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Methylation impact analysis of erythropoietin (EPO) Gene to hypoxia inducible factor-1α (HIF-1α) activity

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Abstract:
Erythropoietin (EPO) is a glycoprotein hormone that play a role as key regulator in the production of red blood cells. The promoter region of *EPO* is methylated in normoxic (non-hypoxia) condition, but not in hypoxic condition. Methylation of the *EPO* enhancer region decline the transcription activity of EPO gene. The aim of this study is to investigate how different methylation percentage affected on the regulation and transcriptional activity of EPO gene. The DNA sequence of erythropoietin gene and protein sequence was retrieved from the sequence database of NCBI. DNA structure was constructed using 3D-DART web server and modeling structure of HIF1 predicted using SWISS-MODEL web server. Methylated DNA sequence of EPO gene using performed with YASARA View software and docking of EPO gene and transcription factor HIF1 analyzed by using HADDOCK webserver. Our result showed that binding energy in 46% methylated DNA was higher (-161,45 kcal/mol) than in unmethylated DNA (-194,16 kcal/mol) and 8% methylated DNA (-175,94 kcal/mol). So, we presume that a silencing mechanism of the Epo gene by methylation is correlated with the binding energy, which is required for interaction. A higher methylation percentage correlates with a higher binding energy which can cause an unstable interaction between DNA and transcription factor. In conclution, methylation of promoter and enhancer region of Epo gene leads to silencing.

Keywords: EPO, HIF-1, methylation, promoter, transcription.

Background:
DNA methylation is an important component of the epigenetic regulation of gene expression in eukaryotic cells. Methylation of CpG dinucleotides within transcriptional regulatory elements often results in reduced expression or silencing of genes **[1].** CpG islands defined as genomic regions of more than 200 bases with a GC content of at least 50%, typically occurs at or near the transcription start site (TSS) **[2].** CpG islands methylation is positively associated with the interference of gene transcription by blocking transciption factor binding or by bringing about chromatin alterations **[3].**

Erythropoietin (EPO) is a 34-kDa glycoprotein hormone that is mainly produced in the fetal liver and adult kidney. Expressions of EPO and its receptor (EPOR) have been described in various cancers under hypoxic condition **[4].** Recent data suggest that EPO and EPOR are involved in several growth-stimulating pathways including proliferation, anti-apoptotic, angiogenesis and invasion of cancer cells **[5].**

EPO gene expression is under the control of hypoxia-inducible factors-1 (HIF-1) and hepatocyte nuclear factor transcription factors **[6].** Induction of *EPO* occurs under hypoxic conditions, and activated through binding of the hypoxia-induced factor 1 α (HIF1α) and aryl hydrocarbon receptor nuclear translocator (ARNT; also termed HIF1β) to the enhancer region of EPO gene **[7].** The binding site of HIF1 has been reported to be in the enhancer region (sequence TACGTGCT) located at the 3' UTR region of the *EPO* gene **[8].**

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A CpG near the regulatory region of EPO gene is hemimethylated in normoxic, but not in hypoxic **[9].** In normal mammary cells and breast cancer cell lines (not under hypoxic condition), methylation of regulatory elements of EPO gene is 8% and 46% respectively **[10].** The aim of our study was to investigate the effect of different methylation percentage of EPO gene regulatory elements to HIF1 activity as a transcriptional factor of EPO gene.

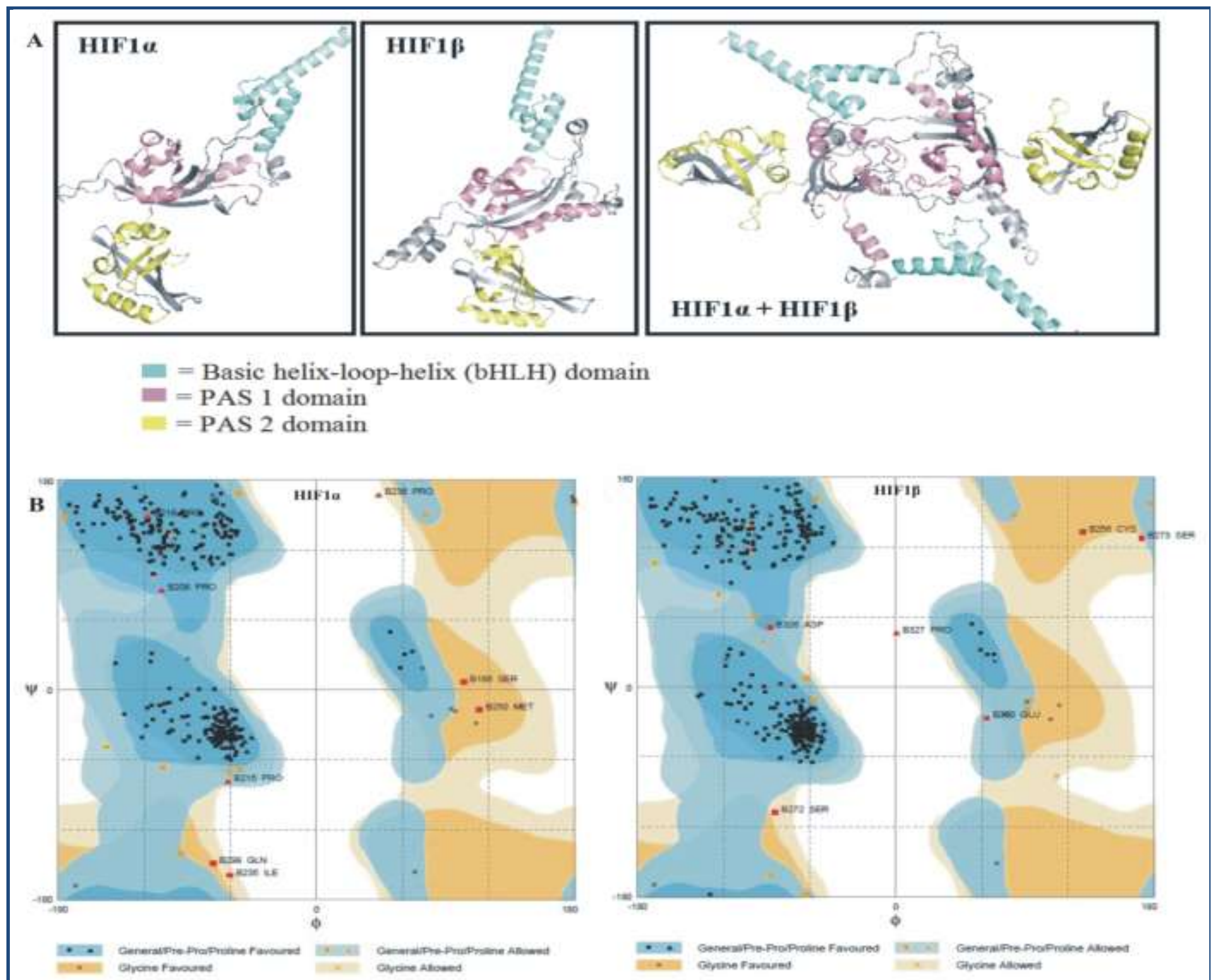

Figure 1: 3-dimensional (3D) model of HIF1α and HIF1β **(A)** by using SWISS MODEL homology modelling, and the result of 3D structure validation using Ramachandran plot analysis **(B).**

Methodology:

Nucleotide sequence and protein structure retrieval

The DNA sequence of Epo gene is GI:298358534, and the protein sequences of HIF-1α and HIF-1β are GI:2498017 and GI:114163, 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) **[11].**

This study has gained ethical clearance from research ethics committee, Faculty of Medicine, Brawijaya University, as a member of ethics committee in Indonesia.

CpG island prediction

The EPO gene sequence was scanned for the distribution of CpG islands using CpG Island Searcher with the parameters set as lower limits: percentage of G and C bases (%GC) = 55%, ratio of observed to statistically expected CpG frequencies (ObsCpG/ExpCpG) = 0.65, frequency of bases in the island = 200 bp and gap between adjacent islands = 100 bp.

Protein and DNA structure modeling

Modeling structure of HIF1α and HIF-1β predicted using SWISS-MODEL web server **[12]** by homology modelling method. DNA structure was constructed using 3DNA-Driven DNA Analysis and Rebuilding Tool (3D-DART) web server **[13].** The enhancer region of EPO gene (5'-3' sequence) was given as input. The 'fiber' module initially developed a DNA structure and a corresponding base pair parameter file was generated using 'find_pair' and 'analyze' modules. The parameter file was then subsequently utilized to introduce 'local' and 'global' bends in the DNA structure with default

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settings of roll, tilt and twist. Finally, the DNA structure file in PDB format was returned with the help of 'rebuild' module of 3DNA.

Structure manipulation and energy minimization
Methylation involves the addition of CH_3 group in cytosines of the EPO gene regulatory elements. Percent of methylation is 0% (cells under hypoxic condition), 8% (normal cells), and 46% (cancer cells under non-hypoxic condition). Yet Another Scientific Artificial Reality Aplication (YASARA) View was extensively used to perform this operation. 'Build' utility was employed to introduce methyl group and subsequently the bond orders were corrected using 'Adjust bond order' utility. The resultant structure files were then energy minimized using YASARA Energy Minimization server.

Computational docking
The docking simulation of EPO gene promoter with transcriptional factor HIF1 performed using High Ambiguity Driven biomolecular DOCKing (HADDOCK) **[14]** engine and HEX software **[15]**. Docking protocol consists of three stages visualization. A rigid-body energy minimization, a semi-flexible refinement in torsional angle space and a finishing refinement in explicit solvent. After execution of each of these stages, the docked conformations are scored and ranked by the scoring function to facilitate the selection of the best conformations and subsequently employed in the next stage. The best docked conformers can be recovered by inspection of HADDOCK score which takes into account the weighted sum of van der Waals, electrostatic, desolvation and restraint violation energies together with buried surface area.

Interaction of HIF1 and EPO gene analysis
The result of computational docking then visualized using CHIMERA software **[16]** and analyzed using NUCPLOT **[17].** The input to NUCPLOT is a file in PDB format. The program identifies which atoms belong to the protein and other ligands and which to the nucleic acid. Protein residues and water molecules interacting with DNA atoms are then identified from a list of hydrogen bonds, van der Waals contacts and covalent bonds that generated automatically by the HBPLUS program which calculates hydrogen bonds and van der Waals contacts for a given PDB file.

Results & Discussion:

Protein structure analysis
Modelling protein and DNA structure have an important role to study interaction between HIF1 and EPO gene enhancer. Protein 3-dimensional structure **(Figure 1a)** were generated using SWISS-MODEL server. From several predicted structure for HIF1α and HIF1β, the best model was selected after Ramachandran plot analysis. The best model was picked based on highest percentages of residues in most favoured regions and lowest percentages scores in outlier region. The stereochemical quality of predicted HIF1α and HIF1β structure were analyzed through residue-by-residue geometry and overall geometry of protein structures using the RAMPAGE server **[18].** Ramachandran plots were drawn for these protein structure. In ramachandran plots **(Figure 1b),** the most favoured regions are indicated by dark blue patches, while bright blue areas show allowed regions.

It was observed that HIF1α has 94.2% number of residues in favoured region, 3.4% residues in allowed region, and 2.5% residues in outlier region. In the case of HIF1β, plot analysis revealed 93.9% residues in favoured region, 4.5% residues in allowed region, and 1.6% residues in outlier region. Protein 3-dimensional structures are fundamental as the biological activity of a protein is accomplished by it's 3-dimensional structure.

Docking analysis
The result of docking analysis including HADDOCK score, RMSD, binding energy, van der Waals energy, electrostatic energy, desolvation energy and restraints violation energy showed in **Table 1 (see supplementary material).** Our result showed that binding energy in 46% methylated DNA was higher (-161,45 kcal/mol) than in unmethylated DNA (-194,16 kcal/mol) and 8% methylated DNA (-175,94 kcal/mol). Suggested that higher binding energy required for interaction caused HIF1 more difficult to bind to EPO gene enhancer, even if HIF1 can bind to EPO enhancer, the interaction might be not stable. In contrast, the lower energy required for the interaction between HIF1 and unmethylated EPO gene enhancer made the interaction become strong and stable, suggested it's lead HIF1 binds strongly to EPO gene enhancer and stimulated transcription of EPO gene This study conducted opinion that methylation has an important role in the regulation of EPO gene trascription. Irvine *et al* **[19]** confirmed that the methylation of DNA had a local effect on transcription. They also showed that acetylated histones were found to be associated with unmethylated DNA and were nearly absent from methylated DNA regions. These methylation effects were local and there is no preferential interaction if both the partners (histone tails and DNA) were methylated. Kumar *et al* **[20]** also found that DNA normally being a negatively charged biomolecule, if methylated, it additives the negativity of the DNA thereby eliminating the role of methylated histones tails to interact physically. So we presume that methylation isn't only interfere binding process between HIF1 to EPO gene enhancer, but also correlated with the interaction of histone tails to DNA.

The result of docking interaction analysis also showed that amino acid and nucleotide that directly involved in the interaction is different in each percentage of methylation **(Figure 2).** In the interaction between unmethylated EPO gene enhancer and HIF1, formed 3 hydrogen bonds between nucleotide and amino acids, that are THY6 → Ser184; ADE7 → Lys187; and CYT → Ser13, Ser16. Hydrogen bonds that formed between 8% methylated EPO gene and HIF1 is between CYT5 → Ser184; CYT11 → Lys41, Lys187, Thr195; GUA12 → Lys41; and GUA14 → Asp9. In the interaction between 46% methylated EPO gene and HIF1, it's formed 5 hydrogen bonds, that is GUA9 → Ser37, His38; GUA12 → His38; CYT14 → Lys41; ADE15 → Lys41; CYT16 → Ser196. The more hydrogen bonds that formed between amino acids and nucleotide, the binding energy required for the inetarction is higher (**Table 1).**

Epigenetic alterations of tumor suppressor genes contribute essentially to tumor development and tumor progression in cancers. Epigenetic gene silencing is mediated by aberrant methylation of CpG island promoters. Methylation provides 2 levels of control, both dependent on DNA/protein interactions.

Methylation of 3′ CpGs in the proximal promoter blocks the binding of essential *trans*-acting proteins, thereby indirectly repressing transcription. High-density CpG methylation in the 5′-UTR permits the binding of a methyl-CpG binding protein that either directly represses transcription or recruits corepressors, histone deacetylases, or both **[9].**

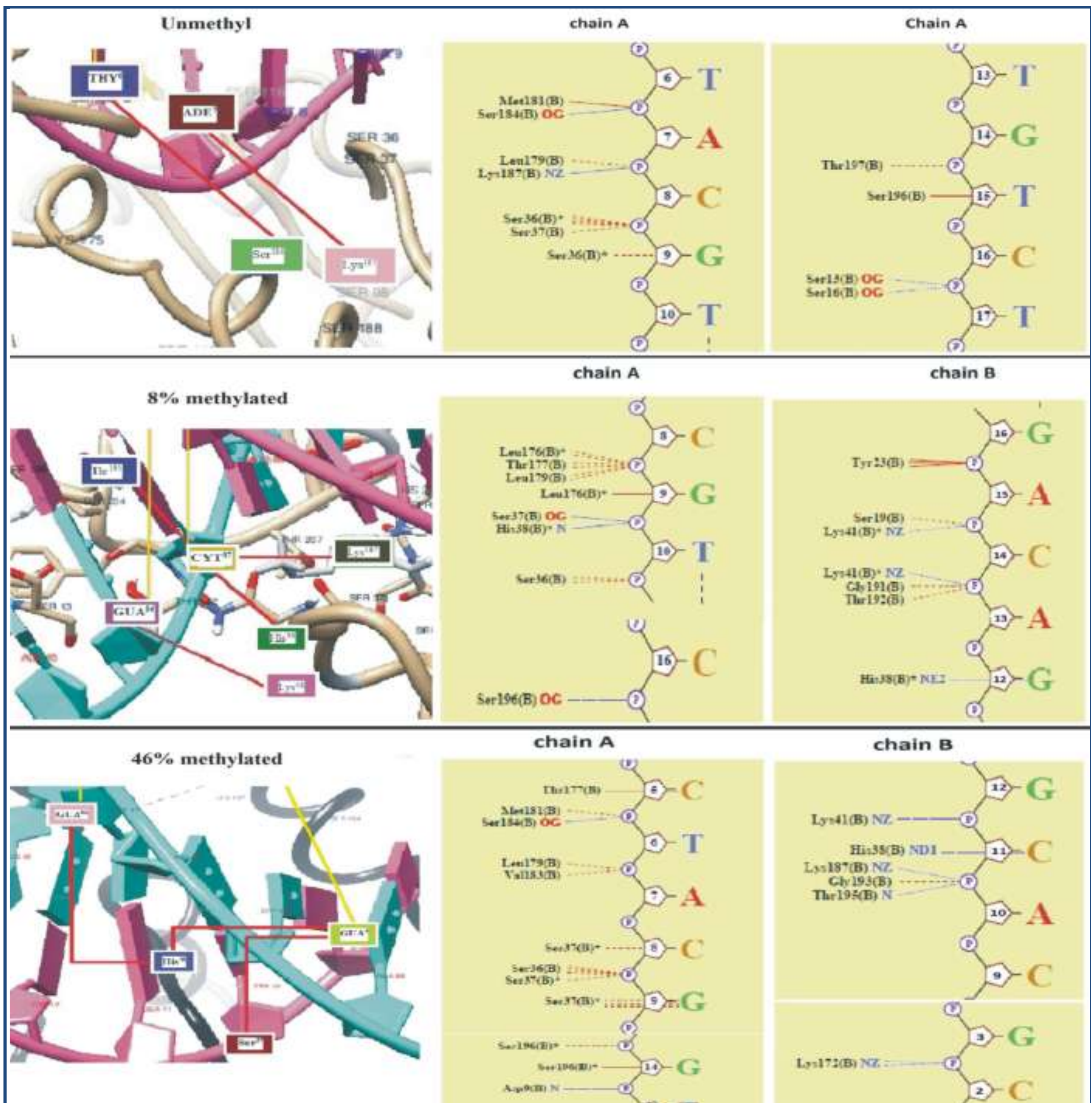

Figure 2 Interaction between EPO gene enhancer and hypoxia inducible factor-1 (HIF-1). The number of hydrogen bonds that formed between EPO gene enhacer and HIF-1 are different in each percentage of methylation (0%, 8%, 46%; 3 hydrogen bonds, 4 hydrogen bonds, 5 hydrogen bonds, respectively).

Conclusion:
In summary, we presume that a silencing mechanism of the Epo gene by methylation is correlated with the binding energy, which is required for interaction. A higher methylation percentage correlates with a higher binding energy which can cause an unstable interaction between DNA and transcription factor. In conclution, methylation of promoter and enhancer region of Epo gene leads to silencing.

Conflict of interest statement:
The authors declare that there is no conflict of interest.

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Supplementary material:

Table 1: Results of docking analysis between HIF1 and EPO gene enhancer

Parameter	Unmethylated	8% methylated	46% methylated
HADDOCK score	-57.8 +/- 7.4	-61.0 +/- 7.1	-55.1 +/- 7.7
RMSD (Å)	8.5 +/- 0.1	4.2 +/- 0.5	4.2 +/- 0.2
Binding energy (kcal/mol)	-194,16	-175,94	**-161,45**
van der Waals energy (kcal/mol)	-51.8 +/- 3.2	-52.1 +/- 5.8	-53.9 +/- 12.8
Electrostatic energy (kJ/mol)	-375.8 +/- 52.4	-357.1 +/- 48.0	-339.1 +/- 24.3
Desolvation energy	46.3 +/- 11.0	45.3 +/- 9.8	42.9 +/- 4.4
Restraints violation energy	228.7 +/- 50.62	172.7 +/- 34.44	237 +/- 26.16

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