Relative expression of aldehyde dehydrogenase 1 family member A1 in different malignancies of human glioma cells

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Abstract. Aldehyde dehydrogenase 1A1 (ALDH1A1) is a cancer stem cell (CSC) marker that plays a role in the differentiation and metastasis of human glioma cells. In this study, we aimed to analyze the expression of ALDH1A1 in different grades of malignancy of human glioma cells. Samples were collected from 32 patients at Cipto Mangunkusumo Hospital, resulting in 19 low-grade glioma (LGG) specimens, 11 high-grade glioma (HGG) specimens, and 2 normal brain specimens. Isolation of RNA was performed prior to the measurement of the relative quantification of ALDH1A1 by using the quantitative reverse transcription polymerase chain reaction (RT-qPCR). There was a trend of higher overexpression of ALDH1A1 in HGG compared to LGG in humans. However, this finding did not reach the level of statistical significance. ALDH1A1 may be a potential marker for malignancy classification. Therefore, further research is needed.

1. Introduction
Gliomas are the most common group of primary brain tumors among adults, contributing to 80% of all malignant primary brain tumors and 30% of all brain and central nervous system (CNS) tumors [1,2]. The World Health Organization classified glioma histologically into Grade I, Grade II, Grade III, and Grade IV, in which Grades I and II are referred to as low-grade glioma (LGG) while Grades III and IV are referred to as high-grade glioma (HGG) [2]. The survival rate of patients with glioma varies with malignancy, with higher five-year survival in non-malignant brain tumors compared to malignant brain tumors. Among malignant brain tumors, there is a decreasing trend of the survival rate when the diagnosis is made at an advanced age [3].

Conventional treatments for glioma, which include surgery, radiotherapy, and chemotherapy, do not give satisfactory results [4]. Although resection improves survival rate and relieves symptoms in various glioma malignancies [5,6], tumor progression occurs in LGGs and HGGs at rates of 29% and 36%, respectively. Furthermore, malignant transformation of LGGs was found in 20% of the cases [7]. Radiation therapy can benefit both levels of glioma malignancy [6], however, different outcomes arise from gliomas with the same classification. Eyre et al. found that the response rate of LGGs to
radiotherapy was 79% [8] whereas another study reported a response rate of 46% [9]. Moreover, chemotherapy may lead to chemoresistance in gliomas [10].

The ability to proliferate and create new tumors differs in cancer cells in the CNS. Most cancer cells have a limited ability to proliferate; however, the presence of cancer stem cells (CSCs) in organs allows cancer cells to have self-renewal, differentiation, and tumor-initiating functions [11,12]. The formation of new tumors can be detected through the expression of transcription factors [11]. One such example of a CSC marker in adult cancers is aldehyde dehydrogenase 1A1 (ALDH1A1) [12]. ALDH1A1 is an enzyme that metabolizes aldehydes to form carboxylic acids [13]. In cancer, ALDH1A1 depicts cancer stemness of various tumors, and its level correlates with patients’ outcomes [14]. This enzyme is located in human hematopoietic stem cells and CSCs of glioma, esophageal squamous cell carcinoma, colorectal cancer, ovarian cancer, lung adenocarcinoma, prostate cancer, and pancreatic cancer cells [13-20]. Unfortunately, there is little evidence of the role of ALDH1A1 in gliomas.

Thus, in this study, we aimed to provide evidence of the role of ALDH1A1 in glioma among Indonesian patients and to analyze the correlation of the relative expression of ALDH1A1 with different malignancies of human glioma cells. As tumors under the same classification respond differently toward radiotherapy, this study supports a molecular approach for treating patients with gliomas.

2. Methods
This cross-sectional study was performed at the Laboratory for Molecular Biology for Oxidative Stress Studies, Department of Biochemistry & Molecular Biology, Faculty of Medicine, Universitas Indonesia. The inclusion criteria were patients diagnosed on the basis of radiological examination with glioma, who underwent tumor dissection and signed informed consent. Confirmation of the degree of malignancy was conducted histologically according to the WHO grading using hematoxylin and eosin staining. Patients were excluded if none of the brain cells demonstrated glioma Grades I–IV based on the WHO classification. All patients provided written informed consent and the protocols were approved by the ethical committee of the Faculty of Medicine, Universitas Indonesia.

Samples consisted of 19 LGG tissue specimens, 11 HGG tissue specimens, and 2 normal brain tissue specimens collected from patients admitted to Cipto Mangunkusumo Hospital for brain surgery. Normal glioma samples from a deeper tumor location were obtained from patients with glioma during normal brain surgery in a small amount. As soon as the glioma samples were collected, they were homogenized using a Polytron Homogenizer and stored at −80°C until use to prevent RNA degradation.

RNAs were isolated from glioma cells using the Geneaid kit according to the manufacturer’s instructions. Reverse transcription of mRNA and amplification of cDNA were performed using the KAPA SYBR® FAST One-Step qRT-PCR Master Mix (2x) kit manufactured by KAPA Biosystems. Quantification of the expression of ALDH1A1 mRNA was performed using a PCRmax Eco 48 Real-Time PCR System (United Kingdom). The protocol comprised reverse transcription at 42 °C for 5 min and enzyme activation at 95 °C for 5 min, 40 PCR cycles with denaturation at 95 °C for 10 s, annealing at 60 °C for 40 s, and extension at 72 °C for 30 s, followed by analysis of melting curves at 95 °C for 15 s, at 55 °C for 15 s, and at 95 °C for 15 s, and finally incubation at 50 °C for 2 min.

In the amplification of ALDH1A1, the following primers were used: TGTTAGCTGATGCCGACTTG (forward) and TTCTTAGCCCGCTCAACACT (reverse) [20]. The housekeeping gene in this experiment was 18S rRNA, which used primers of AAACGGCTACCACATCCAAG (forward) and GGCCTCAGTAAACCAGCATCA (reverse) [21]. The suitability and specificity of the primers were evaluated by analyzing one-peak presentation in the melting curve. This experiment also used Aqua Bidest as a negative control to prevent false-positive results. All experiments were performed in triplicate.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) provided a ΔCT value, which contributed to the Livak methods (2−ΔΔCT) [22] to measure the relative expression of ALDH1A1.
mRNA in glioma cells. The relative expression of ALDH1A1 mRNA in all samples was normalized to 18S rRNA and was presented as a ratio with respect to the ALDH1A1 expression in normal brain cells. Analysis of mRNA expression data was performed using IBM® SPSS Statistics® statistical software (standard version 23.0). Unpaired t-tests were applied to assess the correlation between ALDH1A1 mRNA expression and different malignancies of glioma if the data was normally distributed. For data that were not normally distributed, the Mann–Whitney test was used. Statistical significance was set at \( p < 0.05 \).

3. Results
Thirty glioma samples were collected from patients undergoing surgery at Cipto Mangunkusumo Hospital, which comprised 19 LGG samples and 11 HGG samples. Patient characteristics are summarized in Table 1.

| Characteristic                          | Total | Percentage (%) |
|----------------------------------------|-------|----------------|
| Age                                    |       |                |
| \( \leq 50 \) years old               | 24    | 80.00          |
| >50 years old                         | 6     | 20.00          |
| Degree of glioma malignancy           |       |                |
| Grade I                                | 1     | 3.33           |
| Grade II                               | 18    | 60.00          |
| Grade III                              | 6     | 20.00          |
| Grade IV                               | 5     | 16.67          |
| Glioma pathological anatomy           |       |                |
| Oligodendroglioma                      | 1     | 3.33           |
| Astrocytoma                            | 20    | 66.67          |
| GBM                                    | 4     | 13.33          |
| Mixed glioma                           | 5     | 16.67          |

Hematoxylin and eosin staining confirmed the histological features of the glioma samples. Figure 1A depicts oligodendroglioma Grade II (OII), which falls into the LGG classification. Oligodendroglioma Grade II is characterized by moderate cellularity, isomorphic round to oval nuclei, and branching vessels, as shown in Figure 1A. The characteristics of oligodendroglioma Grade II also include a clear perinuclear halo, branching vessels, and calcification. By contrast, Figure 1B presents an HGG sample, specifically an anaplastic astrocytoma Grade III with increased cellularity, nuclear pleomorphism, and nuclear atypia. Mitotic activity was not clearly seen.

![Figure 1. Histopathological appearance of LGG (A) and HGG (B) confirmed by hematoxylin and eosin staining.](image)
The mean relative expression of ALDH1A1 was 0.8950 (SE = 0.22200) in LGG and 2.0523 (SE = 0.85868) in HGG (Fig. 2). The 95% confidence interval of the mean for LGG was 0.4286–1.3614, whereas for HGG it ranged from 0.1390 to 3.9655. The median relative expression of ALDH1A1 in LGG and HGG was 0.4265 and 0.8703, respectively. Although the expression of ALDH1A1 mRNA was higher in HGG than in LGG, an unpaired t-test indicated that the difference was not statistically significant ($p = 0.977$).

**Figure 2.** (A) ALDH1A1 expression in different degrees of glioma malignancy. (B) Simple boxplot of the relative expression of ALDH1A1 in LGGs and HGGs. Note the outliers of ALDH1A1 expression in HGG. *Anaplastic astrocytoma Grade III. Oligoastrocytoma Grade III.
Of the LGG specimens, the relative expression of ALDH1A1 mRNA was increased in 31.6% of the cases. The same relative expression of ALDH1A1 mRNA was seen among 5.3% of the LGG samples. In addition, 63.1% of the LGG samples did not show any improvements in their expressions.

Interestingly, increases in the relative expression of ALDH1A1 were found in 45.5% of the HGG samples. Increasing ALDH1A1 mRNA expression varied among the HGG samples. ALDH1A1 mRNA expression increased 1.2–3.5-fold, 3.6–5.6-fold, and 5.6–9.4-fold from normal values of 27.3%, 9.1%, and 9.1%, respectively. However, 54.5% of the HGG samples did not show an increased ALDH1A1 mRNA expression. Table 2 summarizes the relative expression of ALDH1A1 mRNA in LGG and HGG samples.

Table 2. Expression (%) of ALDH1A1 relative to the ALDH1A1 expression in normal human brains.

| Percentage sample with relative expression of ALDH1A1 | Low | Same | High |
|------------------------------------------------------|-----|------|------|
| Degree of glioma malignancy                          |     |      |      |
| Low grade                                            | <0.9x | 0.9–1.1x | 1.2–3.5x | 3.6–5.6x | 5.6–9.4x |
| High grade                                           | 63.1 | 5.3  | 31.6 | - | - |

4. Discussion

The presence of CSCs may contribute to self-renewal, differentiation, and tumor-initiating functions in cancer [11,12]. CSCs in glioblastoma account for tumor recurrence as well as resistance of glioma toward drugs and irradiation [24]. The response of glioma under the same histological classification toward radiation therapy can vary. Thus, a biomolecular approach is needed. Detecting CSCs through the use of a biomarker, such as ALDH1A1, may lead to a better knowledge about glioma progression and a better management strategy for patients with glioma.

Previously, Li et al. assessed proteomic profiles between glioblastoma multiforme (GBM) and normal brain tissues using four normal brain tissue samples [25]. Schwartz et al. in their research compared the expression of peptides and proteins in primary brain tumor and nontumor brain tissues using five normal brain tissue samples [26]. This contrasts with our study where we only used two normal brain tissue samples because of the difficulty in collection.

In our study, the mean relative expression of ALDH1A1 mRNA in HGGs was two times higher than that in normal brain cells and LGGs. Our results suggest that the higher the expression of ALDH1A1, the higher the degree of glioma malignancy, although the relationship was not significant statistically.

Interestingly, Xu et al. [13] also reported a significant overexpression of ALDH1A1 in HGGs compared to LGGs. The high level of ALDH1A1 mRNA expression suggested stemness of glioma. HGGs had a high level of ALDH1A1 that contributed to a high level of matrix metalloproteinase-2 (MMP-2), MMP-7, and MMP-9. MMPs play various roles in degrading extracellular matrix proteins to allow for tumor cell invasion [13]. Stemness due to a high expression of ALDH1A1 has also been reported in other types of cancer. Yang et al. [14] reported that a high expression of ALDH1A1 in esophageal squamous carcinoma was related to invasion and metastasis. In vitro experiments showed a high level of vimentin, indicating an epithelial-mesenchymal transition phenotype, and a high level of MMPs, which led to the degradation of extracellular matrix proteins to provide entry for cancer cell proliferation and invasion [14]. Overexpression of ALDH1A1 was predicted to mediate glioma resistance toward chemotherapy in HGG. In line with this, Schäfer et al. showed that glioblastoma expressing ALDH1A1 and O6-MG DNA methyltransferase (MGMT) were resistant to temozolomide (TMZ), a chemotherapeutic drug that causes DNA lesions and stops the cell cycle at the G2/M stage. Knocking down ALDH1A1 with shRNA in glioma cells treated by TMZ resensitized ALDH1A1 and MGMT+ GBM cells toward TMZ, decreased clonogenicity, and increased cytotoxicity of TMZ-resistant cells [27]. This study supports the relationship of high levels of ALDH1A1 with chemotherapy-resistant glioma cells.
The mechanism of TMZ resistance in glioma remains unknown. However, Olivia et al. in their research suggested that a significant increase in CcO and complex II/III activity may enhance electron transport chain efficiency, reduce proton and electron leakage in mitochondria, and reduce production of reactive oxygen species (ROS) in mitochondria [28]. In TMZ-sensitive glioma whose production of ROS is high, ROS oxidizes unsaturated lipids, generating bioactive molecules such as reactive aldehyde [29]. As the expression of ALDH1A1 was higher in TMZ-resistant glioma, this may support the role of ALDH1A1 in protecting cells against cytotoxic drugs through detoxification of reactive aldehyde to avoid DNA damage caused by TMZ.

Moreover, overexpression of ALDH1A1 in HGG was believed to promote radioresistance. Cojoc et al. in a recent study reported that the inhibition of WNT/β-catenin signaling in prostate cancer cells led to the inhibition of ALDH+ cells and radiosensitization [30].

Some LGG samples had a higher expression of ALDH1A1 than HGG, even though these values were still lower than the highest value of ALDH1A1 expression in HGG. This suggests the progression of LGG into HGG. Glioblastoma may arise from primary tumors, known as de novo glioblastoma, or from progression of primary tumors, which is termed secondary glioblastoma [1-31]. A study in Romania between 2006 and 2009 of 110 cases reported 10% transformation of LGG into malignant glioma [32]. Similarly, Sanai et al. reported transformation from LGG into malignant tumors in 20 out of 104 patients [7].

The role of ALDH1A1 in predicting patients’ outcomes is still controversial. Adam et al. found that a high expression of ALDH1A1 in tumor cells that coexpress GFAP correlated significantly with better survival of patients with glioblastoma [33]. In contrast, a high expression of ALDH1A1 is also reported to predict poor prognosis of patients with GBM [27].

Moreover, some HGGs had a lower rate of ALDH1A1 expression than LGGs; however, this was still higher than the lowest ALDH1A1 expression value in the LGG. This might indicate a good prognosis of these HGG samples for surgery and therapy. Alternatively, it is possible that normal brain tissue was taken along with the glioma brain samples. Among patients with GBM who underwent a median follow-up of 11 months following surgery and were not lost to follow-up, 4 out of 196 patients were alive with no disease [34]. The good prognostic group of patients with recurrent GBM (NIH Recurrent GBM Scale Score: 0) showed longer median survival of 10.8 months compared to the intermediate (NIH Recurrent GBM Scale Score: 1-2) and poor (NIH Recurrent GBM Scale Score: 3) prognostic groups with 4.5-month and 1.0-month median survival, respectively [35].

Further experiments are needed with more brain samples along with a cohort study of patients’ responses and survival rates following surgery and therapy to provide evidence of the role of ALDH1A1 in patients’ outcomes. In addition, tests of the role of ALDH1A1 in hypoxia conditions of glioma are needed.

5. Conclusion
Expression of ALDH1A1 in HGG tends to be higher than that in LGG, even though the difference was not statistically significant in our study. Our findings suggest a role of ALDH1A1 in cancer stemness, resistance toward chemotherapy, promotion of radioresistance, and cancer transformation.

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