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Original Article

Impact of anemia on erythropoietin and erythropoietin receptor expression: correlation with the proliferation of breast cancer cells

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Key Words
Anemia; Breast cancer; Erythropoietin; Erythropoietin receptor; Non-anemia; Proliferation

Abstract

Objective: The aim of this research is to investigate the relationship between anemia and erythropoietin (Epo) and erythropoietin receptor (EpoR) expression. This study also investigated the relationship between Epo and EpoR expression level and the proliferation rate of cancer cells.

Methods: 20 samples of breast cancer tissues were divided into two groups; anemic group (from patiens with Hb level < 12) and non-anemic group (from patients with Hb level > 12). All samples were analyzed by using immunofluorescence staining in order to examine Epo and EpoR expression. Proliferation of cancer cells were analyzed by using Hematoxylin-Eosin staining.

Results: Anemic breast cancer group represented higher Epo and EpoR expression than the non-anemic group. The results also indicated that in anemic samples expression levels of Epo and EpoR were negatively correlated with the number of cancer cells. In contrast, Epo and EpoR expression levels from non-anemic samples were positively correlated with the number of cancer cells.

Conclusion: These results conducted that anemia is a crucial factor of hypoxic condition. Hypoxia led by anemia cause a different control mechanism of Epo and EpoR expression and cancer cell proliferation.

INTRODUCTION

Anemia (hemoglobin < 12 g/dl) is a frequent complication of malignant diseases and cancer therapy. Anemia may be caused from bleeding, nutritional deficiencies, bone marrow damage, and the malignant process itself [1]. Anemia has been found not only to reduce the quality of life, but also to reduce the duration of survival. In cancer patients, anemia has been hypothesized to lead to tumor hypoxia [2]. Tumor hypoxia has been hypothesized to lead tumor growth and resistance to therapy because it leads to angiogenesis, genetic mutations, resistance of apoptosis [3].

Erythropoietin (Epo) is a glycoprotein hormone which -in the normoxic condition- is produced in adult kidney and the fetal liver that regulates growth, differentiation, and survival of erythroid progenitors [4]. Epo circulates in the blood vessels and binds to Epo receptors (EpoR). EpoR is a type-1 cytokine receptor family expressed in erythroid progenitor cells to accelerate viability, proliferation and differentiation, resulting in an increased number of erythrocytes [5]

When tumor tissue experiences hypoxic conditions, cancer cells can synthesize Epo by activation of hypoxia inducible factor-1α (HIF-1α). During hypoxic conditions, HIF-1α dimer with constitutively expressed HIF-1β form a transcription factor that regulates the adaptive response to oxygen deprivation in the cell and transcribes several target genes associated with cell survival and proliferation, one of them being Epo [6, 7]. Binding Epo to EpoR on the cell membrane causes a conformational change that brings EpoR associated with Janus family tyrosine kinase-2 (JAK2)

molecules. To regulate cell survival, the EpoR-activated JAK2/STAT5 (signal transducer and activator of transcription) pathway triggers the transcription of several genes for survival and prevention of apoptosis [8].

The result from previous study showed that Epo and HIF-1α expression of all cancer stages were not significantly different. Another result showed that the cancer patient with or without anemia was not affected by HIF-1α expression; however, it was stimulated by downstream genes of HIF-1α, such as VEGF [9]. But it was still unclear how anemia could be affected by the Epo gene that was induced by HIF-1α.

The promoter region of Epo was methylated in normoxic (non-hypoxia) condition, but not in hypoxic condition. Methylation of the Epo enhancer region will decline the transcription activity of Epo gene [10]. The region of Epo promoter between –288 to +81 is a HIF-1 binding site. A study by Yin and Blanchard [11] showed that methylation of the HIF-1 binding site on the Epo promoter declined Epo gene expression by inhibiting activity of HIF-1 as transcription factor. However, the mechanism this action need more proper examination. In the present work, it was aimed to investigate the correlation between anemia with Epo and EpoR expression and how methylation affect the regulation and transcriptional activity of Epo

MATERIALS AND METHODS

Subject

This study was mainly conducted in the Central Laboratory of Life Sciences, Brawijaya University, Malang, and East Java, Indonesia. Samples were taken from patients with breast cancer in Dharmais Hospital Jakarta from 2008 until 2011. All samples used for this study were taken from breast cancer patients who have never got any therapy with a Epo stimulating agent (ESA). The total number of samples was 20, which were divided into two groups. First group consist of breast cancer tissues from anemic patients (10 samples), and second group consist of breast cancer tissues from non-anemic patients (10 samples). Consent forms were obtained from all participants. The study achieved ethical clearance from the Research Ethics Committee, Faculty of Medicine, Brawijaya University, Indonesia (157A/EC/KEPK-S3JK/06/11, 2011).

Tissue preparation

Breast cancer tissues were firstly fixed with formalin (10%). Afterwards, tissues were dehydrated with ethanol (96%) for 5 h, then cleared using xylene for 2 h, and infiltrated using liquid paraffin at 56-57ºC for 3 h. Tissues were then embedded with paraffin and sectioned with 5 µm thicknesses.

Histopathological analysis

We analyzed histopathological profile of breast cancer tissue by hematoxylin-eosin (HE) staining. Deparafinization of samples was conducted by using xylene and dehydration done by using alcohol series (absolute ethanol, 90 and 70%) respectively. Then slides were washed with phosphate buffered saline (PBS) for 15 min. Thereafter slides were soaked in hematoxylin for 2 min and then washed with running water for 5 min. Afterwards slides were stained with eosin for 10 min and washed with running water for 5 min. Slides were dehydrated again with 70 and 90% ethanol, each for 2 min, followed by absolute ethanol. Then the slides were immersed in xylene for 10 min. Slides were then dried and covered with Enthelan® (Merck, Darmstadt, Germany) and closed with coverglasses. Results of HE staining were observed using BX53 (Olympus) microscope to calculate the number of cancer cells in breast cancer tissue.

Immunofluorescence analysis

Immunofluorescence assays were performed on formalin-fixed paraffin-embedded sections. Five millimeter thick sections deparaffinized in xylene and rehydrated in graded alcohol, were steamed in 0.01 M sodium citrate buffer (pH 6) for 10 min and then washed in PBS (pH 7.4). After blocking with 2% skim milk for 1 h, slides were incubated with the first primary antibodies against EpoR (anti-EpoR; 1:1000 dilution) in 2% skim milk at room temperature for 1 h. First secondary antibody was fluorescein-5-isothiocyanate (FITC)-labelled rabbit anti-mouse IgG (1:1500 dilution) for 1 h at room temperature without light. Then the slides were washed with PBS for 30 min. Sections were then blocked again by using 2% skim milk for 1 h and then incubated with second primary antibody against Epo (anti-Epo; dilution 1:1000) in 2% skim milk at room temperature for 1 h. Sections were then blocked with secondary antibody using rhodamine-labeled goat anti-rabbit IgG (1:1500) for 1 h at room temperature without light. Result of immunofluorescence staining were visualized and analyzed using a confocal laser scanning microscope to determine Epo and EpoR expression in breast cancer tissue.

In silico analysis

The DNA sequence of Epo gene is NG_021471, and the protein sequences of HIF-1α and HIF-1β are Q16665.1 and P27540.1, respectively; retrieved from the sequence database of the National Center for Biotechnology Information (NCBI), United States National Library of Medicine (NLM), National Institutes of Health (NIH). DNA structure was constructed using the 3DNA-Driven Analysis and Rebuilding Tool (3D-DART) web server [12]. Modeling structure of HIF1 was predicted using the

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SWISS-MODEL web server [13, 14], and methylated DNA sequence of Epo gene using YASARA (Yet Another Scientific Artificial Reality Application) View software. Docking of Epo gene and transcription factor HIF1 was analyzed by using HEX software [15, 16] and the details of interaction between Epo gene and HIF1 were detected using LigandScout software [17].

Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed using independent sample t-test via SPSS 15.0 statistical package; P value less than 0.05 was considered statistically significant.

RESULTS

Hemoglobin (Hb) level and the number of erythrocytes of anemic group were declined and it stimulated the increase of Epo and EpoR expression. Otherwise, the number of leukocytes and thrombocytes of anemic group was higher than the non-anemic group (Table 1). Epo and EpoR analyzed by immunofluorescence showed a higher expression, especially in anemia group compared to non-anemia (Fig.1). Interestingly, Epo and EpoR expression in patients with anemia have a different pattern compared to patients with non-anemia. Breast cancer tissue taken from anemia patients showed higher EpoR expression than Epo. In contrast, EpoR expression in non-anemic group was lower than Epo. We found that EpoR expression was significantly higher in anemia (1048.6 ± 99.72) than in non anemia (233.72 ± 44.57). We also found that Epo expression of anemia (990.04 ± 119.35) was higher than non-anemia (630.46 ± 77.17), but there was no significantly different in between (Fig.2).

The result of interaction analysis between Epo and EpoR showed that in anemia and non-anemia group, Epo expression was correlated with EpoR expression with a confidence level of 99%. There was a negative correlation between cancer cell proliferation with Epo and EpoR expression in anemic group. When Epo and EpoR expression is low, afterwards it will be followed by the increase of cancer cell's number, and vice versa. In the contradictory situation with the anemic group, we found a positive correlation between cancer cell proliferation with Epo and EpoR expression of non anemic group. High Epo and EpoR expression will be followed by the increasing number of cancer cells, and vice versa (Fig.3). In addition, there is no significant difference of cancer cell proliferation between anemia and non-anemia group.

In silico analysis show that binding energy in methylated DNA was greater (–859.16 KJ/Mol) than in unmethylated DNA (–8259.22 KJ/Mol) (Fig.4). So, we presume that silencing mechanism of the Epo gene by methylation is correlated with binding energy that is required for docking interaction. In normoxic conditions in which the Epo promoter is methylated, binding energy for interaction is greater but less stable than in unmethylated. In addition, methylation of the Epo promoter gene has impact on changing the docking position between HIF1 and Epo promoter.

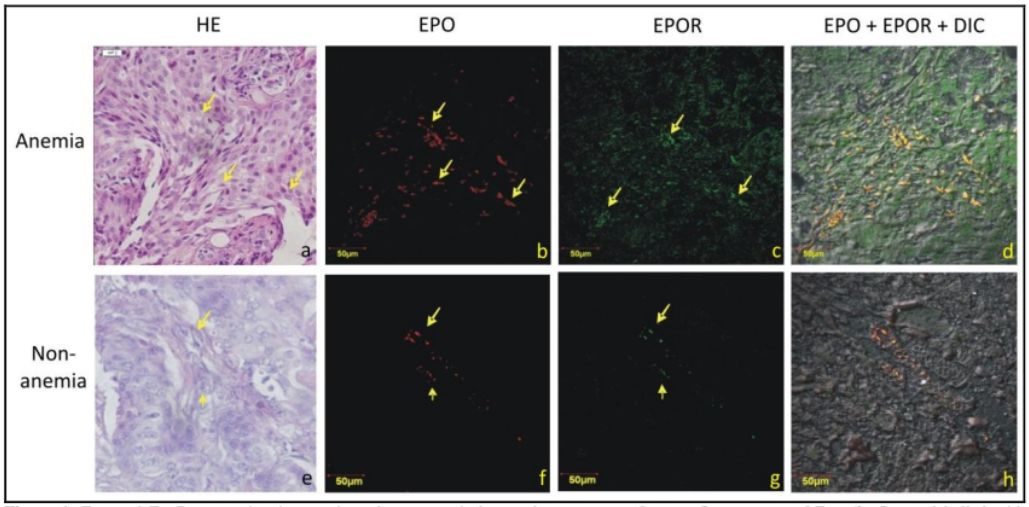


Figure 1. Epo and EpoR expression in anemia and non-anemia human breast cancer. Immunofluorescence of Epo **(b, f)** was labelled with rhodamine, so EPO expression is shown by red color; EpoR expression **(c, g)** was labelled with FITC, so EPOR showed by the green ones. Comparison between HE staining and imunofluorescence staining suggested that Epo and EpoR expression is limited just on cancer cells.

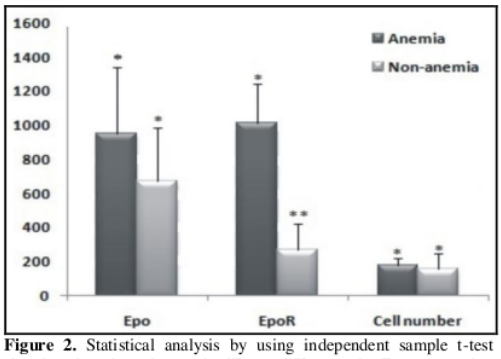
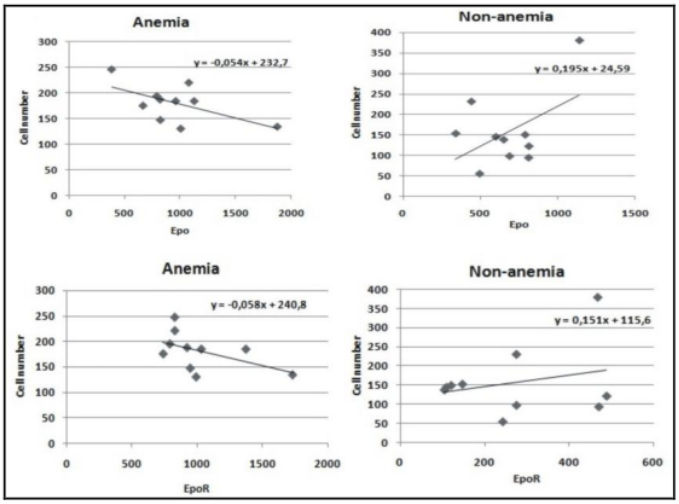


Figure 2. Statistical analysis by using independent sample t-test showing that there is no significant difference in Epo expression between anemia and non-anemia groups, but EpoR expression is significantly different. We also found that there is no significant difference for the number of cancer cells between anemia and non-anemia groups.

Table 1. Hematopoietic data from breat cancer patients (number of patients)

Parameter	Anemia	Non-anemia
Hb (g/dL)	8.76 ± 1.89 (10)	12.98 ± 0.79 (10)
Leukocyte (/µl)	8.51 ± 3.63 x 10^3 (10)	7.44 ± 2.11 x 10^3 (8)
Erythrocyte (/µl)	4.16 ± 0,57 x 10^6 (7)	4.76 ± 0.37 x 10^6 (8)
Thrombocyte (µl)	343.3 ± 168,25 x 10^3 (10)	314.75 ± 62.94 x 10^3 (8)
Hematocrit (%)	29.68 ± 4.9 (9)	38. 76 ± 3.96 (8)

Figure 3.
Regression and correlation analysis between Epo and EpoR expression and cancer cell proliferation (Epo and EpoR are set as independent and cancer cell proliferation is the dependent variable). A negative correlation was recorded in anemia group. The number of cancer cells decrease when Epo and EpoR expressions are high. Otherwise, in non-anemic group, a positive correlation was found, indicating that the number of cancer cell increase when Epo and EpoR expressions are high.



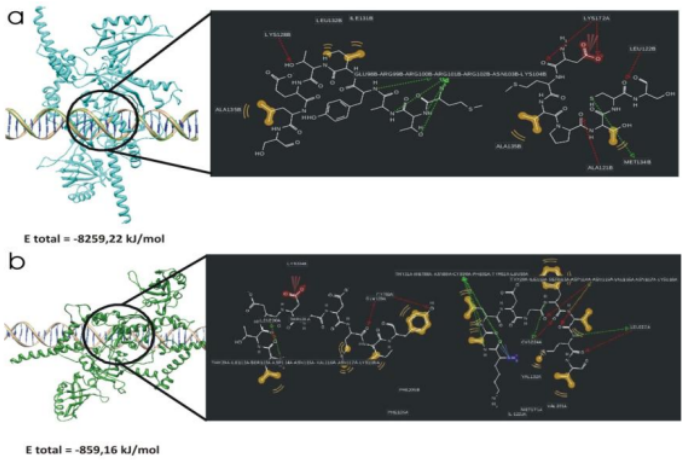


Figure 4.
Interaction of transcription factor HIF1 with Epo gene promoter.
There is a differentiation in the binding energy between unmethylated DNA **(a)** and methylated DNA **(b)**.
Binding energy in unmethylated DNA was found to be greater than in methylated DNA.

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DISCUSSION

In the present study, the high expression of Epo and EpoR in anemic human breast cancer suggested that the condition of the microenvironment of the tumor in anemic group is more hypoxic than in non-anemic group. In solid tumors, adaptive responses to hypoxia are correlated with angiogenesis, enhanced aggressiveness, reduced apoptosis, and poor responses to therapy. The relationship between anemia and hypoxia is complex and influenced by multiple variables. The analyses of Epo and EpoR expression results in anemic and non-anemic groups have different patterns. In anemic group, EpoR expression is higher than Epo, however, in non-anemic group, EpoR expression is lower than Epo. This result was supported by several previous studies in non-hematopoietic tissues, such as non-small cell lung carcinomas [18], human prostate cancer [19], head and neck cancer [20, 21], and colorectal cancer [22], which showed the result that Epo and EpoR expressions have different patterns. Because autonomous Epo expression can mediate autocrine growth of EpoR, expression of Epo and EpoR by tumors of non-hematopoietic tissues may also stimulate cancer [23].

Anemia may cause a change in the control mechanisms of Epo and EpoR expression. A study by Dunst *et al* [24] showed that there was no relationship between Hb levels and angiogenesis; however, a strong correlation between decreased Hb levels and increased angiogenesis was observed in chronic-hypoxic tumors with a low pO_2. Those results support that anemia impacts on tumor hypoxia, and hypoxia causes a mechanism control change of several gene targets involved in angiogenesis. Thus, it can be suggested that all samples of the anemic group, i.e. samples which experienced hypoxic condition, caused a different control mechanism on Epo and EpoR expression.

The decreased number of cancer cells in anemia group when Epo and EpoR expression is high, suggested that therapy using ESA may not cause an increase of cancer cell progression, but in non-anemic breast cancer, ESA therapy may lead to increased progressivity of the cancer cells. The latter was characterized by the higher number of cancer cells with high expression of Epo and EpoR. Hopefully, the results of this study support the research conducted by the National Comprehensive Cancer Network (NCCN) in 2011 reporting a decrease of survival in cancer patients with Hb levels higher than 12 g/dl with ESA therapy, whereas when ESA was provided for cancer patient with Hb level less than 12 g/dl, it will not cause a decline in survival [25].

The five prime untranslated region (5'-UTR) of the Epo gene has a region of high CpG (–cytosine–phosphate–guanine–) density that is methylated in non-hypoxic, but unmethylated in hypoxic conditions. We have presented evidence that methylation of the promoter and 5'-UTR of the human Epo gene contributes to regulation of Epo gene by using HIF-1 as transcriptional factor for the transcription process of itself. Another study by Yin and Blanchard [11] showed that silencing mechanism on Epo gene promoter occurs by two different mechanisms: *(1)* high density methylation of the 5' UTR recruits a methyl-CpG binding protein to the promoter, and *(2)* methylation of CpGs at the proximal promoter blocks the association of nuclear protein. Those previous findings support the present study; methylation on Epo promoter gene blocks the association of nuclear protein to the promoter by high energy which is required for the binding process.

In summary, our findings support the hypothesis that anemia and tumor hypoxia are closely linked to each other although the underlying mechanisms are not yet fully explained. Hypoxia led by anemia seems to cause a different control mechanism of Epo and EpoR expression and cancer cell proliferation.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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