mRNA expression of tumor growth factor-βeta1 in human glioma

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Abstract. Glioma describes a tumor that starts within the spinal cord or brain, and originates from glial cells; gliomas are the most commonly-found malignant brain tumors in Indonesia. Until now, there was no specific molecular biomarker used to distinguish gliomas based on tumor grade. TGF-β1 plays a key role in maintaining tissue homeostasis and cancer progression. Because of this, TGF-β1 has potential as a biomarker for differentiating between low- and high-grade gliomas. Samples were collected from patients with gliomas at the Cipto Mangunkusumo Hospital, Indonesia. The samples were categorized as low- (grades 1 and 2) or high- (grades 3 or 4) grade gliomas through histopathologic examination and based on the guidelines set forth by the World Health Organization. Relative mRNA expression of TGF-β1 was quantified through real-time RT-PCR with 18sRNA as the housekeeping gene. There were 27 glioma patient consisting of 17 low-grade glioma tissues and 10 high-grade glioma and 2 normal brain tissues as controls. There were 13 male and 14 female patients, with 63% <40 years old. There was decreased relative expression of TGF-β1 in high-grade, compared to low-grade gliomas. However, this difference was not statistically significant. The role of TGF-β1 as a definite biomarker was not proven in this study; therefore, more research should be conducted to elaborate the role of TGF-β1 as a biomarker.

1. Introduction
Glioma is the most common type of primary brain tumor throughout the world. In Indonesia its yearly incidence is 3 to 5 out of every 100,000 people [1,2]. Glioma is a common term that is used to describe a primary brain tumor which originates from the supportive cells of the neural network called the glia. These cells promote and maintain the neuron function [3]. Gliomas, like any other tumor, are categorized according to their malignancy, as set forth by the World Health Organization (WHO). We simplified this system by categorizing grades 1 and 2 as low-grade gliomas and grades 3 and 4 as high-grade gliomas. Currently, there are no molecular biomarkers for distinguishing patients with gliomas based on tumor grade. Therefore, research is currently exploring the possibility of hindering gene overexpression that can lead to malignancy, since the glioma progression is regulated by many genes [4].
Transforming growth factor-β (TGF-β) is a regulating cytokine which has many functions within the body, including tissue homeostasis and cancer regulation [5]. TGF-β binds and activates a series of membrane receptors, serine/threonine kinase, which will phosphorylate Smad protein. In clinical settings, Smads are signaling molecules which act as mediators for TGF-β and are believed to be poor prognostic markers for patients with glioblastoma. On the other hand, TGF-β also induces the expression of genes which regulate cell cycles such as plasminogen activator inhibitor and platelet derived growth factor [5]. The vast majority of human cells are responsive to TGF-β; therefore, TGF-β can prevent cells from becoming malignant tumors by regulating cellular adhesion, differentiation, proliferation, survival, and the cellular microenvironment. These actions can affect cancer during many stages of its development, including the premalignant stage, malignant progression, invasion and dissemination, and metastatic colonization. The preventive stage of TGF-β is expressed within the premalignant stage, where TGF-β enforces homeostasis and prevents tumor progression through its autonomous tumor-suppressive actions (apoptosis, differentiation, and cytostasis), or indirectly via the stroma (suppressing inflammation and stroma-derived mitogen) [6].

Some inconsistencies are seen in TGF-β expression throughout different stages and different types of tumors, indicating that increased TGF-β activity in advanced high-grade tumors is associated with poor prognosis in clinical settings [7]. Advanced, high-grade gliomas are able to evade and use TGF-β to their advantage. In such cases TGF-β can have an anti-apoptotic effect. This effect can be credited to two mechanisms exhibited by advanced high-grade tumors: the inactivation of the core component of the TGF-β signaling pathway or downstream alteration which inactivates the tumor-suppressive effects of TGF-β. The latter has a greater effect as it can affect all the downstream processes which involve TGF-β. TGF-β can inhibit apoptosis and can fail to inhibit cell growth. Another important factor that needs to be considered is the fact that TGF-β plays an important role in immune regulation. When the TGF-β pathway is altered, it can cause chronic inflammation, potentially leading to a pro-tumorigenic environment [7].

The aim of this study was to investigate the use of TGF-β as a biomarker for glioma progression, by correlating the relative expression of TGF-β in various glioma grades. We hoped to provide additional information regarding glioma and the possibility of using TGF-β as a molecular marker for treating patients who suffer from glioma, especially in Indonesia.

2. Methods
This cross-sectional study was conducted by members of the Faculty of Medicine at the University of Indonesia (FMUI), Molecular Laboratory for Oxidative Stress Studies, and Department of Biochemistry and Molecular Biology. The tissue samples were obtained from patients diagnosed with gliomas by radiology examination. The tissue samples were resected by a neurosurgeon at Cipto Mangunkusumo Hospital. The inclusion criteria for the sample obtained were based on anatomical pathology; the exclusion criteria included samples not identified through anatomical pathology as gliomas. The ethical committee of Universitas Indonesia approved all aspects of this study.

The samples were homogenized and stored in the deep freezer at −80 °C. Total RNA was isolated using Geneaid, according to the manufacturer’s instructions, and stored at −80 °C to prevent RNA degradation. RNA amplification was carried out using KAPA SYBR® FAST One-Step qRT-PCR Master Mix (KAPA Biosystem) Kit according to the manufacturer’s instructions, and the procedure was conducted using PCRmax Eco 48. This kit allows the amplification and the cDNA synthesis to be conducted in one tube. The reaction protocols were as follows: reverse transcription process at 42 °C for 5 min, enzyme activation at 95 °C for 3 m, 40 PCR cycles which consisted of 3 segments: at 95 °C for 10 s; 55 °C for 40 s; and 72 °C for 30 s. The melt curve analysis consisted of 3 segments: at 95 °C for 15 s; 55 °C for 15 s; and 95 °C for 15 s. Finally, the samples were incubated at 50 °C for 2 min.

The primers used for TGF-β1 gene were: 5’ GCCTTTTCCTGCTTCTATGG 3’ (Forward Primer) and 5’ CTCCGTTGAGCTGAAGCAATA 3’ (Reverse Primer). Here, 18s rRNA acted a housekeeping gene, and the primer for 18s rRNA were: 5’ AAACGGCTACCACATCCAAG 3’ (Forward Primer)
and 5' CCTCAATGGATCCTCGTTA 3' (Reverse Primer). The relative quantification of mRNA was based on the Livak method and was analyzed using an unpaired Mann Whitney test. \( P \) values <0.05 were regarded as significant [8].

3. Results
There were 27 patients diagnosed with glioma, consisting of 13 males and 14 females from Rumah Sakit Cipto Mangunkusumo in Jakarta (Table 1). The cutoff age was 40 years old based on the long-term prognosis of patients with gliomas [9]. From the histopathologic examination, we analyzed all the gliomas samples based on the WHO criteria. An example of a low-grade glioma was oligoastrocytoma grade II, and glioblastoma multiforme was considered a high-grade glioma grade IV (Figure 1).

| Table 1. Statistical data of the 27 glioma samples including sex, age, WHO grade, and glioma cell type. |
|--------------------------------------------------|----------|------------------|
| Characteristic                                  | Total    | Percentage (%)   |
| Sex                                             |          |                  |
| Male                                            | 13       | 48.1             |
| Female                                          | 14       | 51.9             |
| Age                                             |          |                  |
| 40                                              | 17       | 63.0             |
| >40                                             | 10       | 37.0             |
| WHO grading                                     |          |                  |
| Low-grade                                       |          |                  |
| Grade I                                         | 3        | 11.1             |
| Grade II                                        | 14       | 51.9             |
| High-grade                                      |          |                  |
| Grade III                                       | 6        | 22.2             |
| Grade IV                                        | 4        | 14.8             |
| Glioma cell type                                |          |                  |
| Astrocytoma                                     | 17       | 63.0             |
| Oligodendrogliona                               | 1        | 3.7              |
| Oligoastrocytoma                                | 5        | 18.5             |
| Glioblastoma Multiforme                         | 4        | 14.8             |

The image below consists of examples of low- and high-grade gliomas that were used for this experiment.

The relative expression of TGF-\( \beta \)1 mRNA was higher for low-grade, compared to high-grade, gliomas with mean relative expressions of TGF-\( \beta \)1 for high- and low-grade gliomas measured at 0.9438 (SE = 0.27253) and 2.1213 (SE = 0.6583), respectively (Figs. 2 and 3). Independent Sample testing revealed a \( P > 0.05 \) \( (P = 0.186) \), with a Confidence Interval that ranged from \(-0.60928 \) to 2.94625. This indicated that the between-group differences were not statistically significant.
Figure 1. Histopathology of Oligoastrocytoma grade II (low-grade glioma) (A) with nuclear pleomorphism, Glioblastoma Multiforme grade IV (high-grade glioma) (B) with multinucleated giant cell and microvascular proliferation.

Figure 2. Relative TGF-β1 expression for high- and low-grade gliomas ($P > 0.05$).

Figure 3. Distribution of TGF-β1 expression for high- and low-grade gliomas (NS).
4. Discussion

Increased levels of TGF-β1 expression were associated with low-, compared to high-, grade gliomas. TGF-β1 expression has been studied in many different type of cancer, including prostate, lung, skin cancer, and gliomas. Most research has shown that increased levels of TGF-β1 are observed in patients with high-grade gliomas, suggesting that increased gene expression leads to a more aggressive and complex tumor [10]. This might be because of the dual role of TGF-β, as both tumor-suppressor and promoter. A possible reason that the expression of TGF-β was lower in high-, as opposed to low-, grade gliomas is the fact that TGF-β has a dual role which allows it to act as both tumor-suppressor and tumor-promoter [11].

TGF-β is able to act as a tumor-suppressor via (1) downregulation of cyclin dependent kinase (CDK) activity through upregulation of inhibitory protein p15, p21, and p27, and (2) inhibition of cyclin dependent kinase expression. Both of these activities are accomplished due to Smad-mediated transcription activities which are activated by TGF-β signaling via TGF-β receptors I and II. When the receptors are activated, the Smad complex is phosphorylated and enters the nucleus, increasing the production of p15 and p21 [11]. Here, p15 and p21 bind to CDK and prevent its activation. CDK acts as an enzyme and is involved in cell cycles since there are several important checkpoints before mitosis can occur. CDK allows the cell to pass these checkpoints. The protein p15 acts as a CDK4 inhibitor, thus, preventing formation of Cyclin D/CDK4 (G1-CDK 4) which is an important enzyme during the G1 phase of the cell cycle. This inhibition effectively disrupts the cell cycle. p21 is an inhibitory protein which performs a similar function to p15. In addition, p21 can bind to CDK 1 and CDK 2. The Cyclin B/CDK 1 complex is essential during mitosis, catalyzing many functions such as the formation of the mitotic spindle, chromosome condensation, and breakdown of the nuclear membrane. Cyclin A/CDK 2 (S-CDK) is another complex which plays a role during the S phase of the cell cycle where genetic material is replicated. p21 acts as an inhibitor, that prevents the formation of these complexes. This provides the tumor-suppressive capabilities of TGF-β [12]. These could be reasons why we observed a lower expression of TGF-β in high-grade, compared to low-grade, gliomas in our study.

Apart from cell cycle inhibition, TGF-β can also induce apoptosis. TGF-β upregulates pro-apoptotic proteins such as TGF-β inducible early response gene (TIEG1), inositol-5-phosphatase (SHIP), and death-associated protein kinase (DAPK). TIEG1 increases oxidative stress, while DAPK causes the release of cytochrome c, inducing cell apoptosis. SHIP inhibits the PI3K–Akt pro-survival pathway. TGF-β can also work through Smad4 and activate the SAPK/JNK pathway. The result of this is the formation of numerous pro-apoptotic genes. TGF-β also independently promotes the production of pro-apoptotic genes such as caspase 3 and 8. This is an essential TGF-β tumor-suppressive effect, because although some cells are resistant to TGF-β growth inhibition, such as those in high-grade tumors, they are still susceptible to TGF-β-induced apoptosis via the translocation of mitochondrial protein apoptosis-related protein in the TGF-β signaling pathway (ARTS) to the nucleus [13]. ARTS is a septin-like mitochondrial protein which mediates TGF-β-induced apoptosis. It functions by acting as an X-linked IAP (XIAP) antagonist, which promotes cell apoptosis. Inhibitors of apoptosis (IAP) are some of the best studied caspase inhibitors; these proteins contain domains that directly inhibit caspase activity [13]. In many cases, the TGF-β signaling depends on the Smad pathway. A key component of this is the TGF-β Receptor II (TβRII) that allows the ligand, TGF-β, to bind to the cell [12,13]. Mutation in TβRI, TβRII, or decreased expression and phosphorylation of these components have been implicated in human cancers. Mutations within TβRII are frequently found in tumors with microsatellite instabilities and can results in decreased expression of TGF-β, potentially associated with poor prognosis [14]. Mice model experiments indicated that genetic deletion or downregulation of TGF-β signaling resulted in a more malignant tumors in the breast, pancreas, intestine, and colon [15]. Alteration of TGF-β in stratified epithelium can cause spontaneous squamous cell carcinoma in the genital region through destabilization of homeostasis [16]. This information supports our results wherein TGF-β mRNA expression was higher in low-grade gliomas; this means, compared to high-grade gliomas, there was more apoptosis in low-grade gliomas. Previous studies also proved that apoptosis was higher in low-, compared to high-, grade gliomas [17].
However, TGF-β has also been a tumor-promoter gene in many studies. The role of TGF-β in carcinogenesis is unclear. Accumulation of genetic alterations within the TGF-β signaling pathway is believed to be the cause of the pro-oncogenic nature of TGF-β. A study conducted by Padua & Masague in 2009 concluded that the removal of a downstream tumor-suppressor arm activated the tumor-suppressive effects, while at the same time increasing tumor progression and metastasis [18]. A key element of high-grade tumors is their ability to metastasize, and TGF-β signaling is important for epithelial mesenchymal transition. These changes cause loss of cell polarity, cadherin down regulation, and increasing invasion and metastatic capabilities of the cell. TGF-β also downregulates tumor-suppressive microRNA, leading to both increased metastasis and a positive feedback loop which upregulates TGF-β expression; leading to an even higher rate of metastasis [11].

At the microenvironment level, TGF-β exerts pro-tumor effects by remodeling the tumor microenvironment and stroma interactions. The stroma consists mainly of extracellular proteins and various cell types, including mesenchymal cells, nerve cells, immune cells, endothelial cells, and bone marrow-derived stem cells. All of these cells have TGF-β receptors; this means that TGF-β affects microenvironment fibrosis, immune cell infiltration, and angiogenesis. It is important to note that microenvironmental changes can contribute to both non-tumor and pro-tumor environments [18]. TGF-β controls immune surveillance and maintains tissue homeostasis through its effects on the proliferation, the differentiation, and the survival of immune cells [11]. Research using genetically-modified mice with defective TβRII showed T-cell differentiation along with autoimmune diseases. For example, T-cell specific blockade of TGF-β signaling allowed the formation of tumor-specific cytotoxic T lymphocytes [19]. TGF-β blocks this pathway through transcription repression of genes, inhibiting T-cell proliferation and repressing cytopotoxic genes which favor tumor progression. TGF-β also suppresses the activity of dendritic cells, natural killer cells, and creates a pro-inflammatory environment. This results in the recruitment of macrophages and neutrophils that secrete tumor-promoting cytokines, including TGF-β, resulting in a positive feedback mechanism [20].

The TGF-β signaling pathway plays an important role in normal angiogenesis by promoting the production of pro-angiogenic growth factor. As the tumor grows, it has an increased need for nutrients. This need quickly outgrows which a normal vasculature can provide; thus, the tumor cells induce angiogenesis to fulfill their need for nutritive support. High levels of TGF-β increase vascular density in cases of prostate cancer, hepatocellular carcinoma, and renal cell carcinoma [21]. Chinese research involving genetically-modified hamster ovary cells which produced an excessive amount of TGF-β1 resulted in increased angiogenesis. When TGF-β neutralizing antibody was introduced to the system, it inhibited angiogenesis processes [22].

Unfortunately, the expression of TGF-β varies considerably among patient and tumor grades, making it difficult to identify its role in malignancies. In the past, the signaling intensity and the relationship to the Smad pathway might have explained the transition of TGF-β from tumor-suppressor to promoter. Currently, it is believed that the Smad-dependent pathway mediates growth inhibition while the Smad-independent pathway probably contributes to tumor progression in early stages of tumor development [22]. The findings suggested that the Smad-dependent pathway inhibited cellular growth, as indicated by higher expression in cases of low-grade glioma.

There has been much research of TGF-β as a tumor-suppressor and promoter; however, there is still much to learn regarding when TFG-β acts as a tumor-suppressor or tumor-promoter. As mentioned above, TGF-β has many roles in tumor progression and inhibition, and these transitions occur alongside decreased or altered responses toward TGF-β. This, combined with genetic mutation of tumor cells, makes predicting the effects of TGF-β difficult.

5. Conclusion

Relative expression of TGF-β was higher in patients with low-grade glioma, compared to those with high-grade glioma; however, the difference was not statistically significant. Based on this study, TGF-β holds unproved potential for use as a molecular biomarker.
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