Clinical and genetic markers of erythropoietin deficiency anemia in chronic kidney disease (predialysis) patients

Nava Yugavathy1, Hasniza Zaman Huri*2, Lim Soo Kun1, Abdul Halim Bin Abdul Gafor3, Wong Muh Geot4,7, Sunita Bavanandan5 & Wong Hing Seng6

1Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia
2Department of Pharmacy, Faculty of Pharmacy, University of Malaya, Kuala Lumpur 50603, Malaysia
3Department of Medicine, National University of Malaysia, Bangi 43600, Malaysia
4Department of Renal Medicine, Royal North Shore Hospital, NSW, Australia
5Department of Nephrology, Hospital Kuala Lumpur, Kuala Lumpur 50586, Malaysia
6Department of Nephrology, Hospital Selayang, Selangor 68100, Malaysia
7Department of Renal and Metabolic, The George Institute for Global Health, University of New South Wales, NSW, Australia

* Author for correspondence: Tel.: +61 2 8535; Fax: +603 7967 4964; hasnizazh@um.edu.my

Aim: To determine the clinical and genetic markers associated with erythropoietin deficiency anemia in predialysis individuals. Materials & methods: Patients were categorized into cases and control group. Demographic characteristics and clinical parameters were obtained from medical record review and serum EPO and ferritin were obtained with ELISA. HIF-1α (rs2057482), IL-1β (rs1143627) and EPO (rs1617640) gene polymorphism were genotyped. Results: Female gender, glomerular filtration rate, treatment with hematins, anticoagulant and diuretic were strong predictors of EPO-deficient anemia in predialysis chronic kidney disease patients. Genetic polymorphism in the HIF-1α recessive model was associated with non-EPO-deficiency, followed by EPO recessive allele associated with low-serum erythropoietin and IL-1β recessive model with low hemoglobin level. Conclusion: EPO-deficiency anemia can be diagnosed more conveniently in the presence of biomarkers.

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Keywords: EPO gene • EPO deficiency • genetic polymorphism • HIF-1α • IL-1β • predialysis • renal anemia

Chronic kidney disease (CKD) is often accompanied by anemia, defined as hemoglobin (Hb) less than 12.0 g/l for women and 13.0 g/l for men and a target of more than 13.0 g/l may increase risk of serious adverse event [1]. There is a strong correlation of glomerular filtration rate (GFR) with reduction in Hb concentration, which is more pronounced in individuals with advanced CKD [2]. Anemia can lead to debilitating symptoms resulting in significant morbidities [3], poor quality of life [2] and risk associated with blood transfusion [4] in individuals with CKD. The hypothesis of erythropoietin (EPO) deficiency is the predominant cause of anemia in CKD is yet to be accepted [5]. There can be many other contributing factors including chronic inflammatory state resulted in upregulation of hepardin [6] that affects intestinal iron absorption and utilization, water soluble vitamin (folate and vitamin B12) deficiency, blood loss and hyperparathyroidism [7]. In addition, pharmacological treatment such as concomitant use of renin-angiotensin system blockers (RAS-blockers) have direct narrow suppression on erythropoiesis [8]. Epidemiological evidence suggests correction of anemia with recombinant-EPO can lead to improvement in quality of life and all-cause mortality [9–11], but randomized controlled trials in the past decades have demonstrated harm in over correction of anemia and supra-physiological dose of EPO (US Normal Hematocrit Trial [12], CREATE Study [13], CHOIR Study [14] and TREAT Study [15]).

The pathophysiology of ineffective EPO production in CKD is mainly due to dysfunction or loss of renal interstitial fibroblast in response to tissue hypoxia superimposed with chronic inflammation leading to kidney fibrosis. Several pro-inflammatory and probiotic pathways including NF-KB, TGFβ/Smad and GATA-2 signaling pathways are activated resulting in a cascade of downstream signaling, many of which have direct and indirect
suppression effect on EPO-regulatory genes [16,17]. Other studies postulated EPO-deficient anemia might be due to efferent sympathetic denervation of the kidney causing the loss of EPO production [18].

Hypoxia inducible factor (HIF) consists of subtype \(HIF-1\alpha\) and \(HIF-1\beta\) that controls a wide range of hypoxia response including erythropoiesis and angiogenesis [19,20]. The levels of HIF changes according to oxygen concentration through prolyl hydroxylase dehydrogenase (PHD) enzyme. In hypoxic state, PHD level decreases, causing accumulation of \(HIF-1\alpha\) thus, inducing erythropoietin expression and receptor upregulation [21]. Activation of HIF by PHD inhibition is being investigated as a novel therapeutic strategy to treat anemia in both dialysis and nondialysis CKD patients. Studies have associated \(HIF-1\alpha\) polymorphism to diabetes Type 2, heart diseases, renal carcinoma, cancer and more [22–24]. The rs2057482 polymorphism is located in the 3’ untranslated region of the \(HIF-1\alpha\) mRNA, thus, polymorphism in this region is more likely to compromise in response to tissue hypoxia.

Elevated levels of pro-inflammatory cytokines are often associated with impaired production of EPO. Studies have shown pro-inflammatory cytokines impair the erythroid colony formation in response to erythropoietin and downregulate the membrane-bound erythropoietin receptor [25,26]. In addition, clinical studies have also reported several cytokines including \(IL-1\beta\) showing significant blunted erythropoietin response independent of age and sex [27,28]. The \(IL-1\beta\)-511C/T (rs1143627) single-nucleotide polymorphism (SNP) has been associated with a variety of diseases in which inflammation plays an important role [29]. Jeong et al. (2008) reported that the \(IL-1\beta\)-511CC genotype was significantly associated with lower erythropoietin resistance index (ERI) values in hemodialysis patients [30].

The main determinant of EPO synthesis is the regulation of its gene, which is often related to oxygen tension. The oxygen dependant control of EPO production is regulated by HIF binding to hypoxia responsible element thus activating EPO gene transcription [31]. EPO-deficiency anemia and diabetic nephropathy is a major trait of impaired kidney function where the latter can be inheritable. In vitro experiments by Tong et al. have shown that a SNP from G to T in EPO promoter (rs1617640) can alter the EPO mRNA levels [32]. In a study conducted on chronic hepatitis C undergoing viral therapy, the T allele of rs1617640 was associated with higher levels of EPO in the vitreous body fluid on nondiabetic patients compared with G allele. Moreover, the G allele was independently associated with Hb decline during the antiviral therapy [33].

Although genetic polymorphisms are linked with anemia through its direct or indirect effects on erythropoiesis, inflammation and hypoxia, their association with EPO-deficiency anemia in predialysis CKD remain largely unknown. In this study, we aim to classify EPO-deficiency anemia and non-EPO-deficiency anemia patients. We also investigated the association of EPO-deficiency anemia with SNP in \(HIF-1\alpha\) (rs2057482), \(IL-1\beta\) (rs1143627) and \(EPO\) (rs1617640), as well as demographic, clinical markers in individuals with predialysis CKD [34]. To our knowledge, this is the first study to describe the association of these clinical and genetic markers in EPO-deficient predialysis CKD patients.

**Materials & methods**

**Study subjects**

This is a prospective, multicenter, cross sectional study involving 445 individuals with stage 3–5 CKD as per Kidney Disease Improving Global Outcomes (KDIGO) classification [34]. Only predialysis stage 5 CKD individuals are included in the study. Written informed consent were obtained from all eligible participants, and the study is conducted in accordance to the Declaration of Helsinki [35]. Blood samples were collected from three centres, namely University Malaya Medical Centre, Hospital Kuala Lumpur and Hospital Selayang. This study was approved by the Medical Ethics Committee of University Malaya Medical Centre (reference number: 20156–1384) and NIH by Ministry of Health (Ministry of Health Medical Research Ethics Committee Reference Number: NMRR-18-296-39684). Demographic data, clinical and biochemical parameters were obtained from patient electronic database in each center.

The inclusion criteria are adults of 18 years old or older with stable CKD stage 3 (GFR: 30–59 ml/min/1.73 m²), stage 4 (GFR: 15–29 ml/min/1.73 m²) or stage 5 (predialysis) (GFR: <15 ml/min/1.73 m²) according KDIGO (2012) guidelines [34]. Patients were categorized into anemia (Hb: <12.0 g/dl) and nonanemia (Hb: >12.0 g/dl) according to European Best Practice Guidelines (2004) [36]. Pregnant women, patients receiving dialysis or individuals with evidence of blood loss 2 months prior to consent, hypothyroidism (patients on thyroid hormone replacement therapy), autoimmune diseases, malignant disease, hemoglobinopathies, leukemia, patients who received chemo therapy, radio therapy or immune therapy and acute kidney diseases were excluded from the study.
Genotyping
Three candidate genes HIF-1α (rs2057482), IL-1β (rs1143627) and EPO (rs1617640) were chosen based on three different causative pathways that might affect EPO production based on literature. All the SNP has minor allele frequency of more than 10% based on database SNP (dbSNP). To the best of our knowledge, these SNPs are naive to erythropoietin deficiency studies in predialysis patients and have potential functional effects. Blood samples were collected using EDTA tubes, serum was separated immediately and stored at -20°C for future ELISA analysis. Genomic DNA was extracted using QIAGEN QIAamp DNA blood mini kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity was measured using NanoDrop spectrophotometry using absorbance at 260 nm (A260) and 280 nm (A280). All polymorphisms were analyzed by real-time (RT)-PCR using the TaqMan fluorogenic 5' nuclease assay (Applied Biosystems, CA, USA). Amplification was carried out in a StepOnePlus™ RT-PCR System (Applied Biosystems), with 96-well microplates containing 10 ng of genomic DNA, 20× Taqman SNP genotyping assay mix and 2× TaqMan Universal PCR Master mix per well. Initial enzyme activation (10 min, at 95°C) followed by 40 cycles of denaturation (15 s at 95°C) and probe annealing/extension (1 min at 60°C). Allelic discrimination was performed by measuring fluorescence emitted by both VIC and FAM dyes in each well (60 s) and computing the results into the Applied Biosystems StepOnePlus RT-PCR Software (StepOnePlus software).

ELISA
Serum EPO and serum ferritin were measured using Enzyme-Linked Immunosorbent Assay (Cloud-Clone Corp., TX, USA). The manufacturers reported normal range from healthy blood donor’s serum was 72.4–638 pg/ml and 61–285 ng/ml, respectively. Average duplicate readings for each standard, control and samples were used to construct standard curve. The best-fit-curve graph of optical density (OD) value of standard (x-axis) against known concentration of standard (y-axis) was constructed using Curve Expert Professional 2.6.4.

Statistical analysis
All continuous variables were analyzed using Mann–Whitney U test due to deviation from normal distribution. Results are expressed in median with interquartile ranges. Categorical variables in the clinical, genetic and demographic parameters were compared using Pearson chi-square test and expressed in percentages. A multivariate analysis was done to test the association of significant baseline characteristics. The genotype frequencies were tested for Hardy–Weinberg equilibrium using a standard chi-square test. For stratified analysis, clinical markers were divided into tertile 1, tertile 2 and tertile 3 (T1–T3) where Hb level (less than 10.0 g/dl, 10.0–12.0 g/dl and more than 12.0 g/dl), serum EPO (less than 72.4 pg/ml, 72.4–638 pg/ml and more than 638 pg/ml), serum ferritin (less than 100 ng/ml, 100–500 ng/ml and more than 500 ng/ml). Logistic regression was used to obtain the odds ratio (OR) and 95% CI between genetic polymorphism and clinical markers and plotted using forest plot. All statistical analysis was carried out using SPSS software version 21.

Results
Demographic & clinical characteristics
Since EPO-deficiency is diagnosis of exclusion, patients were carefully categorized into each group according to the flow chart below (Figure 1). The baseline demographic and clinical characteristics of patients are tabulated in Table 1. Of the 445 patients analyzed, 53.5% were categorized into EPO-deficiency (case) and 46.5% into non-EPO-deficiency (control). Age, ethnicity, comorbidities and treatment of concomitant RAS blockers were similar in between these groups. The ethnicity of the patient in this study represents the Malaysian predominant population. There are 30.8% more female patients in case group compared with control group and a lower median GFR. Patients receiving hematinic and diuretic treatment are higher in case group by 46.2 and 21.8% respectively. Anticoagulant on the other hand is lower in cases group by 11.8%. Variables that were significant at p < 0.25 were used for multivariate analysis. It is found that female gender, GFR, concomitant use of hematinic, anticoagulant and diuretics were significant predictors of EPO-deficiency (Table 2).

Genetic markers
The genotype distribution was in Hardy-Weinberg equilibrium for all three SNPs in both groups. All three SNPs were classified into its additive, dominant and recessive models (Table 3). The frequency of HIF-1α polymorphism (CC, TT and CT genotypes) and recessive allele (TT+CT vs CC genotypes) is significantly lesser in cases than
Haemoglobin normal >12.0 g/dl

Haemoglobin low <12.0 g/dl

ESA treated (case)

Non-ESA treated (control)

ESAs treated (case)

Non-ESA treated

Normal serum EPO: >72.4 pg/ml (control)

Low serum EPO: <72.4 pg/ml (case)

Figure 1. Flowchart showing patient grouping; (case): EPO deficiency, (control): non-EPO deficiency.

Table 1. Demographics and clinical characteristics of EPO-deficiency patients (case) in comparison with non-EPO deficiency patients (control).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Case</th>
<th>Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>238 (53.5%)</td>
<td>207 (46.5%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>68 (60.8–77.0)</td>
<td>69 (62.0–76.0)</td>
<td>0.859‡</td>
</tr>
<tr>
<td>Gender (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– Male</td>
<td>98 (41.2)</td>
<td>149 (72.0)</td>
<td></td>
</tr>
<tr>
<td>– Female</td>
<td>140 (58.8)</td>
<td>58 (28.0)</td>
<td>0.001†</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– Malay</td>
<td>106 (45.1)</td>
<td>95 (46.1)</td>
<td></td>
</tr>
<tr>
<td>– Chinese</td>
<td>81 (34.5)</td>
<td>76 (36.9)</td>
<td>0.539†</td>
</tr>
<tr>
<td>– Indian</td>
<td>48 (20.4)</td>
<td>35 (17.0)</td>
<td></td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum ferritin (ng/ml)</td>
<td>171.5 (70.2–402.4)</td>
<td>139.0 (83.8–382.6)</td>
<td>0.680‡</td>
</tr>
<tr>
<td>GFR (ml/min/1.73 m²)</td>
<td>21.0 (12.0–33.25)</td>
<td>35.0 (26.0–44.0)</td>
<td>0.001‡</td>
</tr>
<tr>
<td>Comorbidities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>173 (73.0)</td>
<td>132 (64.7)</td>
<td>0.060†</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>212 (89.5)</td>
<td>180 (88.2)</td>
<td>0.686‡</td>
</tr>
<tr>
<td>Dyslipidemia (%)</td>
<td>114 (48.3)</td>
<td>104 (51.0)</td>
<td>0.577†</td>
</tr>
<tr>
<td>Heart diseases (%)</td>
<td>62 (26.2)</td>
<td>53 (25.7)</td>
<td>0.918†</td>
</tr>
<tr>
<td>Drug treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematinics (%)</td>
<td>133 (73.1)</td>
<td>49 (26.9)</td>
<td>0.001†</td>
</tr>
<tr>
<td>RAAS inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– ACEI (%)</td>
<td>66 (27.7)</td>
<td>65 (31.6)</td>
<td>0.380†</td>
</tr>
<tr>
<td>– ARB (%)</td>
<td>62 (26.1)</td>
<td>74 (35.9)</td>
<td>0.024†</td>
</tr>
<tr>
<td>Anticoagulants (%)</td>
<td>77 (32.4)</td>
<td>91 (44.2)</td>
<td>0.010†</td>
</tr>
<tr>
<td>Diuretics (%)</td>
<td>119 (50.0)</td>
<td>58 (28.2)</td>
<td>0.001†</td>
</tr>
</tbody>
</table>

Significant p-value <0.05 were bolded.

†Anova test.

‡Pearson chi-square test.

AR: Angiotensin Receptor Blocker; GFR: Glomerular filtration rate.
Table 2. Multivariate analysis of demographic and clinical characteristics in EPO-deficiency patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Comparison</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>female vs male</td>
<td>3.051 (1.897–4.907)</td>
<td>0.001</td>
</tr>
<tr>
<td>GFR (ml/min/1.73 m²)</td>
<td>per 15 ml/min/1.73 m² decrease</td>
<td>0.995 (0.961–1.090)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hematinics</td>
<td>vs not treated</td>
<td>2.296 (1.392–3.787)</td>
<td>0.001</td>
</tr>
<tr>
<td>Anticoagulants</td>
<td>vs not treated</td>
<td>0.453 (0.274–0.746)</td>
<td>0.002</td>
</tr>
<tr>
<td>Diuretics</td>
<td>vs not treated</td>
<td>1.811 (1.094–2.997)</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Significant p-value values were bolded.
GFR: Glomerular filtration rate; OR: Odds ratio.

Table 3. Genotype distribution of HIF-1α, IL-1β and EPO gene alleles in cases and control group.

<table>
<thead>
<tr>
<th>Gene (SNP)</th>
<th>Genotype</th>
<th>Case (n = 238) (%)</th>
<th>Control (n = 207) (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α (rs2057482)</td>
<td>CC</td>
<td>135 (56.9)</td>
<td>95 (45.8)</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>14 (6.0)</td>
<td>20 (9.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>89 (37.2)</td>
<td>92 (44.3)</td>
<td></td>
</tr>
<tr>
<td>Dominant</td>
<td>(CC, CT vs TT)</td>
<td>224 (94.0)</td>
<td>189 (91.1)</td>
<td>0.263</td>
</tr>
<tr>
<td>Recessive</td>
<td>(TT, CT vs CC)</td>
<td>103 (43.3)</td>
<td>112 (54.2)</td>
<td>0.028</td>
</tr>
<tr>
<td>IL-1β (rs1143627)</td>
<td>GG</td>
<td>70 (29.5)</td>
<td>53 (25.5)</td>
<td>0.440</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>62 (25.2)</td>
<td>55 (26.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>108 (45.2)</td>
<td>99 (47.8)</td>
<td></td>
</tr>
<tr>
<td>Dominant</td>
<td>(GG, GA vs AA)</td>
<td>179 (75.2)</td>
<td>148 (71.7)</td>
<td>0.595</td>
</tr>
<tr>
<td>Recessive</td>
<td>(AA, GA vs GG)</td>
<td>174 (73.4)</td>
<td>147 (71.0)</td>
<td>0.433</td>
</tr>
<tr>
<td>EPO (rs1617640)</td>
<td>AA</td>
<td>135 (56.9)</td>
<td>105 (50.8)</td>
<td>0.206</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>20 (8.3)</td>
<td>18 (8.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>82 (34.7)</td>
<td>84 (40.5)</td>
<td></td>
</tr>
<tr>
<td>Dominant</td>
<td>(AA, AC vs CC)</td>
<td>217 (91.7)</td>
<td>188 (90.7)</td>
<td>0.737</td>
</tr>
<tr>
<td>Recessive</td>
<td>(AC, CC vs AA)</td>
<td>102 (43.1)</td>
<td>100 (48.1)</td>
<td>0.316</td>
</tr>
</tbody>
</table>

Significant p < 0.05 in bold. A one-way ANOVA test was used to determine the p-value.

those in the control group. Surprisingly, EPO (rs161764) and IL-1β (rs2057482) polymorphism did not differ much between the two groups. All three genes were then analyzed to study the correlation between deemed as reference range and SNP polymorphism of the predialysis cohort (Figure 2). After adjusting with age, gender and ethnicity, the recessive model of EPO contributes significantly to low level of serum EPO, less than 72.4 pg/ml (OR:1.605 [95% CI: 1.041–2.457]; p = 0.032). Moreover, the recessive variant of IL-1β (AA+GA) is associated with Hb less than 10 g/dl (OR: 1.941 [95% CI: 1.029–3.660]; p = 0.041). To our surprise, all three SNPs did not show any significant correlation with CKD stages and serum ferritin. It was also noted, comorbidities such as diabetes, hypertension, dyslipidemia and heart diseases often related to CKD does not have any significant association with the genetic models investigated (Supplementary Table 1).

Discussion
In our study, we described a wide range of clinical and genetic markers prospectively in a cohort of individuals with EPO-deficiency anemia predialysis stage 3–5 CKD. The novel finding of this study is that in individuals with predialysis, recessive gene polymorphism of EPO (rs1617640) is associated with lower level of serum EPO. According to Hoogendoorn et al. [37] a third of promoter variants might alter gene expression resulting in phenotypic variation. Our finding is consistent with this notion as we can see direct relation to serum EPO as allele A was substituted with allele C. In contrast to our finding, in European-American the dominant allele A is associated with elevated level of EPO in the mouse models of diabetic eye, vitreous body of human eye and kidney complications [38]. This promoter region has been linked with severity of diabetic retinopathy, anemia in mild kidney disease patients [34,35] and now low serum EPO in predialysis.

The recessive gene of IL-1β (rs1143627) is found to be associated with low Hb (<10 g/dl). Inflammation plays a major role in anemia of chronic diseases as IL-1β suppresses the colony formation of erythroid progenitors in bone marrow inhibiting erythropoiesis. Evidently, during an acute phase inflammatory response, IL-1β increases the
Figure 2. Association between HIF-1α, IL-1β and EPO gene models and clinical risk factors of EPO-deficiency anemia. Logistic Regression test was used to determine the OR and p-value. Significant p-value marked with a circle.

CKD: Chronic kidney disease; EPO: Erythropoietin; Hb: Hemoglobin; OR: Odds ratio; T1: Tertile 1; T2: Tertile 2; T3: Tertile 3.

production of both heavy and light subunits of ferritin [38]. Previous study on AA Amyloidosis patients also showed similar result as Hb less than 11.0 g/dl was highly correlated with the recessive allele T [39]. It was also reported circulating IL-1β correlates negatively with Hb and the recessive allele might be contributing to its increased production [39]. This inverse relationship observed is in line with Faquin et al. [39] who reported IL-1β inhibits the production of EPO at mRNA level.

In hypoxic conditions, HIF-1α is often elevated to increase the production of EPO by targeting EPO gene upregulation [35]. The association between HIF-1α polymorphism and EPO-deficiency anemia might be more complicated than merely adaptation to stimulate EPO production. To our surprise, the recessive gene polymorphism
of HIF-1α (rs2057482) 3'UTR region is associated with non-EPO-deficiency cohort. Patients grouped in cases had significantly lower frequency of T allele compared with the control group. To justify our findings, we postulated the recessive (TT + CT) allele might be responsible for protection against EPO-deficiency anemia. In contrast, a study evaluating the relationship between the functional coding region of HIF-1α and acute kidney injury reported T allele carriers had higher odds of adverse event including dialysis requirement [39] which indicates T allele might be a risk allele. To the best of our knowledge this is the first study which reports rs2057482 polymorphism as protective marker of EPO-deficiency anemia in predialysis patients.

Besides genetic polymorphism, we also found several potential clinical markers associated with EPO-deficiency anemia in predialysis CKD. Female gender, renal function (GFR), concomitant drug treatment; hematinics, diuretics and anticoagulant are the factors associated with EPO-deficiency. It was also reported earlier; female patients were at higher risk to ESA resistance due to the difference observed in iron released from reticuloendothelial cells compared with male gender [37,38]. Renal function (GFR) is often a confounding factor in renal anemia and one of the hypothesis promulgated is that as the renal function declines, there is a progressive and slow reduction in Hb which becomes evident at GFR below 60 ml/min. Our data suggest, GFR below 33.3 ml/min which is the case's upper quartile is highly predictive of EPO-deficiency anemia as compared with the control group's upper quartile 44.0 ml/min. Rao et al. stressed on the importance of nutritional status with EPO responsiveness which is in line with our cohort's treatment as majority of the EPO-deficient group patients were on hematinics [7]. This will help to improve the cohort's responsiveness to endogenous or recombinant EPO in future.

Comorbidities and concurrent drug treatment was also explored in our study to determine the association with EPO-deficiency anemia. Type 2 diabetes is one of the most commonly associated comorbidity with CKD and anemia [40]. Although the number of diabetics is slightly higher in EPO-deficient group, it was not significant enough to be a marker. In fact, none of the comorbidities studied were significant markers for EPO-deficiency anemia in predialysis. However, the usage of certain concomitant drugs to treat these comorbidities might play a role as predictor. Although the exact mechanism is unknown and to be studied in the future, hematinics and diuretics were found to be a risk factor in this cohort. Anticoagulant on the other hand plays a protective role. Interestingly, although studies have reported RAS blockers can cause impairment of erythropoiesis by suppressing the angiotensin pathway, our findings did not show statistical significance with this treatment.

In summary, our study focuses on anemia with EPO-deficiency as the potential underlying mechanism in CKD stage 3–5. These findings demonstrate EPO-deficiency is the result of complex relationship between genetic polymorphism and clinical factors.

Conclusion
To our knowledge, this is the only study trying to classify EPO-deficiency patients and strengthening it with clinical and genetic markers. EPO gene (rs1617640) polymorphism is associated with low serum EPO in predialysis CKD patients. Besides that, the recessive HIF-1α gene (rs2057482) model is associated EPO-deficiency in our study cohort. We also observed, the recessive gene model of IL-1β (rs1143627) is associated with Hb less than 10 g/dl raising a possible explanation on how all three genes polymorphism can be related to EPO-deficiency anemia in predialysis patients. At last, female patients (CKD 3–5) with some modifiable treatments should be given extra attention. With these insight, we provide a better understanding to categorize high risk patients.

Future perspective
In near future, such new knowledge of biomarkers obtained from this study can be used in clinical practice: to precisely identify high risk patients; etiology and pathophysiology of EPO-deficiency can be understood profoundly; and generate individualized treatment to reduce adverse effect and provide better quality of life.

Author contributions
N Yugavathy [1] was responsible for clinical sample collection, laboratory work, interpretation of data and drafting the whole manuscript. HZ Huri [2] and LS Kun [1] for study grant, conception and design. AHBA Gafor [3] and WM Geot [4,7], were responsible for data interpretation ideas and revision of manuscript. S Bavanandan [5] and WH Seng [6] were responsible for revision of manuscript and provided on-site assistance for sample collection.
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Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Summary points

### Predialysis chronic kidney disease
- Chronic kidney disease (CKD) patients often suffer from anemia with unknown etiology. It is often misdiagnosed or neglected which will affect their quality of life.
- Many studies have been conducted on dialysis patients in order to improve patient’s treatment and life.
- There are many causes to anemia in CKD patients and the most hypothetical one is erythropoietin (EPO)-deficiency.

### EPO-deficiency anemia
- EPO-deficiency is a condition whereby the kidney cells are unable to compensate the hypoxia rectifying mechanism either by not producing sufficient EPO or unable to utilize the available EPO to support red blood cell production.
- In this study, we have categorized patients into EPO-deficient as case and non-EPO deficient as control by interpreting hemoglobin (Hb) level, serum erythropoietin and treatment that corrects iron-deficiency anemia; hematinics.

### Demographic & clinical markers
- Our demographic data are age, sex and ethnicity.
- Clinical parameters that are often associated with the current patient population is serum ferritin, Hb, serum EPO and glomerular filtration rate.
- Comorbidities such as hypertension, dyslipidemia, diabetes and cardiovascular diseases were also analyzed.
- Concurrent drug treatment that is often related to anemia and EPO production, renin-angiotensin system blockers. Other general treatment including diuretics and antiplatelet was also analyzed.

### Genetic markers
- **HIF-1α** controls a wide range of hypoxia response including erythropoiesis and angiogenesis. The rs2057482 polymorphism is located in the 3’ untranslated region of the gene’s mRNA thus polymorphism in this region is more likely to compromise in response to tissue hypoxia.
- Elevated levels of pro-inflammatory cytokines are often associated with impaired production of EPO. **IL-1β rs1143627** has been associated with a variety of diseases in which inflammation plays an important role including ESA treatment resistance.
- The main determinant of EPO synthesis is the regulation of its gene which is often related to oxygen tension, therefore, single nucleotide polymorphism from promoter region was chosen (rs1617640).

### Conclusion
- Female patients, glomerular filtration rate, treatment with hematinics, diuretics, antiplatelet are strong clinical predictors of EPO-deficiency anemia.
- **EPO** gene (rs1617640) polymorphism is associated with low serum EPO in predialysis CKD patients.
- Recessive **HIF-1α** gene (rs2057482) model is protective marker against EPO-deficiency.
- Recessive gene model of **IL-1β** (rs1143627) is associated with low Hb.
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