and not enough to give correct statistical results.

Another study as Akin et al., 2011compared C3435T polymorphism with the response in ITP children in Turkey but patients were given high-dose methylprednisolone at a dose of 30 mg/kg/day for 3 days and 20 mg/kg/day for 4 days. They found no significant difference in C34353T genotype and allele distribution between responders and non- responders.(Akin et al., 2011) As regard G2677T Polymorphism, we also found there was no statistical significant difference or special distribution in genotypes or alleles between groups of partial or complete response, early or late responders, dependent or non-dependent group. These results differ from Xuan et al., 2014who found that homozygous mutant genotype had significantly better response to 28 days GCs therapy compared with wild genotype which had GCs poor response (Xuan et al., 2014).

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