The −1355G/C Polymorphism of Ferroportin (*FPN1*) Gene Among Adolescent Girls With Iron Deficiency Anemia

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Abstract

Ferroportin is a cellular iron exporter protein that has been identified in human and plays an important role for iron homeostasis in tissues. The −1355G/C polymorphism in ferroportin *(FPN1)* gene promoter region leads to increase in ferroportin expression and iron export, decreases in hemoglobin and serum ferritin levels that manifest as iron deficiency anemia (IDA). The aim of this study was to investigate the *FPN1* −1355G/C polymorphism as a risk factor of IDA. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) with B*fa*1 restriction enzyme was used to determine the −1355G/C polymorphism among 20 adolescent girls with anemia (cases) and 52 healthy controls. The blood samples were collected and analyzed for complete blood count. Serum ferritin levels were measured by ELISA. All of the data was obtained by statistically analyzed by the chi-square test or Fisher's exact test, independent sample t-test and Mann-Whitney Test. Genotypes distribution showed no significant differences between IDA group (CC 38.5%, CG 53.8%, and GG 7.7%) and control group (CC 36.5%, 55.8% CG, 7.7%). The odds ratio (OR) of C allele having IDA was 2.036 (95% CI, 0.518-7.995, p=0.249). Subjects carrying the C allele in the IDA group had lower serum ferritin levels than the non-IDA group (p=0.000). This study suggests that the C allele of *FPN1* −1355G/C genetic polymorphism is a risk factor for iron deficiency anemia among adolescent girls.

Key words: Polymorphism, iron deficiency anemia, ferritin serum levels, ferroportin, *FPN1* −1355G/C

Introduction

The prevalence of iron deficiency varies based on age, sex, physiological condition, pathological, environmental, socioeconomic and life stage (World Health Organization, 2001 & Deegan *et al*., 2005). One of the most vulnerable groups are young women and this is proven by the high prevalence of iron deficiency anemia in adolescent girls. According to World Health Organization, the incidence of anemia in non-pregnant women of reproductive age in Indonesia is 33.1% (World Health Organization, 2008).

In Indonesia, there are 20 provinces that have anemia prevalence greater than national prevalence (14.7%), one of which includes the Special Region of Yogyakarta with the prevalence of 15% (Ministry of Health Republic of Indonesia, 2008). The results of the study conducted by Yogyakarta City Health Department and Faculty of Medicine Universitas Gadjah Mada in 2012 showed that 34% of 280 high school female students were anemic (City of Goverment of Jogjakarta, 2013). These data indicate that anemia is still a public health problem in Indonesia, especially in Yogyakarta. The prevalence of anemia in adolescent girls in Islamic boarding school in Surabaya was 65.5% (Wahyuni *et al*., 2002). Another study in the Islamic boarding school of Tarbiyah Islamiyah Candung West Sumatra found the prevalence of anemia was 39.6% (Isniati, 2007).

Ferroportin (*FPN1*) or *SLC40A1* is one of important genes in iron metabolism that encodes ferroportin for iron absorption, release, and recycle inside the body. Ferroportin (FPN) is a cellular iron exporter protein that has been identified in human and plays an important role for iron homeostasis in tissues (Nemeth *et al*., 2004; Cui *et al*., 2009). Ferroportin expression is also regulated by *FPN1* gene promoter region. In-silico examination of the G/C nucleotide transversion in the *FPN1* gene promoter at positions -1355 showed a new activation of transcription factors FOXC1 bound to the 5'-iron responsive element (IRE), thus the translational process of ferroportin still occur (Hallendorf, 2008). Increased expression of ferroportin cause increased iron export to lower serum ferritin.

The variant of *FPN1* −1355G/C in the promoter region leads to increase in ferroportin expression and iron export, increases in cellular iron needs, decreases in hemoglobin and serum ferritin levels that manifest as iron deficiency anemia. In Indonesia, research related to *FPN1* −1355G/C gene polymorphism was conducted in pregnant women in Surakarta. The genotype frequency of GC and CC were 100% in pregnant women with iron deficiency anemia (IDA) and 95.2% in pregnant women without IDA. Subjects carrying C allele had a risk of 1.6 times higher to experience IDA than subjects carrying G allele (Istiqomah *et al*., 2013).

This research investigated the effect of *FPN1* −1355G/C gene polymorphism on the incidence of iron deficiency anemia in adolescent girls.

Materials and Methods

This research was a case-control study with protocol approval by The Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (No. Ref: KE/FK/887/EC, October 2 2013). The study participants were adolescent girls at four Islamic boarding schools in Yogyakarta and Sleman Regency. Ethical approval for this study was granted from The parents or caregivers were informed about the objectives of the study and their informed consent was obtained. The girls were also informed and the objectives and procedures in this study were explained. Those who fulfilled the inclusion criteria and gave their assent were recruited to join the study.

Before the recruitment of study participants, a screening for anemia was conducted among adolescent girls. Postmenarcheal girls with anemia (Hb < 12.0 g/dL), aged 14-19 years, Javanese, not suffering from any major illness or disease at the time of data collection, were recruited to join the study. The study participants were interviewed using a questionnaire about the history of the disease. A healthy and non-anemic adolescent girls were recruited as a control group.

The sample size was based on a requirement for case-control study. The minimum sample in each group is 41 adolescent girls so that the total sample is at least 82 adolescent girls. After screening of hemoglobin levels, subject with anemia (cases) were only 20 subjects, and the number of control group was 52 subjects.

Ferritin serum level assay

Hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were determined by the automated hematology analyzer (Sysmex XT 2000i). Serum ferritin levels were determined using the a commercial ELISA DRG® Ferritin ELISA kit (EIA-1872; DRG International, Inc., USA).

DNA extraction and FPN1 −1355G/C *genotyping*

DNA extraction was carried out in Biochemistry Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. Genomic DNA was extracted from peripheral leukocytes using The Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, USA). Genotyping was determined by PCR-RFLP. Briefly, to amplify *FPN1* gene, forward primer: 5'-GTA GAC CTT TGG GGC TCC TG-3' and the reverse primer: 5'-TGG AGG GTG AGG TGA ATG AC-3' were used [Panton, 2008]. The final reaction volume of 30 µL contain of 2 µL DNA, 15 µL of master mix PCR (2x PCR buffer, 150 mM of dNTP, and 0,5 U of Taq DNA polymerase), 2 µL of primers (1 µL of primer forward and 1 µL of primer reverse) and 11 µL of nuclease free water. PCR amplification was performed in a PCR thermocycler (Esco Thermal Cycler, Esco Technologies, Inc.). The reaction conditions for amplification includes an initial denaturation at 95° C for 5 min followed by 35 cycles each consisting of denaturation at 95° C for 5 min, annealing at 60° C for 2 min, and extension at 72° C for 1 min. Final extension occurred at 72° C for 10 min. The PCR program ran for 2 hours and 55 min. These primers yielded PCR products of 288 bp in length.

Subsequently, the amplicons were digested with restriction enzyme B*fa*I (FspBI; Thermo Fisher Scientific Inc. #ER1761, Waltham, USA). PCR products were incubated for 16 h at 37° C to allow complete digestión and separated by 3% (w/v) agarose gel electrophoresis stained with ethidium bromide at 100 V for 55 min in 0.5x TBE buffer solution. The DNA fragments were visualized by ultraviolet light transilumination (Carestream Gel Logic 212 Pro).

Statistical analysis

Categorical variables were analyzed by Chi-Square or Fisher's exact test. An independent t-test used to examine numeric variables with normal distribution and Mann-Whitney as alternative for data not normally distributed. Deviation of of the genotype frequencies from the Hardy-Weinberg equilibrium (HWE) was assessed by chi-square test. A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

A total of 20 anemia subjects and 52 healthy subjects (control) were eligible for analysis. Characteristics of both groups are shown in Table 1.

Table 1. Subjects characteristic between anemia and control groups

Variables	Anemia (n=20)	Control (n=52)	*p*-value
Age (years)	16.0 {2}	16.0 {2}	0.830*
Hemoglobin (g/dL)	11.6 {1.0}	13.3 {1.2}	0.000*
Hematocrit (%)	35.9 {3.0}	40.2 {2.6}	0.000*
MCV (fL)	74.3 {8.6}	85.1 {3.4}	0.000*
MCH (pg/cell)	23.8 {3.9}	28.5 {2.5}	0.000*
MCHC (g/dL)	31.7 {1.3}	33.3 {1.2}	0.000*
Serum Ferritin (ng/mL)[a]	12.9 ± 12.2	37.1 ± 18.5	0,000†

Data are expressed as mean ± S.D[a] or median {interquartile range (IQR), †Independent Sampel T-test; *Mann-Whitney Test (Wahyuni, 2014).

Twenty anemic subjects were divided into categories of iron deficiency anemia (IDA) and non-iron deficiency anemia (non-IDA) group based on the levels of hemoglobin and serum ferritin levels. Iron deficiency anemia was defined as anemia subjects who had serum ferritin levels < 15.0 ng/mL. Hemoglobin levels, hematocrit levels, all red blood cell indices were not different between two groups. There was a significant difference in serum ferritin levels in both groups (p = 0.002). Serum ferritin levels were lower in subjects in the IDA group compared to the non-IDA group (Table 2).

Table 2. Mean of hemoglobin, hematocrit, all red blood cell indices, serum feritin levels between IDA and non-IDA groups

Variables	IDA (n=13)	Non-IDA (n=7)	*p value*
Hemoglobin/dL)[a]	11.6 {1.2}	11.6 {0.8}	1.000*
Hematocrit (%)	35.3 ± 2.3	35.1 ± 2.1	0.878†
MCV (fL)	75.4 ± 7.0	70.2 ± 10.9	0.207†
MCH (pg/cell) [a]	23.5 {2.7}	22.4 {6.2}	0.500*
MCHC (g/dL)[a]	31.7 {1.8}	32.1 {1.6}	0.551*
Serum ferritin (ng/mL)	5.6 ± 4.0	26.4 ± 10.5	0.002†

Data are expressed as mean ± S.D. or median {interquartile range (IQR)}[a], †Independent Sampel T-test, *Mann-Whitney Test (Wahyuni, 2014).

Genotyping of *FPN1* −1355G/C gene polymorphism found all of the possible genotypes which were CC, CG, and GG. The CC genotype (mutan homozygous) was designated by a single band at 285 bp, the CG genotype by a three band at 285 bp and 255 bp and 30 bp, and GG genotype by a double band at 255 bp and 30 bp. The result for the genotyping is shown in Figure 1.

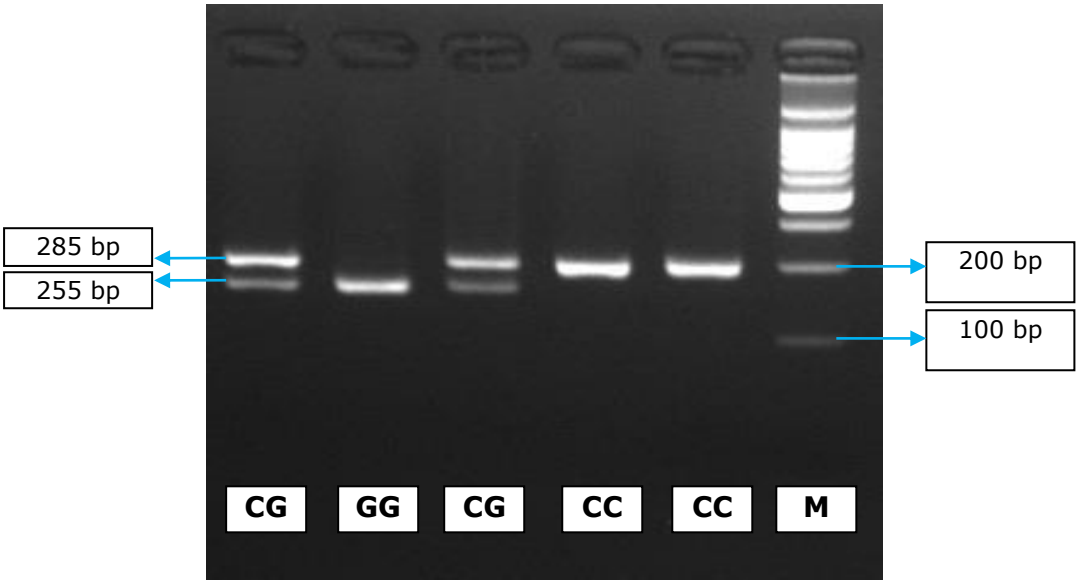

Figure 1. The genotyping result of -1355G/C in *FPN1* gene. M = 100 bp Marker (Geneaid); GG genotype = *wild type* (255 bp, 30 bp (unseen), CG = heterozygous mutant (285 bp, 255 bp and 30 bp (unseen) and CC = homozygous mutant (285 bp) [Wahyuni, 2014].

Genotype and allele frequencies between groups are summarized in Table 3 and Table 4.

Table 3. Genotype frequency of *FPN1* -1355G/C polymorphism between IDA and control groups

		IDA (n=13)	Control (n=52)	*p-value*	OR (95% CI)
Genotypes	CC	5 (38.5%)	19 (36.5%)	0.906*	-
	CG	7 (53.8%)	29 (55.8%)		
	GG	1 (7.7%)	4 (7.7%)		
	CC	5 (38.5%)	19 (36.5%)	1.000*	0.921
	CG+GG	8 (61.5%)	33 (63.5%)		(0.263-3.221)
Allele	C	17 (65.4%)	67 (64.4%)	0.927*	1.043
	G	9 (34.6%)	37 (35.6%)		(0.423-2.571)

Data are expressed as number (%), **Chi-Square Test. OR: Odds Ratio* [Wahyuni, 2014].

Table 4. *FPN1* -1355G/C genotypes and allele distribution between IDA and Non-IDA groups

		IDA (n=13)	Non-IDA (n=7)	*p-value* OR (95% CI)
Genotypes	CC	14 (53.8%)	3 (21.4%)	0.108#
	CG	11 (42.3%)	9 (64.3%)	
	GG	1 (3.8%)	2 (4.3%)	
	CG+CC	12 (92.3%)	7 (100.0%)	0.650* OR = 0.632 (0.448-0.890)
Allele	C	19 (73.1%)	8 (57.3%)	0.249*
	G	7 (26.9%)	6 (42. 9%)	OR = 2.036 (0.518-7.995)

Data are expressed as number (%), # *Chi-Square Test, OR: Odds Ratio*, *Fisher Exact Test (Wahyuni, 2014).

The frequencies of the C and G allele in the IDA group were 65.4% and 34.6%, respectively. Meanwhile, the frequency of the C allele was 64.4% and G allele was 35.6% in the control group. The frequency of CC genotype was higher (38.5%) on the IDA group than the control group (36.5%), although not significantly different (p = 1.000). Based on HWE calculation, this study found that the genotype distributions among cases and control were consistent within HWE (p=0.351). It means that the genotype distribution among subject of this present study had no HWE deviation.

The distribution of genotype frequencies in the IDA group and non-IDA did not differ significantly (p = 0.108). Twelve subjects (92.3%) of the IDA group had C allele. The GG genotype was found only in 1 subject of IDA group and was not found in the non-IDA group.

The independent t-test indicates there was no relationship between allele C with the mean of Hb, MCV, MCH and MCHC (p > 0.05). The mean of serum ferritin levels were lower in subjects carrying C allele in IDA groups (CG and CC) compared to non-IDA group (p = 0.000) (Table 5).

Table 5. The mean difference of hemoglobin levels, hematocrit, all red blood cell indices, serum feritin levels based on genotype between IDA and non-IDA groups

Variables	IDA		Non-IDA		p
	GG (n= 1)	CG+CC (n= 12)	GG (n= 0)	CG+CC (n= 7)	
Hemoglobin (g/dL) [a]	10.6	11.6 (7.5-11.9)	-	11.6 (9.8-11.9)	0.515*
MCV (fL)	77.0	75.3 ± 7.31		70.2 ± 10.9	0.453†
MCH (pg/cell)	24.1	23.4 ± 3.3		22.4 ± 3.7	0.804†
MCHC (g/dL) [a]	31.3	31.7 (25.6-32.6)		32.1 (30.7-33.7)	0.591*
Serum Ferritin (ng/mL)	9.3	5.3 ± 4.0		26.4 ± 10.5	0.000†

Data are mean ± S.D. or median (minimum-maximum)[a] for data not normally distributed, †Independent Sampel T-test, *Mann-Whitney Test (Wahyuni, 2014).

Ferroportin gene has a promoter region or transcription factor binding site (TFBS) bindings that play a role in gene expression at the transcriptional levels. Variations of TFBSs can disrupt the transcription and regulation of gene. The binding of specific transcription factors to gene promoter directly regulate and initiate transcription of a gene. Sequence variation in the promoter gene and will alter identification and binding of transcription factors that ultimately may affect gene expression (Guo & Jamison, 2005).

The in silico examination with the techniques of non-redundant Jaspar CORE database shows that the presence of the variant in the 5'UTR genes will disrupt transcription factors binding sites (TFBSs). This analysis proves that the *FPN1* variant-1355G/C will produce a new TFBSs FOXC1 bound to the iron-regulatory elements (IRE) that persists ferroportin protein translation activity that causes an increase in the export of iron and low ferritin levels in cells (Hallendorf, 2008).

Another study reported the frequency of CC genotype was 50%, CG genotype was 46.2% CG and GG genotype was 3.8% in the population of pregnant women (n = 74). The odds ratios (OR) of the C allele for the incidence of anemia was 1.008 (CI 95%, 0.471-2.156, p = 0.983), whereas the OR for IDA was 1.600 (CI, 95%, 0.296-8.653, p = 0.710). The OR of subjects carrying the C allele were 1.600 and indicates a

risk 1.6 times or 61.5% probability of suffering IDA compared to subjects with allele G (Istiqomah *et al*., 2013).

The C allele as a risk factor IDA was analyzed to assess its correlation with hemoglobin levels and erythrocyte indices. The results showed that there was no correlation between IDA and non-IDA groups of these parameters (p = 0.515). Subjects carrying the C allele in the IDA group had lower serum ferritin levels (5.3 ± 4.0) than non-IDA group (26.4 ± 10.5) (p = 0.000).

The previous study reported that *FPN1* variants may be categorized into two classes: variants that have a gain in function and those that result in a loss of function. Variants that result in a gain of function preserve the ability to activate the iron-response proteins (IRPs) and iron is exported from the cells and ferritin is depleted. Loss of function variants inhibit IRP activation activity and cause only a slight decrease in serum ferritin levels (Hallendorf, 2008). In this study, the frequency of CG and CC genotypes were 92.3% in the IDA group (OR = 0.923, 95% CI, 0.789-1.080, p = 1.000). The OR for the incidence of IDA was 2.036 (95% CI, 0.518-7.995, p = 0.249), while the OR for genotypes carrying C allele (CG and CC) for the occurrence of anemia was 1.583 (95% CI, 0.166-15.094). Based on the OR value, the C allele is a risk factor for iron deficiency anemia. Subjects carrying the C allele had a risk of 2 times or 67.06% probability to experience iron deficiency anemia compared to subjects with the G allele. Thus, our results indicate that the presence of variability -1355G/C of *FPN1* gene may increase the risk of iron deficiency anemia in adolescent girls (OR = 2.036, 95% CI, 0.518-7.995, p = 0.249).

Conclusions

This study suggests that the C allele of *FPN1* −1355G/C gene polymorphism is a risk factor for iron deficiency anemia among adolescent girls. Subjects carrying the C allele in the IDA group had lower serum ferritin levels than non-IDA group.

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