Effect of hypoxia on ALDH1A1 expression in MCF-7 human breast cancer cells and its correlation with Oct-4 pluripotency gene expression

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Abstract. High aldehyde dehydrogenase (ALDH) activity is a marker of breast cancer stem cells (BCSCs). Our previous studies have indicated that, under hypoxia, stemness markers, such as Oct-4, could be modulated according to the ALDH1A1 expression in BCSC ALDH⁺ cells, thereby increasing their pluripotency. This study aimed to analyze the correlation between ALDH1A1 expression and Oct-4 pluripotency gene expression in MCF-7 human breast cancer cells under hypoxia in comparison with that in BCSC ALDH⁺ cells. This study determined the mRNA levels of ALDH1A1, Oct-4, and HIF-1α in MCF-7 breast cancer cells under hypoxia using one-step qRT-PCR, followed by normalization using 18S rRNA. Oct-4 expression was also analyzed via its correlation with ALDH1A1 expression. ALDH1A1 was expressed at a significantly higher level in 6-h hypoxic cells than in the corresponding normoxic cells, albeit the expression level started declining with the incubation time. Oct-4 and HIF-1α mRNA were also expressed at significantly higher levels in 6- and 24-h hypoxic cells, followed by decline in a time-dependent manner. The expression level pattern of these three genes peaked at 6 h under hypoxia, followed by a progressive decline, thereby confirming that the cells went into a hypoxic state. Hypoxia may alter the stemness properties of MCF-7 cells by increasing ALDH1A1 expression along with Oct-4 pluripotency gene expression. This study can contribute to further research on the implications of ALDH1A1 and hypoxia in breast cancer therapy.

1. Introduction
Breast cancer is one of the most common types of cancers among women worldwide and the second leading cause of cancer-related mortalities. Its global incidence is rising and is predicted to continue rising
despite all current efforts to prevent it [1,2]. Recent studies have reported that cancer stem cells (CSCs) may be responsible for resistance to conventional therapies, which contribute to metastatic ability and cancer recurrence [3]. Accumulating experimental evidence revealed that CSCs may share several common properties with embryonic stem cells (ESCs), including properties like self-renewal and pluripotency [4]. Among some of the transcription factors involved, OCT4 (also called POU5F1) plays a pivotal role as the master regulator or gatekeeper of self-renewal and pluripotency [5].

In the past decade, much effort has been expended to define biomarkers that may enable identification of breast CSCs (BCSCs). BCSCs were originally based on cell-surface marker expression (CD24$^-$/lowCD44$^+$). More recently, the high activity of human aldehyde dehydrogenase (ALDH) has been proposed as a BCSC marker [6-8]. ALDH—a superfamily comprising 19 isoforms—plays an important role in the irreversible oxidation of acetaldehyde. ALDH1A1 belongs to the ALDH1A subfamily, which plays an important role in the retinoic signaling pathway. ALDH1A1 acts as a detoxifying enzyme and catalyzes the oxidation of retinal to retinoic acid, thus contributing to the characteristic of self-protection in stem cells. Moreover, it also influences stemness regulation in terms of cell proliferation and differentiation [7,9,10].

Accumulating experimental evidence has revealed that, clinically, tumor hypoxia is an independent prognostic factor for poor patient survival. Hypoxic tumor cells seem to be more aggressive, with reduced apoptosis [11,12]. Recent studies have reported that hypoxia can inhibit differentiation of ESCs and progenitor cells; moreover, hypoxic tumor cells seem to be poorly differentiated and express stem cell markers. These interesting observations have developed a new paradigm that tumor hypoxia may facilitate the emergence of malignant clones by maintaining CSCs in their undifferentiated stem cell state, which permits self-renewal and uninterrupted accumulation of genetic and epigenetic changes over a duration [13,15].

Globally, the most commonly used breast cancer cell line is MCF-7, which was established in 1973 at the Michigan Cancer Foundation [16]. Some studies have reported that, although a cell line may be clonally derived, they contain a cellular hierarchy that represents different stages of cellular differentiation. Experimental studies have shown that compared with high ALDH activity BCSCs, MCF-7 cells exhibit low ALDH activity [16,17]. In our previous study, we found that under hypoxia, stemness markers, such as Oct-4, could be modulated in accordance with the ALDH1A1 expression in BCSC ALDH$^+$ cells. Therefore, the present study aimed to analyze the expression of ALDH1A1 and to confirm its pluripotency in comparison with Oct-4 expression in MCF-7 cells under hypoxia.

2. Materials and Methods

2.1. Cell Culture

The human breast cancer cell line MCF-7 was obtained from the Cell Culture Laboratory for CSCs, Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. The obtained cells were cultured in non-serum high glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco®) supplemented with 1% penicillin–streptomycin, 1% gentamycin, and 1% amphotericin B under standard conditions (37°C, 20% O$_2$, 5% CO$_2$). The medium was changed every 2-3 days. Whenever the culture suspension reached 80%–90% confluency, the cells were subcultured into 2 cell groups: hypoxic and normoxic. The hypoxic cell group was incubated under hypoxic conditions (37°C, 5% O$_2$, 20% CO$_2$) for 6, 24, and 48 h, respectively, whereas the normoxic cell group was incubated under standard conditions (37°C, 20% O$_2$, 5% CO$_2$) for 6, 24, and 48 h, respectively.

2.2. Primer Design

Primers for ALDH1A1, ALDH1A3, and Oct-4 were designed and used in our previous study (unpublished data). Sequences of the said primers are presented in Table 1.
2.3. RNA Isolation and One-step qRT-PCR
Total RNA samples were extracted using the Tripure Isolation Kit (Roche®), according to the manufacturer’s instructions. Total RNA was then quantified using spectrophotometry at a 260-nm wavelength (Varioskan™ Flash Multi-mode Reader, Thermo Scientific). Quantitative real-time PCR was performed using the KAPA SYBR® FAST One-Step qRT-PCR Kit (Kapa Biosystems®) in the 7500 Fast Real-Time PCR (Applied Biosystems®), according to the manufacturer’s instruction. All sets of reactions were measured in triplicates. The measured gene expression was recorded in C_T, and the values were then normalized to the respective 18S rRNA gene expression as a reference. Furthermore, melting curve analysis was also performed for each sample to evaluate the authenticity of the products.

Table 1. Primers, amplicons, and annealing temperature of ALDH1A1, Oct-4, HIF-1α, and 18S genes

| Gene    | Forward Primer | Reverse Primer | Amplicon | Annealing Temperature |
|---------|----------------|----------------|----------|-----------------------|
| ALDH1A1 | 5’-TTGGAAGATAGGCCC TGCAC-3’ | 5’-GGAGGAAACCCTG CCTCTTTT-3’ | 117 bp  | 60°C                  |
| Oct-4   | 5’-GAGGGAGTGCC CAGGCCCTCTTCTCTCTCTT-3’ | 5’-AGCCTTACCCA CTCTCTCTCT-3’ | 459 bp  | 57°C                  |
| HIF-1α  | 5’-GGCGCAGAAC GACAAGAA AAAGGTTAA-3’ | 5’-AGTGGCAACTGATGA GGAA G-3’ | 122 bp  | 54.6°C                |
| 18S     | 5’-AAACGGCTAC CACACTTCCA AAAG-3’ | 5’-CCTCCAAATGG ATCTCTCHTTA -3’ | 155 bp  | 59°C                  |

2.4. Electrophoresis
To verify the PCR products, 2% agarose gel electrophoresis was performed, and the products were visualized using the GelRed Stain (Biotium®).
2.5. Statistical Analysis
Data are expressed as relative expression ± standard error. Statistical differences between the two groups were analyzed using the SPSS Software Version 20.0.0.0 and the Mann–Whitney U-test, whereas correlation was measured using Pearson’s test. P < 0.05 was considered statistically significant.

3. Results
3.1. ALDH1A1 mRNA expression level in hypoxic and normoxic MCF-7 cells
To investigate the expression of ALDH1A1, qRT-PCR was performed for hypoxic and normoxic cells. Figure 1 depicts that ALDH1A1 was expressed at significantly higher levels in 6-h hypoxic cells than in the respective normoxic cells. However, the gene expression declined to similar levels at 24 and 48 h for the hypoxic cells as compared with that for the respective normoxic cells.

3.2. Oct-4 mRNA expression level in hypoxic and normoxic MCF-7 cells
To identify differences between hypoxic and normoxic cells with regard to pluripotency, the Oct-4 expression was measured using RT-PCR. Figure 2 depicts significantly high expression of Oct-4 in the 6- and 24-h hypoxic cells.

3.3. HIF-1α mRNA expression level in hypoxic and normoxic MCF-7 cells
To confirm whether the cells were in hypoxic state, the HIF-1α expression was measured using RT-PCR. This study observed that HIF-1α was expressed at higher levels in 6- and 24-h hypoxic cells than in the respective normoxic cells, albeit at a lower level in 48-h hypoxic cells than in the respective normoxic cells (Figure 3).

3.4. Correlation between ALDH1A1 and Oct-4 mRNA expression level
The results from the statistical analyses between ALDH1A1 and Oct-4 mRNA expression levels in 6-, 24-, and 48-h hypoxic MCF-7 cells indicate a high positive correlation (Figure 4).
Figure 1. ALDH1A1 mRNA expression level in hypoxic and normoxic cells

The results of qRT-PCR, represented as $C_T$, were normalized using 18s rRNA. (A) The melting curve of ALDH1A1 shows a single peak at 77°C. (B) The relative expression of ALDH1A1 is significantly higher in 6-h hypoxic cells than in 6-h normoxic cells ($P < 0.05$). (C) Electrophoresis with a 100-bp DNA ladder showed that a 117 bp-long cDNA was synthesized and amplified using PCR, thereby confirming ALDH1A1 as the PCR product.

* $P < 0.05$. 
The mRNA of 6-, 24-, and 48-h hypoxic and normoxic cells was isolated for performing RT-PCR. The \( C_T \) values obtained were then normalized using 18s rRNA. (A) The melting curve of Oct-4 samples showed a single peak at 83°C. (B) Relative expression of Oct-4 was higher in 6- and 24-h hypoxic cells.
than in the respective normoxic cells (P < 0.05). (C) Electrophoresis with a 100-bp DNA ladder and a 459 bp-long cDNA was synthesized and amplified using PCR to confirm Oct-4 as the PCR product.

Figure 3. HIF-1α gene expression in hypoxic and normoxic cells

The mRNA of 6-, 24-, and 48-hypoxic and normoxic cells was isolated and subjected to RT-PCR. The Ct values obtained were then normalized using 18s rRNA. (A) The melting curve of HIF-1α samples showed a single peak at 79.5°C. (B) The relative expression level of HIF-1α higher in 6- and 24-h hypoxic cells than in the respective normoxic cells (P < 0.05), followed by a decrease at 48 h of treatment. (C) Electrophoresis using a 100-bp DNA ladder indicated a 122 bp-long cDNA, thereby confirming HIF-1α as the PCR product.
Figure 4. Analysis of the correlation between ALDH1A1 and Oct-4 mRNA expression levels in hypoxic MCF-7 cells

The expression levels of ALDH1A1 in comparison with those of Oct-4 under 6, 24, and 48 h of hypoxia, as calculated statistically using the Pearson’s correlation test. The result indicated a high positive correlation between these genes ($R^2 = 0.843; P = 0.017$).

4. Discussion
Hypoxic tumor cells seem to be more aggressive than normoxic cells, with reduced apoptosis [11,12]. Recent studies have reported that hypoxia can inhibit the differentiation of ESCs and progenitor cells and that hypoxic tumor cells tend to be poorly differentiated and express stem cell markers [13,15]. Moreover, hypoxia exhibits the potential to exert significant effects on the maintenance and evolution of CSCs [11]. Several studies have reported that Oct-4 pluripotent genes are higher expressed under hypoxia in BCSCs [11,18,19]. Our previous study (data not presented) also confirmed that Oct-4 pluripotent genes could be modulated in accordance with the ALDH1A1 expression in BCSC ALDH$^+$ cells under hypoxia. Nevertheless, only little is understood about the effect of hypoxia on ALDH1 expression in non-BCSCs.

The present study recorded higher expression of ALDH1A1 in 6-h hypoxic MCF-7 than in the respective normoxic cells; however, the expression level progressively decreased with time. The HIF-1α expression level also confirmed this finding, indicating that the cells had undergone hypoxia. This result indicates that the ALDH activity in MCF-7 cells could be modulated under hypoxia. These effects may be attributed to the role of ALDH in regulating cell proliferation and self-protection through the hypoxia-inducible factor (HIF) pathway even in cancer cells with low ALDH activity, such as MCF-7, similar to that in high ALDH BCSCs [13,20].

In accordance with ALDH1A1, the Oct-4 pluripotent gene was also affected. Our findings revealed significantly higher expression of Oct-4 in 6- and 24-h hypoxic MCF-7 cells than in the respective normoxic cells. However, the trendline started to decrease after 24 h of hypoxia. Interestingly, our study revealed a high positive correlation between the expression levels of ALDH1A1 and Oct-4 pluripotency genes under hypoxia, similar to that observed in BCSCs ALDH$^+$.

Our cumulative findings lead to the inference that hypoxia may enhance the stemness property of MCF-7 human breast cancer cells by upregulating the ALDH1A1 activity. Further studies are required to confirm the specific pathways altered by these genes as well as their interaction with each other to understand the significance of their role in the prognosis and treatment of breast cancer.
Thus, we propose that the high expression of ALDH1A1 in human breast cancer cells MCF-7 under hypoxia may enhance the expression of the Oct-4 pluripotency gene, as noted in BCSC ALDH+ in this study.

5. Conclusion
Based on the findings, this study suggest that hypoxia possibly alters the stemness properties of MCF-7 cells by increasing the expression of ALDH1A1 along with the expression of the Oct-4 pluripotency gene, thus implying that our data may be associated with the MCF-7 properties of the ALDH activity. Our study can help contribute to further research on the implications of ALDH1A1 and hypoxia in breast cancer therapy.

Conflicts of Interest
The authors declare no conflicts of interest.

Acknowledgement
This study was funded by the Hibah PITTA 2017, Universitas Indonesia.

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