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Association of single nucleotide polymorphism at BMP2 gene with iron deficiency status among anaemic patients in Hospital Universiti Sains Malaysia

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Abstract

Background Iron deficiency contributes for over half of all anaemia cases, especially among women and children. Iron deficiency anaemia remains a serious public health concern worldwide. The aim of this study is to determine the association between the single nucleotide polymorphism rs235756 in the bone morphogenetic protein 2 (BMP2) gene and iron deficiency status.

Results 104 total anaemic samples were selected from Hospital Universiti Sains Malaysia. ARMS-PCR was performed to genotype the rs235756 SNP in the 104 samples. The genotype distribution of BMP2 rs235756 showed that AG genotypes had the highest frequency 51 (86.4%) followed by GG 6 (10.2%) and AA 2 (3.4%) in IDA group, whereas AG 42 (93.3%), AA 2 (4.4%) and GG 1 (2.2%) were found in the other anaemia group. The minor allele frequency in BMP2 rs235756 from this study (0.514) was not similar to the East Asian (EAS) population (0.135); however, the allelic frequency showed significant association between these two. The mean of total iron binding capacity level differed significantly between homozygous-dominant AA and AG + GG genotypes ($P < 0.05$) but no significant difference for the mean of haematological parameter, ferritin and serum iron.

Conclusions In future clinical settings, this finding can potentially be as a guide in the early prediction for IDA patients through the genetic testing.

Keywords BMP2 gene, Iron deficiency anaemia, rs235756, Single nucleotide polymorphisms

Introduction

Anaemia has affected almost 30% of the world population and iron deficiency contributes about half of the cases [1]. The prevalence of iron deficiency anaemia (IDA) is highest in children less than 5 years old, pregnant women and women in reproductive age [2]. This condition occurs due to changing and increasing physiological needs among human according to sex and specific life stages development. The rate of the disease is also high in developing countries due to common situation in those countries such as lack of nutrients, chronic diseases and parasitic infection like malaria or intestinal worm

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colonization [3, 4], while for high income countries, those who are affected with IDA mostly due to certain eating habits such as vegetarian diet, heavy menstrual bleeding or malabsorption syndromes [5, 6]. A frequent blood donors and patients with chronic inflammatory conditions also have high risk of getting iron deficiency anaemia [7, 8].

IDA is usually associated with nutritional factors; however, recent research suggest that genetic variations may also contribute to this condition [9]. This is supported by genome-wide association study (GWAS) that has identified few single nucleotide polymorphisms (SNPs) of genes such as Tmprss6, HAMP and BMP2 genes that are associated with iron imbalance by interfering the components in iron regulation [10–12].

Bone morphogenic protein 2 (BMP2) gene is located on chromosome 20, involved in hepcidin expression by activating the bone morphogenic protein 6 BMP/SMAD signalling pathway [13] with the action of haemojuvelin. Hepcidin is a protein synthesized in liver involved in iron homeostasis [14]. Low amount of hepcidin secretion causes iron overload that leads to haemochromatosis, while high amount of it causes anaemia [15, 16].

The presence of SNPs on BMP2 gene has been proven to affect the hepcidin regulation and cause haemochromatosis [17–19], while there were insufficient studies on the association between hepcidin regulation and iron deficiency anaemia [20]. The gold standard for iron deficiency diagnosis is serum ferritin level [21]. Thus, we aim to study the association between BMP2 rs235756 SNP and iron deficiency status among anaemic patients in Hospital USM using serum ferritin level as the indicator.

Materials and methods

This study involved 104 anaemic patients from Hospital Universiti Sains Malaysia (Hospital USM). Full blood count (FBC) parameters, such as RBC count, Hb, MCV, MCH, MCHC, and HCT, were determined using XN-1000 automated haematology analyser (Sysmex Corporation, Kobe, Japan) from Haematology Laboratory, School of Medical Science, USM. Serum ferritin, serum iron and TIBC level were measured using ARCHITECT automated chemistry analyser (Abbott, Illinois, USA).

The DNA samples were obtained from archived DNAs of the previous study [22]. The DNA concentrations were measured using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and the DNA purity was evaluated by the absorbance ratios of A260/A280. Then, all DNA samples were stored at -20°C until use.

ARMS-PCR was performed and optimized to detect rs235756 using following primers:

Forward outer: 3' CATAGAGCAGGGCCCAGA
AGCT 5'

Reverse outer: 3'TCAGGGTACTCACGAAAG
AGAGA 5'

Forward inner (G allele): 3' GAAGACTAAGAATTC
TAGAATCCTCTCG 5'

Reverse inner (A allele): 3' AAGATTTTCCTTTGG
GCACCTGTTGGT 5'

Amplification was performed in a 20 μl reaction volume containing 50 ng genomic DNA, 0.4 μM of each primer, 250 μM dNTPs, 1.5 mM MgCl_2 and 1U Taq DNA polymerase. PCR amplification was performed with a 5-min initial denaturation step at 95°C followed by 30 s at 94°C for denaturation, 30 s at 59°C for annealing, 30 s at 72°C for extension, and a final extension step at 72°C for 5 min. The PCR was performed for 30 cycles. 2% agarose was used to separate the products [20].

All statistical analyses were performed using SPSS software version 24.0 (IBM Corporation, Armonk, NY, USA).

Results

Demographic distribution of patients

A total of 104 anaemic patient samples with Hb level <12.0 g/dL and <13.0 g/dL for male and female, respectively, were selected for this study as shown in Table 1.

Genotyping of rs235756 via ARMS-PCR

The gel electrophoresis of ARMS-PCR of the genotyping is shown in Fig. 1. The homozygous dominant or wild type (AA) was predicted to produce bands at 140 base pair and mutant-type homozygous recessive (GG) at 184 base pair (bp), and heterozygous (AG) would produce bands at both 140 bp and 184 bp as well as internal control band of 270 bp (Fig. 1).

Table 1 Demographic data of patients

Characteristics	Frequency (n)	Percentage (%)
Sex		
Male	55	52.9
Female	49	49.1
Ethnicity		
Malay	102	98.1
Chinese	2	1.9

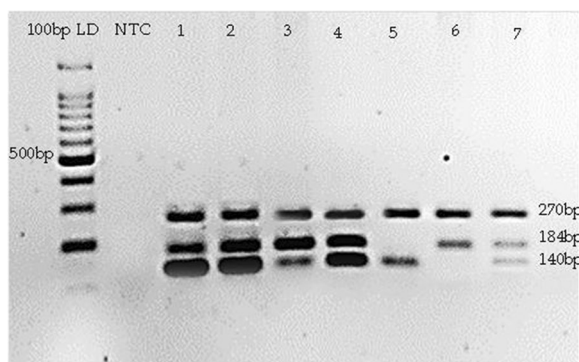


Fig. 1 ARMS-PCR gel electrophoresis for SNP rs235756 genotyping. Lane 5: homozygous dominant (AA), Lane 1–4, 7: heterozygous AG, Lane 6: homozygous recessive (GG). (Abbreviations: bp: base pair, LD: ladder, NTC: non-template control)

Table 2 Genotypes distribution of *BMP2* (rs235756) polymorphism in iron deficiency anaemia and other anaemia group

Genotypes	Iron deficiency anaemia group		Other anaemia group	
	n = 59	%	n = 45	%
AA	2	3.4	2	4.4
AG	51	86.4	42	93.3
GG	6	10.2	1	2.2

Genotype distribution of *BMP2* rs235756 gene polymorphism

Out of 104 total anaemic samples, only 59 samples fulfilled the criteria of iron deficiency anaemia, following the guideline of the indicator serum ferritin level which is below 15 ng/mL [1]. The overall frequency of the SNP genotyping result for all the patients before classification into iron deficiency anaemia and other types of anaemia was calculated (Table 2).

Analysis of population genetics of *BMP2* rs235756

The data obtained from the study were used to analyse the comparison of MAF and HWE P value between the

Table 4 Comparison of allelic frequency of *BMP2* (rs235756) between observed and reference study

Types of population	Allele frequency n (%)		P value
	A allele	G allele	
Observed study	101	107	<0.001
Reference study	872	136	

P value obtained by Pearson’s chi-squared test (2x2 table)

The 0.05 level of significance was used, and P < 0.05 is statistically significant

current study and reference population (East Asian) at <https://gene-calc.pl/hardy-weinberg-page> as shown in Table 3. MAF is the frequency of the second most common allele which is G allele *BMP2* rs235756 that occurs in a population. The current study showed a higher value of MAF (0.514) compared to reference study (0.135). However, the HWE P value of the current study (<0.001) was not statistically significant for consistent distribution with Hardy–Weinberg law (P > 0.05) as compared to the reference population (0.559).

The allelic frequency of *BMP2* (rs235756) between observed and reference study was analysed using Pearson’s chi-squared test (Table 4). At a significance level 0.05, the P value obtained for the A and G allele frequency from both studies was statistically significant (P < 0.001) and thus could be concluded that there was association between both alleles frequency with observed and reference study.

The mean difference of haematological and biochemical parameters between the genotype

The mean difference of Hb, RBC, MCV, MCH, MCHC, HCT, ferritin iron and TIBC level of patients is between homozygous-dominant AA and heterozygous AG + homozygous GG. The mean difference was analysed using independent t test. There was a significant difference for the mean of TIBC level between homozygous-dominant AA and heterozygous AG + homozygous GG genotypes (P < 0.05) but there is no significant difference for the mean of Hb, RBCs, MCV, MCH, MCHC, HCT, ferritin and iron (P > 0.05) summarized in Table 5.

Table 3 MAF and HWE P value comparison between observed and reference study from genotype frequency of *BMP2* (rs235756)

Type of genotype	Genotype frequency n (%)	Observed MAF	HWE P value	Reference population n (%)	Reference MAF	HWE P value
Homozygous-dominant AA	4 (3.8)	0.514	<0.001	380 (75.4)	0.135	0.559
Heterozygous AG	93 (89.4)			112 (22.2)		
Homozygous-recessive GG	7 (6.7)			12 (2.4)		

HWE Hardy–Weinberg equilibrium, MAF minor allele frequency

The 0.05 level of significance was used to calculate HWE P value. P > 0.05 indicates consistent distribution Hardy–Weinberg law

Table 5 Mean difference of haematological and biochemical parameters between the genotype

Parameter (Mean ± SD)	AA n=4	AG+GG n=100	P value
Hb (g/dL)	10.250 ± 1.511	10.268 ± 1.791	0.593
RBCs (× 1012/L)	3.913 ± 1.597	4.618 ± 1.034	0.443
MCV (fL)	81.375 ± 17.177	70.087 ± 10.138	0.281
MCH (pg)	25.275 ± 5.099	22.723 ± 3.694	0.392
MCHC (g/dL)	32.525 ± 2.480	32.340 ± 1.945	0.892
HCT (%)	33.800 ± 5.494	31.734 ± 5.676	0.511
Ferritin (ng/mL)	96.933 ± 102.889	140.578 ± 190.529	0.472
Iron (µmol/L)	8.850 ± 5.869	12.886 ± 10.791	0.502
TIBC (µmol/L)	31.900 ± 4.101	50.471 ± 16.714	0.030

Hb haemoglobin, RBC red blood cells, MCV mean cell volume, MCH mean cell haemoglobin, MCHC mean corpuscular haemoglobin concentration, HCT haematocrit, TIBC total iron binding capacity

P value obtained by independent t test. The 0.05 level of significance was used, and $P < 0.05$ is statistically significant

Discussion

Anaemia has always become a burden in the world population, and iron deficiency contributes half of the cases. In the past, iron deficiency was thought to be associated with dietary diet and environmental factors only. This condition always occurs especially among people who have strict diet, vegetarian, have problems with iron absorption in the body and among women who are pregnant and having heavy menstruation [23, 24]. Environmental factor such as poor sanitation also can increase the infection rate and affect the ability of body to absorb iron, thus leading to this condition [25]. These factors are believed to be the main cause; however, with the advancement of the technologies and the understanding of the underlying mechanism about iron metabolism disorder, it is revealed that genetic factors also can partially contribute to iron deficiency development [26, 27]. This is supported by the studies that have been done across the population around the globe, where the geographic and ethnicity differences have affected the genetic variation that involves in iron deficiency, especially in Asia and African populations [28].

Many studies have been done to study the association between SNPs that locate on the specific gene with the increasing risk of iron metabolism disorder. There are few genes that involve in iron metabolism pathways such as TMPRSS6, FTL, HAMP, and BMP2 gene that associate with different kinds of diseases [19]. BMP2 gene, that is located on chromosome 20, involves in hepcidin regulation that plays a big role in iron metabolism. Over-induced hepcidin can lead to anaemia, and lack of hepcidin can cause iron overload. Thus, variant of BMP2 gene is linked to most hemochromatosis cases, as it

indirectly involves in C282Y mutation that modifies the HFE [18]. Other than that, study by Milet and colleagues found a significant association between the SNP of BMP2 gene (rs235756) with the pre-therapeutic serum ferritin level [29]. However, the association was related to hemochromatosis disease and not iron deficiency anaemia [17, 19, 30]. As there were lacks of studies between rs235756 on BMP2 gene with serum ferritin level and iron deficiency anaemia, therefore, this study investigated the association between this SNP with the iron deficiency status among anaemic patients in Hospital USM, Kubang Kerian, Kelantan, Malaysia.

According to this study, taken serum ferritin level as the indicator for iron deficiency, there was no significant association between BMP2 gene rs235756 with serum ferritin level, and no association with increased risk of iron deficiency in the 104 anaemic subjects. Generally, there was also no association between the homozygous GG genotype and recessive G allele with the serum ferritin level. This finding is consistent with previous study done by An and colleagues [31], where there was no association between BMP2 rs235756 with serum ferritin and risk of iron deficiency. However, this finding is contradicted with another study done by Milet and colleagues [17], where there was a significant association between increased risk of iron deficiency anaemia with serum ferritin even though there was no association between the iron deficiency risk with BMP2 rs235756.

This result may be affected by the existence of interactions with other genetic variation, demographics, or clinical factors that will affect the genotype and phenotype of the polymorphism. In this study, the demographic data including the sex, age, and ethnicity are completely difference with the previous study that has been done. In this study, 52.9% of the subjects were male. The age mean of the subject is 24.82 (SEM: 2.531) years with 95% of them being Malays. However, studies by Al-Amer et al. [20] and An et al. [31] showed that all their subjects were female, where the age of the subjects in Al-Amer et al. studies was between young females, while An et al. use elder female in their study. Female may be chosen because they are prone to have low ferritin level due to iron deficiency that is caused by multiple factors such as menstruation, diets and others. Low level of oestrogen level in female also may affect the hepcidin regulation that cause abnormalities in serum ferritin especially in menopause elder female [32], thus supporting the study by An et al. According to the study by Yusof and team, in Malaysia, Indian has the highest prevalence of anaemia (45%), followed by Malay and Chinese, respectively [33]. However, our data only consist of 98.1% and 1.9% of Malays and Chinese due to the distribution rate of the population around Hospital USM.

If the Indian race was present in the study, as one of the major races that exhibit the highest prevalence towards the disease, it may contribute to different genotype distributions compared to the result that was obtained in the study.

Individuals are considered anaemic when the haemoglobin level is less than 13.0 g/dL in male, and 12.0 g/dL in female generally [34]. Apart from that, other haematological parameters such as RBCs count, MCV, MCH, MCHC and Hct level also contribute to anaemia identification. Iron deficiency anaemia has certain characteristics of haemoglobin parameters, such as microcytic (MCV < 80 fL), hypochromia (MCH < 27 pg/cell) and anisocytosis (RDW > 15.2%) [35, 36]. It also possesses certain abnormalities in biochemical parameters that involve in iron markers such as serum ferritin, serum iron and TIBC level [35, 36]. Among anaemic samples, serum ferritin level is the best indicator for iron deficiency and low ferritin level is widely used for iron deficiency anaemia diagnosis [35]. Ferritin is very sensitive to iron deficiency, so it can be detected when the iron stores start to exhaust and before the amount of serum iron starts to deplete [37]. Thus, even though the serum iron and TIBC still remain normal, decreases in ferritin level indicate an acute phase of iron deficiency anaemia [38].

In this study, we analysed all the anaemic samples collected generally without grouping it into their respective types and determine the association between haematological and biochemical parameters with the anaemic status. This study demonstrated the mean of Hb was 10.29 ± 0.17 g/dL, RBC was $4.59 \pm 0.1 \times 10^{12}/L$ and haematocrit was $31.81 \pm 0.56\%$; all below the normal value indicate the samples are anaemic. The mean MCV was 70.52 ± 1.04 fL, and MCH was 21.82 ± 0.37 pg/cell. Low MCV below 83.0 fL and low MCH below 27 pg/cell are defined as hypochromic microcytic anaemia. The expression of the parameters is due to low amount of stored iron that reduces the concentration of haemoglobin, thus producing a paler red blood cell, and insufficient amount of haemoglobin reduces the size of newly produced red blood cells [39]. These are proved by studies by Johnson Wimbley and Graham [40], Tong et al. [41] and Rabindrakumar et al. [42] that show the same parameters value with our study.

The mean value of ferritin was 138.90 ± 18.42 ng/mL ranging from 0.9 to 732.10 ng/mL. The mean value was quite high due to the wide range of ferritin level among the samples. The limitation of serum ferritin is that it is an acute phase protein. Its value can remain normal or elevate in a response of infections and inflammation such as inflammatory bowel disease, chronic kidney disease and other conditions [43]. The correlation between serum

ferritin with other clinical parameters shows that there was statistically significant negative correlation between ferritin with Hb, RBCs, haematocrit and TIBC, and not statistical significance with MCV, MCH and serum iron. These findings contradict with the theory as ferritin level must have positive correlation with Hb, RBCs, haematocrit, MCV, MCH and negative correlation with TIBC [44]. Previous study also reported a statistically significant positive correlation ($P < 0.05$) between ferritin level and each of Hb, RBCs, haematocrit, MCV and MCH values [45].

ARMS-PCR was used in determining the BMP2 rs235756 genotypes in the sample. This method can amplify the specific allele of interest and is time efficient, so it is suitable to be used due to limited time frame allocated for this study. The observed genotype distribution in this study showed that 89.4% of the patients had heterozygous AG followed by 6.7% and 3.8% for homozygous-recessive GG and homozygous-dominant AA, respectively. This finding is comparable with Al-Amer et al. [20] where they found that 88% had heterozygous AG followed by 5% for both for homozygous-recessive GG and homozygous-dominant AA, respectively, in their study. However, comparing the minor allele frequency (MAF) and HWE P value between this studies with reference population obtained from Ensembl genome study showed obvious difference between the genotype distribution. In reference population, homozygous AA comprises 75.4% of the population followed by 22.2% of heterozygous AG and 2.4% of homozygous-recessive GG.

MAF is the second most frequent allele occurs in a given population, which is allele G, while HWE stands for Hardy-Weinberg equilibrium is used to estimate the number of homozygous and heterozygous variant carriers based on its allele frequency in populations that are not evolving [46]. Our observed MAF (0.514) is higher compared to reference MAF of East Asia (0.135). SNPs with MAF frequency more than 5% are classified as a common variant in the population and are suitable to be used in disease research [47]. HWE P value analysis of the reference population is 0.559 and shows consistent distribution ($P > 0.05$), as they are not likely to represent genotyping errors. However, our data have HWE P value less than 0.05. This may be due to imbalance genotype distribution of our sample where the heterozygous genotypes are the highest, compared to the reference population [48]. If the HWE P value of the reference population also do not meet the criteria of $P > 0.05$, the gene allele frequencies may change and the population may evolve for that gene [49]. Comparison between allele A and G between this study and the reference population

shows a statistically significant association ($P < 0.05$) between both.

The patients were divided into two groups: iron deficiency anaemia ($n = 59$) and other types of anaemia ($n = 45$), according to the serum ferritin level of the patients. The distribution of the genotype is likely the same with overall genotype distribution. The only difference is that iron deficiency anaemia group has higher frequency of homozygous-recessive GG (11.9%) compared to other type of anaemia group (2.2%). The higher frequency of homozygous-recessive GG genotype in iron deficiency groups compared to other may indicate that this minor genotype involves in susceptibility of the disease. This value is supported again by the study done by Al-Amer et al., where the homozygous-recessive GG of the iron deficiency group (13.79%) was higher than normal group (2%) and found association between the major allele with the disease in both group ($P < 0.05$). In study done by Milet et al. [17], major A allele of BMP2 rs235756 has association with higher ferritin level even though it is a study about hemochromatosis.

Study by Al-Amer et al. [20] shows that the OR 95% CI for the homozygous-recessive GG of rs235756 in BMP2 was 29.3 (1.494–575.401) and there was a significant association ($P < 0.026$) between the gene with increased risk of iron deficiency anaemia in female students in the study. However, in this study, the association between the risks of iron deficiency with the genotype cannot be identified. This is because the risk of a disease in a research can only be calculated based on a study that has two different groups which are group that have exposed with the disease and other is not [50]. This study consists of two groups; however, both groups were already exposed with the disease (anaemia) so only frequency of the genotype distribution across the groups could be identified. The distribution of the dominant and recessive allele among clinical parameters also shows no significant association except TIBC with $P < 0.03$. The distribution for G allele exceeds the major allele A because of the genotype distribution that has high frequency of heterozygous AG.

In this study, the impact of ferritin on the recessive allele G was determined and the findings show that there is no significant association between serum ferritin levels with minor genotype studied (AG + GG) compared to major genotype AA with P value > 0.05 . So, it can be concluded that the rs235756 BMP2 does not affect the level of serum ferritin in the body. Very little research has been done to study the relation between rs235756 BMP2 with low serum ferritin and iron deficiency. The only studies that show the association between allele and the serum ferritin level in iron deficiency are as always mentioned before, by Al-Amer et al. [20] and Milet et al.

[29]. The insignificant result in this study may be affected by the imbalanced genotype distribution. Based on the reference population of rs235756 BMP2 in Ensembl genome, it is showed that the dominant genotype AA and major allele A have the highest frequency, followed by heterozygous AG and minor allele G and homozygous GG genotype are the least. However, this study showed heterozygous AG as the most common genotype compared to dominant genotype AA causing the difference between the distribution in this study as compared to the reference population.

Conclusion

The current study demonstrates the substantial roles BMP2 polymorphic variant rs235756 in iron deficiency status among anaemic patients in Hospital USM. In the future research, we hope that with detail preparation and improvement from this study, we may find a significant result that can help in the early prediction for iron deficiency through the genetic testing.

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Author contributions

NAA and NSB contributed to conceptualization; NAA, NSB, SNAMG and SNNAM were involved in methodology; NAA, ZZ and NSB contributed to writing—original draft preparation; NAA, ZZ and NSB were involved in writing—review and editing; and all authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The data presented in this study are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate

This study has been approved by the Research Ethics Committee (Human) Universiti Sains Malaysia with code USM/JEPeM/19090552 and patient consented to participate.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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