Original Article

Study Of Snp Rs1375515 Of Calcium Channel Gene Cacna2d3 In Some Egyptian Females Who Suffer From Iron Deficiency Anemia In The Age Of Childbearing

Heba Mohammad Noh[1], Khadiga Mohammed Abo-Gabal[1], Hanan Ali Taha[1,] Shaimaa Mohamed Zaied[1]

1. Clinical & Chemical Pathology Faculty of Medicine, Beni-Suef Universit, Egypt
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Abstract

Iron deficiency anemia is a worldwide health problem in which environmental, physiologic and genetic factors play important roles. The associations between iron status biomarkers and single nucleotide polymorphisms (SNPs)' known to be related to iron metabolism' were studied in menstruating women. A group of 80 Egyptian menstruating women, a population group at risk of iron deficiency anemia, participated in the study. Hematological and biochemical parameters were analyzed and rs1375515 SNP of CACNA2D3 was selected and genotyped by real time PCR. The associations between genetic and biochemical data were analyzed by (SPSS) version 25. The results showed that there were no significant associations between rs1375515 SNP and iron deficiency anemia in all examined women.

Key words: Iron deficiency anemia - rs1375515 SNP- CACNA2D3 gene-Iron metabolism

INTRODUCTION

Iron levels in biological fluids are essential for normal body function, in oxygen transport and for other important metabolic reactions. Thus, fine regulation of this element is required since departures from its optimal levels produce severe alterations. For example, iron overload triggers the Fenton reaction in which the generation of hydroxyl radicals, causes major tissue damages. By contrast, iron deficiency may cause anemia [1].

Among iron related diseases, Iron deficiency anemia is considered a major worldwide health problem in the 21st century. This fundamental health issue has still not been resolved and continues affecting the health, quality of life and working capacity of approximately two billion people all over the world, especially children and women of child-bearing age [2].

Iron deficiency anemia is influenced by diet, iron intake, blood loss, physiological status and infections. But, not only traditional dietary and host-related factors are determinants of iron levels, but also genetic factors play an important role. Mutations in key proteins involved in iron metabolism, red cell stability, and in iron absorption, have proved to generate severe anaemia and haemochromatosis [3].

Single-nucleotide polymorphisms (SNPs) are the most common type of genetic variation, and they are widely represented in the genome, but only a large portion of them are genuine polymorphisms. About 3 million SNPs from human population samples have been identified, and it is believed that the human genome contains at least 10 million SNPs. Some of these variants could be genetic factors that lead to iron overload or deficiency [4].
A novel SNP (rs1375515), located in an intronic region of a gene that codifies a subunit of a calcium channel CACNA2D3, significantly associated with hemoglobin and ferritin, as well as with different probabilities of belonging

to different iron-clinical phenotypes [5]. Calcium channels are associated with iron homeostasis through the modification of red cell volume that leads to disorders such as liver insufficiency, fibrosing disease and sickle cell anemia. Other studies have shown that these channels could be directly responsible for iron uptake, namely that the L-type voltage dependent calcium channel could be an alternate route for iron to enter different cell types [6] **.** The aim of this case control study was to assess the association between SNP rs1375515 and iron deficiency anemia in some Egyptian women in the age of childbearing period.

Patients and Methods

The current study was a case control study involving 40 female patients, 40 healthy women as a control group. Recruitment of cases was started on August 2018 till December 2018. Patients were admitted from the internal medicine department at Beni-suef University Hospital after taking patient´s consent.

Inclusion criteria

1. Female patients in the childbearing age.
2. Female patients having iron deficiency anemia

Exclusion criteria

1. Pregnant or breast feeding.
2. Having any chronic diseases such as inflammatory bowel disease, crohn's disease, gastric ulcers, or hemorrhagic diseases.

Control group

comprised of 20 healthy women with normal hemoglobin level above 12gm/dl, matching the patient group by age and other parameters.

Both groups were subjected to

1. Thorough History Taking.
2. Full Clinical Examination.
3. Routine Laboratory Investigations:
   a. Complete blood picture
   b. Serum Iron level.
   c. Serum ferritin level.
4. specific laboratory investigations:

Using RT-PCR method to detect

CACNA2D3 (rs1375515) single nucleotide polymorphism using Real Time-polymerase chain reaction (RT-PCR) method.

Statistical methodology

Data collected were reviewed, coding and statistical analysis of collected data were done by using SPSS program (statistical package of social science; SPSS Inc., Chicago, IL, USA) version 25 for Microsoft Windows.

A. Descriptive statistics:

Data was summarized using mean, standard deviation, median, minimum and maximum for quantitative variables and frequencies (number of cases) and relative frequencies (percentages) for categorical variables.

B. Analytic Statistics:

Comparing groups was done using:

a. Chi-square-test (χ^2): for comparison of qualitative data and Fisher exact test for comparison of qualitative data <5 in frequency [7].

b. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated whenever applicable, to test association between genotype and the disease. The significance of the OR was calculated by a 2 by 2 contingency table.
c. MannWhitney U test to determine the significance in the difference between two non- parametric variables [7].
d. The level of significance was taken at p-value of <0.05 with confidence level 95% .

Results

The study was conducted on 80 subjects classified into two groups: group1that included 40 females in childbearing period with IDA has a mean of 27.58(± 5.77)
while group 2 that included 40 age and gender related healthy controls has a mean of 27.9(±5.95). There was no statistically significant difference between the study groups regarding the age (p value = 0.805).
The laboratory data are presented in table (1) and summarized in the following points

Table (1): Comparison between the two studied groups regarding laboratory data

	IDA (n=40)	**Healthy controls (n=40)**	**P**
HGB	8.65±1.63	12.66±0.60	<0.001
HCT	26.64±3.75	36.15±2.89	<0.001
MCV	68.28±6.49	84.60±3.22	<0.001
MCH	21.60±2.97	29.10±1.69	<0.001
Log S.iron	1.45±0.50	1.89±0.13	<0.001
Log Ferritin	1.54±0.54	1.76±0.22	<0.001

Data presented as mean (± SD).

Table (1) shows a statistical significant difference on comparing the anemia related parameters including HGB, HCT, MCV and MCH between the two groups (p value = < 0.001, < 0.001, < 0.001, < 0.001 respectively).

Also, in table 1, both Log (S. iron) and Log (Ferritin) showed statistical significant difference on comparing the two groups (p value = < 0.001, < 0.001 respectively).Serum iron and ferritin were log-transformed prior to analysis because they showed skewed distribution.

Genotype frequency of the studied gene rs1375515 (*CACNA2D3)in the studied groups*

Table (2): Frequency of rs1375515 in the studied groups

			Group		**%Total**	**p**
			IDA	**Healthy control**		
rs1375515	CC	% within Group	11 (27.5%)	14 (35.0%)	62.5%	0.229
	CT	% within Group	16 (40.0%)	18 (45.0%)	95.0%	0.286
	TT	% within Group	13 (32.5%)	8 (20.0%)	52.5%	

Data presented as number and percent

Table (2) shows that there was no statistically significant difference between the two groups as regard to the Frequency of rs1375515 occurrence in the two studied groups and the frequency of CC was 27.5 % in the IDA group and 35.0% in the healthy control group.

Allele Frequencies of the Studied gene (rs1375515 (*CACNA2D3)*)

Table (3): frequency distribution of alleles in the two studied groups

			Group		P
			IDA	**Healthy controls**	
Alleles	C	% within Group	38(47.5%)	46(57.5%)	0.206
	T	% within Group	42(52.5%)	34(42.5%)	

Data presented as number and percent

Table (3) showed that there was no statistically significant difference between the two groups as regard to their alleles (p-value=0.206) and the frequency of the C allele was 47.5 % in the IDA group, 57.5% in the control group.

Table (4) Association between the CACNA2D3 SNP rs1375515 and red blood cells indices in anemia patients

	Gene PCR		
	CC	**CT+TT**	**P value**
HGB	9.36±1.69	8.38±1.56	0.090
HCT	27.19±4.16	26.43±3.65	0.574
MCV	68.36±7.05	68.24±6.4	0.958
MCH	22.00±2.53	21.45±3.15	0.606

Data presented as mean±SD

Table (4) shows that there was no statistically significant association between CACNA2D3 SNP rs1375515 and HGB, HCT, MCV and MCH. P value was 0.090, 0.574, 0.958 and 0.606 respectively.

Table (5) Association between rs1375515 and iron profile in anemia patients

	Gene PCR		
	CC	CT+TT	P value
Log serum iron	1.52±0.53	1.42±0.49	0.568
Log ferritin	1.45±0.54	1.57±0.54	0.556

On comparing log S.iron and log ferritin values between anemic patients with CC genotype and those with CT or TT genotype, there was no statistically significant difference. P value was 0.568 and 0.556 for log serum iron and log

ferritin respectively. Serum iron and ferritin results were log transformed prior to analysis because they showed skewed distribution.

Linear regression analysis

Table (6) linear regression analysis between CACNA2D3 genotype and HGB

HGB	**Coefficient**	**95% CI**		**P value**
		lower	**upper**	
CC	0.981	-0.160-	2.122	0.090
CC+CT	0.044	-1.087-	1.174	0.938

Table (6) shows that there was (no) statistically significant association between rs1375515 SNP and HGB, p value was 0.938.

Table (10) linear regression analysis between CACNA2D3 genotype and HCT

HCT	Coefficient	95% CI		P value
		Lower	upper	
CC	0.760	-1.955-	3.475	0.574
CC+CT	-0.579-	-3.171-	2.013	0.654

Table (10) shows that there was (no) statistically significant association between rs1375515 SNP and HCT, p value was 0.654.

Table (11) linear regression analysis between CACNA2D3 genotype and MCV

MCV	Coefficient	95% CI		P value
		lower	upper	
CC	0.122	-4.590-	4.834	0.958
CC+CT	2.573	-1.839-	6.985	0.245

Table (11) shows that there was (no) statistically significant association between rs1375515 SNP and MCV, p value was 0.245.

Table (12) linear regression analysis between CACNA2D3 genotype and MCH

MCH	Coefficient	95% CI		P value
		lower	upper	
CC	0.552	-1.596	2.700	0.606

CC+CT	-0.023	-2.078	2.032	0.982

Table (12) shows that there was (no) statistically significant association between rs1375515 SNP and MCH, p value was 0.982. .

Table (13) linear regression analysis between CACNA2D3 genotype and Log S.iron

Log S.iron	Coefficient	95% CI		P value
		lower	upper	
CC	0.103	-0.259-	0.465	0.568
CC+CT	-0.094	-0.440	0.251	0.584

Table (13) shows that there was (no) statistically significant association between rs1375515 SNP and Log serum iron, p value was 0.584.

Table (14) linear regression analysis between CACNA2D3 genotype and Log ferritin

Log ferritin	Coefficient	95% CI		P value
		lower	upper	
CC	-0.114	-0.503	0.275	0.556
CC+CT	-0.289	-0.649	0.071	0.112

Table (14) shows that there was (no) statistically significant association between rs1375515 SNP and log ferritin, p value was 0.112.

Discussion

Iron deficiency (ID) is the most common malnutrition disorder around the world. It accounts for 50%–60% of all anemia cases. In Egypt, prevalence of anemia among women in childbearing age in 2016 was 28.5% [8].

According to the Global Burden of Disease Study 2016, Iron deficiency anemia (IDA) is considered one of the five leading causes of *'years lived with disability'* in humans, and the top cause in women. It has been mainly regarded as a public health concern affecting growing children, women during childbearing period, patients with chronic conditions and the elderly [9].

There are multiple physiologic, environmental and pathologic causes of iron deficiency (ID) that lead to IDA. Many risk factors are accused of depleting iron stores in the body, including insufficient dietary intake, malabsorption disorders, helminthic infections, chronic and acute blood loss, and increased body demands during periods of rapid body growth [10].

Not only traditional dietary and host-related factors are determinants of iron levels, but also genetic factors play a significant role. Many studies have evidenced that common allelic variants of certain genes are associated with iron related phenotypes or account for a noticeably percentage of the genetic variation in the levels of iron-related parameters [11].

Several genetic variants within the iron regulatory genes have been associated with imbalances in iron homeostasis, which could lead either to iron deficiency or overload. Genetic variants leading to excess body iron occur mainly in the haemochromatosis (*HFE*) gene but are also seen in hepcidin (hepcidin antimicrobial peptide (*Hamp*)), transferrin receptor 2 (*TFR2*), solute carrier family 40 member 1 (*SLC40A1*), haemojuvelin (*HJV*) and transferrin (*TF*) genes. These loci have important functions in the iron homeostasis pathways. For example, hepcidin regulates iron absorption and release. Genetic polymorphisms in genes involved in the hepcidin suppressive pathway such as *TMPRSS6* (transmembrane protease serine 6), have been associated with low iron status and a condition described as iron-refractory iron deficiency anemia (IRIDA) [12].

CACNA2D3 gene is located on locus 3p21.1 of chromosome 3. It encodes a member of the alpha-2/delta subunit family, a protein in the voltage-dependent calcium channel complex. Calcium channels mediate the influx of calcium ions into the cell upon membrane polarization and consist of a complex of alpha-1, alpha-2/delta, beta, and gamma subunits in a 1:1:1:1 ratio [13].

The CACNA2D3 subunit has been implicated to have a role in several cancers. An 80-fold decrease in expression of CACNA2D3 has been reported in the highly metastatic osteosarcoma cell line MG63-A1, when compared with the parental cell line [14]. CACNA2D3 is highly expressed in neuroblasts and favorable prognosis neuroblastomas, with expression downregulated in unfavorable neuroblastomas [15].

The aim of this study was to detect the association between SNP rs1375515 of *CACNA2D3* gene and iron deficiency anemia in 40 female patients aging from 18 to 40 years old from Intrinsic Medicine department at Beni-Suef university hospital compared to 40 healthy females representing the control group.
The results of this study showed that CT genotype frequency was higher in control group (45.0%) when compared to the patient group (40.0%). However, the difference was not statistically significant (p = 0.286). We found that 40% of cases showed CT genotype frequency, 27.5 % showed CC genotype and 32.5 % showed TT genotype. But there was no statistically significant difference.

In agreement with our study, **Baeza-Richer et al., (2013)** reported that 45% of cases showed CT genotype, 44% showed CC genotype and 1% showed TT genotype. Yet this difference did not reach a statistical significance (P = 0.655) [16].
Also **Blanco-Rojo et al,. 2014** found that genotype frequency of rs1375515 were similar among the studied clusters and no differences were observed [17]. In contrast, **Bertocini et al., (2011)** found that the mean value of TT genotype frequency was higher in anemic group than control group (0.267 versus 0.081) [18].This difference was statistically significant (p= 0.009). Our results revealed that C allele frequency was higher in control group than the patient group 57.5% versus 47.5%. But this difference was not statistically significant (P =0.206).

Baeza-Richer et al., (2013) found that the frequencies of C allele and T allele were 67% and 33% respectively in anemia cases. However, there was no significant association between allelic frequencies in women with anemia relative to controls [16].

Baeza-Richer et al., (2013) reported that rs1375515 showed significant association with the levels of MCV. They also found statistically significant association between the SNP and the levels of hemoglobin. On the contrary, we found that there was no statistically significant association between CACNA2D3 SNP rs1375515 and red blood cells indices in anemia patients including HGB, HCT, MCV and MCH. P values were 0.090, 0.574, 0.958 and 0.606 respectively [16]. In our study, log Serum iron and log ferritin values were compared between anemic patients with CC genotype and those with CT or TT genotype, we found that there was no statistically significant difference. P values were 0.568 and 0.556 for log serum iron and log ferritin respectively. On the other hand, **Baeza-Richer et al., (2013)** declared that there was statistically significant association between the allele T and low levels of log ferritin. Moreover, **Baeza-Richer et al., (2015)** detected those individuals carrying CC or CT genotypes presented the highest mean of log Ferritin, whereas those carrying TT genotype showed the lowest values of this biochemical parameter [5]. Linear regression analysis was calculated between genotype rs1375515 and red blood cell indices including HGB, HCT, MCV, and MCH. It was also calculated with log ferritin and log serum iron. However, no significant associations were found, p values were 0.938, 0.654, 0.245, 0.982, 0.112 and 0.584 respectively. But this was not the case with **Baeza-Richer et al., (2013)**, who presented that this SNP generates significant p-values for regression models considering recessive models, for the variables hemoglobin, log10ferritin, hematocrit and MCV. P values were 0.004, 0.012, 0.018, 0.039 respectively [16].

Conclusion and Recommendations

On conclusion, our results showed that there was no association between CACNA2D3 rs1375515 SNP and iron deficiency anemia in females during childbearing age. This may be contributed to the small sample size. A further study using larger sample size is recommended.

References

1. Breymann, C., Honegger, C., Hösli, I., & Surbek, D. (2017). Diagnosis and treatment of iron-deficiency anaemia in pregnancy and postpartum. *Archives of gynecology and obstetrics*, *296*(6), 1229-1234.
2. Milman, N. (2006). Iron and pregnancy—a delicate balance. *Annals of hematology*, *85*(9), 559-565.
3. Finberg K.E (2009). Iron-refractory iron deficiency anemia, Semin Hematol 46 (2009), 378-386.
4. Forrellat Barrios M, Gautier du Défaix Gómez H, Fernández Del-gado N. Metabolism of iron. Rev Cuba Hematol Inmunol yHemoter. 2000; 16: 149 --- 60.3.
5. Bertoncini, S., Blanco-Rojo, R., Baeza, C., Arroyo-Pardo, E., Vaquero, M. P., & López-Parra, A. M. (2011). A novel SNaPshot assay to detect genetic mutations related to iron metabolism. *Genetic Testing and Molecular Biomarkers*, *15*(3), 173-179.
6. Baeza-Richer, C., Arroyo-Pardo, E., Blanco-Rojo, R., Toxqui, L., Remacha, A., Vaquero, M. P., & López-Parra, A. M. (2015). Genetic contribution to iron status: SNPs related to iron deficiency anaemia and fine mapping of CACNA2D3 calcium channel subunit. *Blood Cells, Molecules, and Diseases*, *55*(4), 273-280.
7. Blanco-Rojo, R., Pérez-Granados, A. M., Toxqui, L., Zazo, P., de la Piedra, C., & Vaquero, M. P. (2013). Relationship between vitamin D deficiency, bone remodelling and iron status in iron-deficient young women consuming an iron-fortified food. *European journal of nutrition*, *52*(2), 695-703.
8. Chan, Y. H. (2003). Biostatistics 102: quantitative data–parametric & non-parametric tests. blood Press, 140(24.08), 79.
9. World Health Organization (2016), Global Health Observatory Data Repository/World Health Statistics .
10. Vos T, Abajobir A, Abbafati C, Abbas K, Abate K (2017).GBD 2016 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016 .;390(10100):1211-1259
11. Albaroudi IN, Khodder M, Al Saadi T, Turk T, Youssef LA (2018). Prevalence, diagnosis, and management of iron deficiency and iron deficiency anemia among Syrian children in a major outpatient center in Damascus, Syria. Avicenna J Med. 2018;8(3):92–103.doi:10.4103/ajm.AJM_169_17
12. McLaren C.E , C.P. Garner, C.C. Constantine, S. McLachlan, C.D.Vulpe, B.M. Snively, V.R. Gordeuk, D.A. Nickerson, J.D. Cook, C. Leiendecker-Foster, K.B. Beckman, J.H. Eckfeldt, L.F. Barcellos, J.A. Murray, P.C. Adams, R.T. Acton, A.A. Killeen and G.D. McLaren (2011). Genome-Wide Association Study Identifies Genetic Loci Associated with Iron Deficiency, Plos One (2011), 6: e17390. doi:10.1371/journal.pone.0017390.
13. Jallow MW, Cerami C, Clark TG, Prentice AM, Campino S (2020). Differences in the frequency of genetic variants associated with iron imbalance among global populations. PLoS One. 2020;15(7):e0235141. Published 2020 Jul 1. doi:10.1371/journal.pone.0235141
14. Palmieri C, Rudraraju B, Monteverde M, et al (2012). Methylation of the calcium channel regulatory subunit α2δ-3 (CACNA2D3) predicts site-specific relapse in oestrogen receptor-positive primary breast carcinomas. Br J Cancer. 2012;107(2):375–381. doi:10.1038/bjc.2012.231
15. Xiong W, Niu PY, Zhu WT, Chen J (2009). Tenascin-C as a prognostic biomarker in osteosarcoma. Chin Med J. 2009;122:2737–2743.
16. Thorell K, Bergman A, Carén H, Nilsson S, Kogner P, Martinsson T, Abel F(2009). Verification of genes differentially expressed in neuroblastoma tumours: a study of potential tumour suppressor genes. BMC Med Genomics. 2009;53:1–13.
17. Baeza-Richer C. , Blanco-Rojo R., Lopez-Parra A.M., A. Brichs, S. Bertoncini, A.M. Perez-Granados, A. Buil, J.M. Soria, E. Arroyo-Pardo, M.P. Vaquero(2013). Identification of a novel quantitative trait nucleotype related to iron status in a calcium channel gene, Dis. Markers 34 (2013) 121–129.
18. Blanco-Rojo, R., Toxqui, L., López-Parra, A. M., Baeza-Richer, C., Pérez-Granados, A. M., Arroyo-Pardo, E., & Vaquero, M. P. (2014). Influence of diet, menstruation and genetic factors on iron status: a cross-sectional study in Spanish women of childbearing age. *International journal of molecular sciences*, *15*(3), 4077-4087.
19. Bertoncini, S., Blanco-Rojo, R., Baeza, C., Arroyo-Pardo, E., Vaquero, M. P., & López-Parra, A. M. (2011). A novel SNaPshot assay to detect genetic mutations related to iron metabolism. *Genetic Testing and Molecular Biomarkers*, *15*(3), 173-179.