Evaluating the potential of NANOG as a glioma malignancy biomarker

F Runtu1, S Ichwan2, S I Wanandi3 and N S Hardiany4*

1Undergraduate student of International Class Program Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
2Department of Neurosurgery Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital, Jakarta, 10430, Indonesia
3Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
4Center of Hypoxia and Oxidative Stress Studies, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
*E-mail: novi.silvia@ui.ac.id

Abstract. To elucidate the role of NANOG in malignancy, the difference in relative expression of NANOG between low- and high-grade gliomas was evaluated. Gene quantification was performed using real-time reverse transcription polymerase chain reaction from 29 isolated RNA samples of glioma resected during the surgery of patients in the Neurosurgery Department of FKUI-RSCM. The relative expression of NANOG in low- and high-grade gliomas was analyzed for normality and compared statistically. Statistical analysis showed no significant difference in NANOG expression between low- and high-grade glioma although there was a tendency for higher expression in the latter. The results support the indication of NANOG as a malignancy biomarker. However, further studies of NANOG expression in malignancy using a greater sample size or other methods may help confirm its role as a cancer biomarker that could be used as a potential therapeutic target or prognosis indicator.

1. Introduction

Glioma is the most common type of primary brain tumor and represents 80% of brain tumors [1]. The World Health Organization (WHO) differentiates gliomas into four groups based on the degree of malignancy: grades I, II, III, and IV. The differentiation is made based on histopathological observations of several features such as mitotic activity, microvascular proliferation, and necrosis [2]. Grading helps in identifying suitable treatments for patients.

High-grade gliomas (i.e., grades III and IV) require multimodal treatment consisting of surgical resection, radiotherapy, and chemotherapy, whereas low grade gliomas (i.e., grades I and II) are generally resolved with surgical intervention [2,3]. Grading also helps in formulating the prognosis of the disease, with grade II patients generally surviving over a five-year period after diagnosis, grades II–III surviving over a two- to three-year period, and grade IV patients (the majority of glioblastoma cases) surviving up to one year [4].

The high mortality in patients with glioma is partly due to the relative inaccessibility of the tumor tissue. Performing resection surgery is risky because there is a possibility of compromising the surrounding brain tissue, which is essential in controlling daily activities [3]. Following resection,
there is a 24% complication rate that increases with subsequent resection surgery. Chemotherapy may not be suitable for all patients because genetic factors can render a patient resistant to chemotherapy. For instance, 50% of glioblastoma patients do not respond to temozolomide (TMZ) therapy, a standard therapy for glioma recommended by the European Organization for Research and the Treatment of Cancer/National Cancer Institute of Canada. This resistance could contribute to the genetic mutation in the MSH6 gene or the action of a demethylating enzyme, such as O6-methylguanine methyltransferase, which dealkylates the alkylating function of TMZ [1,5].

Without early detection of genetic markers such as TMZ resistance, glioma patients would suffer unnecessary financial costs. Hochberg et al. estimated that the cost of TMZ treatment can reach up to $50,000 per year. Considering the cost of chemotherapy, an early biomolecular diagnosis would reduce the financial burden of non-beneficial therapy [1]. Biomolecular markers are therefore useful as diagnostic tools that aid in determining the appropriate therapy for a patient. Due to the increasing discovery of biomarkers and their role in imparting malignancy to tumor cells, WHO has considered the incorporation of molecular markers in the classification in 2016 [4].

NANOG, along with OCT4, SOX2, and KLF4, acts as regulator of embryonic stem cell pluripotency. The NANOG gene is located on chromosome 12 and is named after the fountain of youth in Celtic mythology, Tir Na Nog. This name aptly describes the gene function that codes for a transcription factor that maintains pluripotency and proliferative capacity in embryonic stem cells [6]. In healthy cells, NANOG is only found in epiblast and germ cells. Due to the similarity of cellular characteristics between embryonic stem cells and cancer stem cells, researchers are looking into NANOG as a potential biomarker of cancer cells.

Malignancy and tumor transformation are governed by the genomic changes in expression. Several studies have reported a positive correlation between NANOG expression and cancer cell proliferation. NANOG expression has been found in human tumors in the prostate, cervix, breast, ovary, and oral cavity [7]. Malignancy is due to a cell’s ability to migrate and invade other regions. NANOG promotes epithelial mesenchymal transition (EMT), the transition of epithelial cells to mesenchymal cells that allows for tumor cells to migrate to other regions and metastases [8].

This study examines the role of NANOG in cancer malignancy through a biomolecular approach by evaluating the relative expression of NANOG in low- and high-grade gliomas. We used tissue samples from patients obtained during resection surgery in the hope that NANOG could be considered as a biomarker for tumor grading.

2. Materials and Methods
2.1. Samples collection procedure
We obtained samples of glioma tissue from resection surgery in the FKUI-RSCM Neurosurgery Department. Samples were obtained with an informed consent procedure that was approved by the ethics committee of the Faculty of Medicine at Universitas Indonesia. Samples were obtained from 2014 to 2015. Patients were diagnosed with glioma grade I-IV according to histological examinations. Patients were excluded from the study if an anatomical pathology examination did not indicate glioma based on the WHO classification. Samples were obtained from 29 patients and included samples of high-grade glioma, low-grade glioma, and normal brain tissue.

2.2. Gene product isolation and amplification
Glioma samples were homogenized with Polytron Homogenizer and stored at –80°C prior to isolation. The evaluation of relative genetic expression was conducted by real-time reverse transcription polymerase chain reaction (RT-PCR) to quantify the expression of NANOG mRNA. RNA was isolated using the Geneaid kit.

To evaluate the genetic expression of NANOG, gene amplification was conducted using the KAPA SYBR® FAST One-Step quantitative RT-PCR kit from Kapabiosystem, which allows a reaction mixture to combine reverse transcription of RNA into a cDNA reaction with an amplification reaction
of the targeted DNA. The mixture reaction was prepared according to kit instructions and reacted in the PCRMax EcoTM instrument (U.K.).

The real-time PCR System was set up with the following protocol: reverse transcription at 42°C for 5 min, polymerase activation at 95°C for 5 min, PCR cycling for 40 cycles with denaturation at 95°C for 10 s, annealing at 60°C for 40 s, extension at 72°C for 30 s, followed by analysis with melt curves at 95°C for 15 s, 55°C for 15 s, and 95°C for 15 s, and finally incubation at 50°C for 2 min.

The NANOG primers used for the reaction amplification were described by Tachi et al. with the following sequence 5’–AGCTACAAACAGGTGAAGAC-3’ (forward primer) and 5’–GAGTAAAGGCTGGGGTAGGTA–3’ (reverse primer) [9]. We used 18sRNA as a reference gene for relative gene quantification. The primer for 18sRNA was 5’-AAACGGCTACCATCCAAG-3’ (forward primer) and 5’-CCTCCAATGGATCCTCGTTA-3’ (reverse primer).

2.3. Data analysis
We measured the expression of the targeted gene relative to the expression of a reference gene from the same sample (18sRNA). Relative expression was quantified based on the threshold cycle (Ct) and computed using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen [10]. Relative expression of NANOG between high- and low-grade gliomas was assessed for significant statistical difference (*$P < 0.05$). Statistical analysis was performed using the IBM® SPSS Statistics® statistical software.

3. Results
Of the 29 glioma samples, 11 were high-grade and 18 were low-grade gliomas. Patient age and tumor types are shown in Table 1. Patient age group was classified into ≤50 years old and >50 years because these age groups have been shown to predict overall survival in patients with high-grade gliomas [11]. Figure 1 shows the histopathological features of a sample of high-grade glioma (glioblastoma type IV) and a sample of low-grade glioma (astrocytomas grade II). High-grade gliomas were characterized by anaplasia or undifferentiated states with high mitotic activity and necrosis. The glioblastomas shown are characteristic of malignant tumors with high mitotic activity, indicated by high cellularity and dense chromatin intensity. Cells also have an anaplastic cellular appearance. The characteristic necrosis of malignant tumors is not shown in this microscopic view. The low-grade tumor shows cytologic atypia (Figure 1B), which differentiates it from the higher grade III. The tumor should have an anaplastic appearance and high mitotic activity [4].

| Group       | Number | Percentage |
|-------------|--------|------------|
| ≤50 years   | 25     | 86.2%      |
| >50 years   | 4      | 13.8%      |

| Glioma Malignancy Classification | Number | Percentage |
|----------------------------------|--------|------------|
| Grade I                          | 1      | 3.45%      |
| Grade II                         | 17     | 58.62%     |
| Grade III                        | 6      | 20.69%     |
| Grade IV                         | 5      | 17.24%     |

| Glioma Pathological Anatomy      | Number | Percentage |
|----------------------------------|--------|------------|
| Oligodendroglioma                | 4      | 13.8%      |
| Astrocytoma                      | 20     | 69.0%      |
| Mix glioma                       | 5      | 17.2%      |

Table 1. Characteristics of the 29 glioma samples and patients: patient age, glioma grading classification, and glioma pathology anatomy.
Figure 1. Histopathological features of high- and low-grade glioma samples stained with hematoxylin and eosin stain (magnification, 400×). (A) High-grade glioblastoma grade IV; the microscopic view features malignant histopathology characteristics such as high cellularity, cellular anaplasia, and denser chromatin compared to (B) low-grade astrocytoma grade II, showing cellular atypia.

Statistical analysis showed that two samples were considered outliers in the high-grade glioma due to very high NANOG expression. These outliers were astrocytoma grade III and glioblastoma grade IV (Figure 2A). The non-parametric statistical test showed that there was no significant difference in NANOG expression between the high- and low-grade samples ($P > 0.05$). However, the high-grade gliomas tended to express more NANOG compared to low-grade gliomas (Figure 2B). The mean NANOG relative expression of high-grade gliomas was 23.05 ($N = 11$), whereas the mean expression of low-grade gliomas was 7.53 ($N = 18$). The median NANOG relative expression for the high- and low-grade gliomas were 0.199 and 0.226, respectively.

Figure 2. (A) Distribution of NANOG expression data for high-grade and low-grade glioma showing two samples of high-grade glioma with relative expression exceeding the maximum relative expression of low-grade glioma. Outliers were glioblastoma grade IV and astrocytoma grade III. (B) Mean of relative NANOG expression for high-grade and low-grade glioma samples. High-grade glioma tended to overexpress NANOG compared to low-grade glioma. Error bars represents 95% confidence intervals.
Compared to the relative expression of normal brain tissue, 45% of high-grade glioma samples showed higher expression of NANOG. In 50% of the overexpressed glioma samples, NANOG was expressed at least 50 times more than in normal brain tissues. In low-grade gliomas, 38.8% of samples showed higher NANOG expression compared to normal brain tissues.

4. Discussion
Malignant tumors display characteristics of stem cells such as high proliferative capacity and ability to maintain undifferentiated states or anaplasticity. Genes that are highly expressed in stem cells (e.g., OCT4, SOX2, and NANOG) are hypothesized to impart the stemness property of cells. OCT4 has a role in maintaining pluripotency and self-renewal, SOX2 regulates the self-renewal capacity of stem cells, and NANOG encodes a transcription factor that is responsible for the pluripotency of embryonic stem cells [12].

NANOG maintains pluripotency through multilevel interactions with several proteins. In vitro studies have shown that overexpression of NANOG is correlated with the proliferation of cancer cells. Knockdown of embryonic stem cells markers such as NANOG can arrest xenograft tumor growth [8]. When NANOG is knocked down, genes regulating the cell cycle such as cyclins D1, D2, and D3 are downregulated. In addition, p53, a tumor suppressor gene, is also downregulated, contributing to the growth of tumor cells. Torres et al. suggested that NANOG interacts with Stat3, which forms regulatory pathways with the leukemia inhibitory factor to maintain pluripotency. NANOG interacts with NFkB, downregulating NFkB expression, which increases in activity during embryonic stem cell differentiation [13].

Because of its function in differentiation suppression, NANOG has only been found in germ cells and epiblasts, which are undifferentiated in healthy people. There is a small amount of NANOG in human fibroblast cells [6]. Our current study shows that there is a tendency for NANOG overexpression in malignant tumors. Malignancy is characterized by less differentiated cells and NANOG overexpression in the high-grade gliomas, providing additional evidence to support the role of NANOG in the maintenance of cellular pluripotency in malignant tumor cells.

Niu et al. have investigated the role of NANOG in tumorigenesis and malignancy in gliomas by evaluating its expression in undifferentiated cells. The undifferentiated cells originated from brain tumor cells isolated from the cell line U87 after culture. Their results showed higher expression of NANOG in isolated tumor stem cells compared to the U87 parental cells. They also compared the expression of NANOG (mRNA and protein levels) in high-grade and low-grade glioma samples obtained from 69 patients with primary gliomas of varying pathological grade. The results showed significantly higher expression of NANOG mRNA (F = 50.86, P < 0.01) in high-grade gliomas compared to low-grade gliomas. Western blot analysis also showed positive correlation between NANOG expression and malignancy. Immunohistochemistry demonstrated that NANOG was mainly found in the nucleus, supporting the role of NANOG as a DNA-binding protein transcription factor. In contrast to the current study, Niu et al. study used additional clinical samples and employed different quantification methods.

Despite the use of different gene expression quantification techniques, Niu et al. results are in agreement with the current study, where we found a positive correlation between malignancy and NANOG expression. This study provides additional evidence on the role of NANOG in tumor malignancy. Despite the high number of studies that have evaluated the expression of NANOG and other stem-cell biomarkers in cancer cells, there is still a limited understanding of the role of molecules and histopathological features in malignancy. This area of research should be considered for further study.

Aside from diagnostic use, NANOG has the potential to be used as a prognostic biomarker. Luo et al. studied the expression of the embryonic stem cells markers SOX2, OCT4, NANOG, and NESTIN to associate their expressions to prognosis [8]. Multivariate analysis showed that high expression of OCT4 and NANOG correlated to lower survival rates (P < 0.05) [8]. Another study used NANOG as a prognostic biomarker for the treatment of myeloid leukemia with hedgehog (Hh) signal inhibitor because NANOG acts downstream of the Hh signaling pathway [14]. NANOG has great potential to
be used as a malignancy biomarker for tumor classification and possibly for prognosis and therapy response. Therefore, there should be further attempts to use NANOG as a potential therapeutic target to treat cancer.

5. Conclusion
The study evaluated the potential of NANOG as a glioma biomarker by comparing its relative expression in high- and low-grade gliomas. We found a tendency for higher NANOG expression in high-grade gliomas which are more poorly differentiated. Our results confirm the role of NANOG in maintaining undifferentiated cellular states in malignant gliomas.

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