High concentration of γ-H2AX correlates with a marker of apoptotic suppression and PI3K/Akt pathway upregulation in glioblastoma multiforme

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Received August 30, 2022; Accepted January 13, 2023

DOI: 10.3892/ol.2023.13735

Abstract. Glioblastoma multiforme (GBM) is a very aggressive type of primary brain tumor in adults with a poor prognosis. DNA double-strand breaks are known to be associated with the development of numerous cancer types due to their ability to generate genomic instabilities. In GBM, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is a common pathway that can be activated by exogenous and endogenous factors. Genomic instability may be an endogenous stimulating factor for activation of the PI3K/Akt pathway, which may inhibit the apoptosis of GBM cells. Spontaneous DNA double-strand breaks play an essential role in the survival of GBM cells, and apoptosis levels may reflect survival ability. However, no study has yet been conducted to analyse the association between spontaneous DNA double-strand breaks and apoptosis in patients with GBM prior to treatment. Therefore, the present study examined the concentrations of γ-histone 2AX (γ-H2AX), a sensitive marker of spontaneous DNA double-strand breaks, and cleaved caspase-3, a marker of apoptosis, in patients with GBM. The correlation of γ-H2AX with cleaved caspase-3, PI3K and Akt was also investigated. A total of 26 pre-treatment tumor tissue specimens from patient with GBM were analyzed to determine the concentrations of γ-H2AX, PI3K, Akt and cleaved caspase-3 using sandwich enzyme-linked immunosorbent assays. The results showed a moderate positive correlation between γ-H2AX and PI3K (r=0.52; P=0.007), a moderate positive correlation between γ-H2AX and Akt (r=0.4; P=0.041) and a strong negative correlation between γ-H2AX and cleaved caspase-3 (r=-0.61; P=0.0009). These analyses were also performed in seven tumor tissue specimens from patients with grade I glioma as controls, but no significant correlations were detected. The findings of the present study suggest that a high level of γ-H2AX may affect GBM cell apoptosis via the PI3K/Akt pathway.

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Key words: high-grade glioma, low-grade glioma, DNA double-strand breaks, signal transduction pathways, cleaved caspase-3

Introduction

Glioblastoma multiforme (GBM) is a central nervous system malignancy and the most common glioma subtype, with very aggressive and highly infiltrative behavior (1-3). The incidence of GBM is 12-15% among all intracranial tumors; it is mainly found in the cerebral hemisphere and originates from the supratentorial area in 95% of cases (4,5). Patients with GBM usually have a poor prognosis, with a median survival time of 12-15 months after diagnosis and a 5-year survival rate of only 3-5% (6).

The three main signal transduction pathways involved in GBM are the retinoblastoma pathway, the p53 pathway and the phosphatidylinositol-3-kinase (PI3K) pathway (7). Activation of the PI3K pathway is detected in 88% of cancer types and affects the phosphoinositide-3-kinase regulatory subunit 1 and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α genes. In signal transduction associated with various cellular processes, PI3K has essential roles in the regulation of the metabolism, inflammation, survival, motility and development of cancer cells (8,9). Stimulating factors such as growth factors, cytokines, hormones and other factors, including genomic instability due to excessive non-homologous end joining (NHEJ), also play a role in activation of the PI3K

pathway (10-13). Furthermore, PI3K induces the activation of Akt, which can inhibit the activity of various pro-apoptotic proteins, including Bad, Bax and Bak, and then inhibit the release of cytochrome *c* from the mitochondria. As a result, the activity of caspase-3 is inhibited, and apoptosis does not occur (14-16). Therefore, high levels of Akt impact the survival and progression of malignant cells (17,18).

Caspase-3 is an endoprotease with the main role as an executioner caspase in apoptosis (19). Caspase-3 is activated by caspase-9 and converted to the active form by cleavage at Asp175/Ser176 (19-21). The activation and cleavage of caspase-3 are crucial for programmed cell death. Several studies have shown the role of caspase-3 in the regulation of carcinogenesis in various cancer types, such as breast cancer, gastric and tongue cancer (22-26). Therefore, caspase-3 is a good indicator for the evaluation of cancer progression (19,22).

The apoptosis pathway can be activated by DNA damage, the most threatening type of which is DNA double-strand breaks (27,28). Physiological or pathological processes can cause DNA double-strand breaks to occur. Physiological processes that cause DNA double-strand breaks comprise V(D)J recombination, class-switch recombination, meiosis or DNA replication, in which DNA double-strand breaks occur as a secondary event during the normal cell cycle (29). DNA double-strand breaks can also occur due to exposure to ionizing radiation, oxidizing free radicals, enzymatic processes or cytotoxic agents (30-32).

The physiological response to DNA double-strand breaks includes the detection of DNA damage, the recruitment of factors with a role in repairing the damage and the repair process itself (33). The initial response when DNA double-strand breaks occur is the phosphorylation of histone 2AX (H2AX) to form γ-H2AX, which occurs within 10-30 min after the damage is induced (34,35). γ-H2AX is a sensitive and early indicator of DNA double-strand breaks in which the serine amino acid residue at the 139 position of H2AX is phosphorylated; numerous γ-H2AX molecules are formed in the damaged area (35-37). γ-H2AX serves as a marker for the detection of DNA double-strand breaks and genomic instability, and also indicates the ability of cancer cells to survive (36,38-40). The mechanism for the repair of DNA double-strand breaks can be error-prone, and so may induce genomic instability and genetic mutations that will activate signal transduction, inhibit apoptosis of the GBM cells, and finally lead to rapid progression and aggressive behavior in GBM.

To the best of our knowledge, the association between DNA double-strand breaks and apoptosis in patients with untreated GBM has not been analyzed previously. Therefore, the present study evaluated the correlation between γ-H2AX and cleaved caspase-3, PI3K and Akt in pre-treatment patients with GBM or grade I glioma with the aim of providing an improved understanding of the molecular mechanism underlying the survival of GBM cells.

Materials and methods

Ethics approval and patient consent. The study was approved by the Ethics Committee of The Faculty of Medicine, Universitas Indonesia (ref. no. KET-393/UN2.F1/ETIK/PPM.00.02/2020). The patients and/or their guardians provided written informed consent related to storage and sharing of data and tissue specimens for future research.

Study design. A total of 26 tumor tissue specimens from patients with GBM and 7 tumor tissue specimens from patients with grade I glioma as controls were obtained from Siloam Hospital Lippo Village (Tangerang, Indonesia) and Mochtar Riady Comprehensive Cancer Center Siloam Hospital (Jakarta, Indonesia) between January 2013 and December 2016. All tissue specimens were stored at -80˚C. This study included 14 males and 12 females with GBM (mean age, 49 years; age range, 21-63 years), and 4 males and 3 females with grade I glioma (mean age, 34 years; range, 23-73 years). The inclusion criteria were a diagnosis of GBM, a World Health Organization (WHO) grade of IV and WHO grade I glioma based on the pathological report. None of the patients had received any treatment before biopsy or surgical resection. The concentrations of γ-H2AX, total PI3K, total Akt and cleaved caspase-3 were determined using sandwich enzyme-linked immunosorbent assays (ELISAs). The ELISA kits used in this study were the Human Gamma H2AX ELISA Kit (cat. no. MBS167504; MyBioSource, Inc.), the Human PI3K ELISA Kit (cat. no. CSB-E08417h; Cusabio Technology LLC), the Akt (Total) Human ELISA Kit (cat. no. KHO0101; Invitrogen; Thermo Fisher Scientific, Inc.) and the Caspase-3 (Cleaved) Human ELISA kit (cat. no. KHO1091; Invitrogen, Thermo Fisher Scientific, Inc.). The research was conducted in the Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia (Jakarta, Indonesia).

Protein isolation. Tumor tissues were homogenized on ice using a micro homogenizer with 1-2 ml RIPA lysis and extraction buffer (Thermo Fisher Scientific, Inc.). The extract was incubated on ice for ~15 min and then centrifuged at 14,000 x g for 15 min at room temperature. The supernatant was collected and the concentration of γ-H2AX, total PI3K, total Akt and cleaved caspase-3 in the supernatant was analyzed. The total protein concentration was determined using a BSA protein assay (MilliporeSigma) with measurement of the absorbance at 280 nm.

Sandwich ELISA. A 100-µl sample of the supernatant was put into a microplate well and incubated for 2 h at room temperature, after which the wells were washed four times with 250 µl wash buffer. Next, 100 µl antibody from the ELISA kit was added and the wells were incubated for 1 h at room temperature. After the incubation, the wells were washed four times using 250 µl wash buffer, 100 µl streptavidin-HRP was added and the wells were incubated for 30 min at room temperature. After washing, 100 µl stabilized chromogen was added, followed by incubation for 30 min at room temperature. Next, 100 µl stop solution was added to each well and the absorbance was read at 450 nm. The protein concentration was read from the standard curve. The concentrations of γ-H2AX, total PI3K, total Akt and cleaved caspase-3 were divided by the total protein concentration to standardize them.

Statistical analysis. Statistical analysis was performed on the data obtained, with processing and analysis using SPSS

Table I. Clinicopathological characteristics of patients with GBM and grade I glioma.

Characteristics	GBM (n)	Grade I glioma (n)
Sex		
Male	14	4
Female	12	3
Age, years		
≤40	8	5
>40	18	2
Tumor size, cm		
<4	5	1
≥4	15	3
Unknown	6	3
Surgical type		
Total resection	1	3
Subtotal resection or biopsy	19	1
Unknown	6	3
KPS		
<70	15	0
≥70	11	7

GBM, glioblastoma multiforme; KPS, Karnofsky Performance Scale.

Statistics version 21.0 (IBM Corp.). All data are presented as the mean ± SD. Comparative analyses between GBM and grade I glioma were performed using the independent Student's t-test. The Saphiro-Wilk test was used to determine the normality of distribution. Correlation analyses between variables were conducted using Pearson's correlation test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics. The clinicopathological characteristics of the patients with GBM and grade I glioma included in the present study are shown in Table I.

Comparative analysis of γ-H2AX, total PI3K, total Akt and cleaved caspase-3. The concentrations of γ-H2AX, total PI3K, total Akt and active (cleaved) caspase-3 were measured to obtain information based on their known contributions to particular cellular and molecular mechanisms. Sandwich ELISAs were performed to determine the levels of γ-H2AX, total PI3K, total Akt and cleaved caspase-3 in the GBM and grade I glioma tissues.

The Saphiro-Wilk test showed that the levels of γ-H2AX, total PI3K, total Akt and cleaved caspase-3 were normally distributed. Therefore, a parametric comparative test (independent Student's t-test) was appropriate. Next, a comparative analysis between GBM and grade I glioma was conducted and the results are shown in Fig. 1. In GBM and grade I glioma, the γ-H2AX concentrations were 0.09±0.03 and 0.03±0.01, the total PI3K concentrations were 0.77±0.13 and 0.49±0.22, the total Akt concentrations were 0.07±0.02 and 0.02±0.003, and the cleaved caspase-3 concentrations were 0.02±0.005 and 0.02±0.009, respectively.

Correlation between γ-H2AX and cleaved caspase-3. We hypothesized that spontaneous DNA double-strand breaks might affect the ability of GBM cells to survive through the inhibition of apoptosis. The correlations between γ-H2AX and cleaved caspase-3 concentrations in GBM and grade I glioma were analyzed to investigate this hypothesis using Pearson's correlation test. The results showed a strong negative correlation ($r=-0.61$; $P=0.0009$) in GBM but no statistically significant correlation in grade I glioma (Fig. 2).

Correlation between γ-H2AX and PI3K/Akt. To evaluate the PI3K/Akt pathway as a pathway involved in GBM that may be affected by the DNA double-strand breaks, the correlations between γ-H2AX and PI3K in GBM and grade I glioma were analyzed. The results showed a moderate positive correlation ($r=0.52$; $P=0.007$) between γ-H2AX and PI3K in GBM and no statistically significant correlation in grade I glioma (Fig. 3).

The correlation between γ-H2AX and Akt was also analyzed. A significant positive correlation was detected between γ-H2AX and Akt in GBM ($r=0.40$; $P=0.041$) but no significant correlation was found in grade I glioma (Fig. 4).

Discussion

The ability of cancer cells to survive is an important factor in cancer progression. Apoptosis is a marker of cancer cell survival that can be induced or inhibited by specific signal transduction pathways. In the present study, a significant negative correlation between γ-H2AX and caspase-3 activation in GBM suggested that elevated levels of DNA double-strand breaks were associated with reduced apoptosis. This result also suggested that spontaneous DNA double-strand breaks may contribute to maintaining the tumorigenicity of GBM cells and enabling them to survive. Several previous studies have shown that spontaneous DNA double-strand breaks affect cancer cell survival (41-44). However, to the best of our knowledge, no study has yet been performed on the association between the spontaneous DNA double-strand breaks and the survival of GBM cells.

Spontaneous DNA double-strand breaks are considered to play a role in the progression of GBM, particularly in the survival of GBM cells. The repair mechanism is initiated when the DNA double-strand breaks occur, and includes homologous recombination or NHEJ (45,46). If excessive damage occurs, this may cause the repair processes to proceed incompletely and to be dominated by the NHEJ mechanism, which is considered an error-prone repair pathway (29). A potential explanation for this is that the NHEJ mechanism of repair can take place throughout the cell cycle and does not require any specific template. Therefore, it can immediately repair the damage. The occurrence of DNA double-strand breaks is reflected in the occurrence of erroneous repair mechanisms; if DNA double-strand breaks occur extensively, then the NHEJ mechanism also occurs extensively. This may lead to genomic instability that finally affects the tumorigenicity and aggressive behavior of GBM (10,47,48).

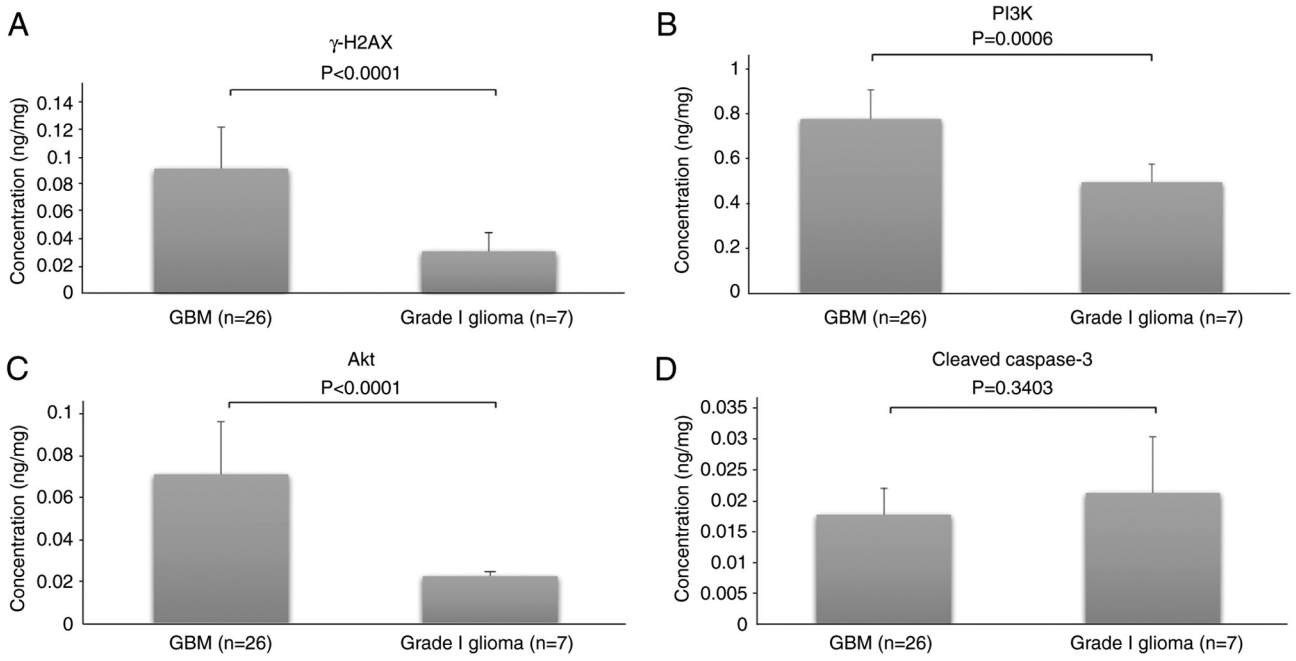

Figure 1. Comparative analysis of four potential markers between GBM and grade I glioma. Comparison of (A) γ-H2AX, (B) total PI3K, (C) total Akt and (D) cleaved caspase-3 concentrations in GBM and grade I glioma. GBM, glioblastoma multiforme; γ-H2AX, γ-histone 2AX; PI3K, phosphatidylinositol-3-kinase.

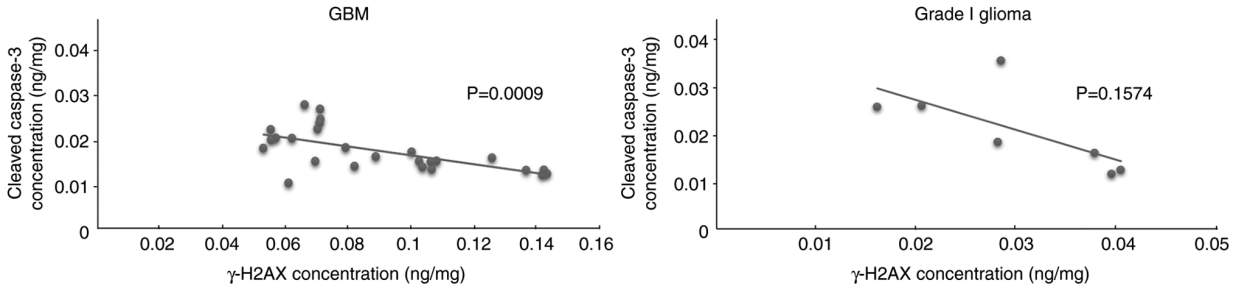

Figure 2. Correlation analysis between γ-H2AX and cleaved caspase-3 in GBM and grade I glioma. GBM, glioblastoma multiforme; γ-H2AX, γ-histone 2AX.

The present study also analyzed the expression of proteins in the PI3K/Akt signal transduction pathway, which has a vital role in the survival of cancer cells. The results showed significant correlations between γ-H2AX and PI3K, as well as between γ-H2AX and Akt, in GBM. These suggest that spontaneous DNA double-strand breaks may activate the PI3K/Akt pathway. The activation of this pathway will suppress the activity of pro-apoptotic proteins and finally inhibit apoptosis in GBM cells (14,17). The results of the present study are consistent with other studies, which have shown that the PI3K/Akt pathway affects various cellular functions, including apoptosis in cancer cells (7,9,14).

As controls, these analyses were also performed in seven tumor tissue specimens from patients with grade I glioma to investigate the potential correlation between γ-H2AX and cleaved caspase-3, which should reflect spontaneous DNA double-strand breaks and apoptosis, respectively. Correlation analyses between γ-H2AX and PI3K/Akt were also conducted. However, no statistically significant correlation was observed in grade I glioma. These findings may strengthen our hypothesis concerning the impact of DNA double-strand breaks on the inhibition of apoptosis through the PI3K/Akt pathway in GBM.

There are several limitations to the present study. First, total PI3K and Akt were examined, which includes both activated and inactivated forms. The determination of the phosphorylated (cleaved) forms, phospho (p)-PI3K and p-Akt, would provide improved information about the role of this pathway, particularly in the inhibition of apoptosis. A further limitation is the lack of experimental data supplying information about the effect of the DNA double-strand breaks on apoptosis through activation of the PI3K/Akt pathway in GBM. Therefore, further experimental study is required to support the current hypothesis, which may be conducted through *in vitro* experiments using a GBM cell line. However, activation of the PI3K/Akt pathway can also stimulate downstream substrates, such as mammalian target of rapamycin, which can promote cell growth, autophagy and proliferation (49). Therefore, further analysis of this pathway is necessary to improve the understanding of the mechanism of survival and progression of GBM cells.

In conclusion, to the best of our knowledge, this is the first study showing a correlation between a high concentration of γ-H2AX and a marker of reduced apoptosis activation in patients with GBM. The concentration of γ-H2AX reflects the occurrence of DNA double-strand breaks that can activate the

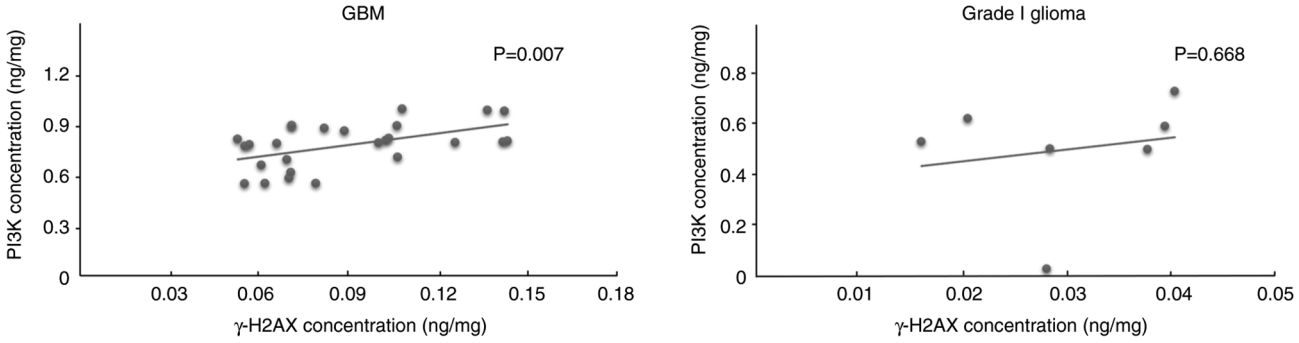

Figure 3. Correlation analysis between γ-H2AX and PI3K in GBM and grade I glioma. GBM, glioblastoma multiforme; γ-H2AX, γ-histone 2AX; PI3K, phosphatidylinositol-3-kinase.

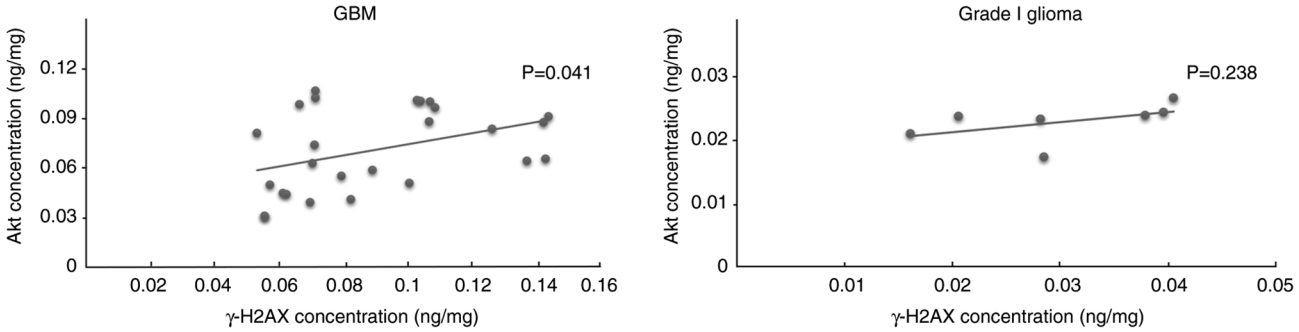

Figure 4. Correlation analysis between γ-H2AX and Akt in GBM and grade I glioma. GBM, glioblastoma multiforme; γ-H2AX, γ-histone 2AX.

PI3K/Akt pathway and affect apoptosis, enabling GBM cells to survive and grow. The present study provides a new academic insight into the spontaneous occurrence of DNA double-strand breaks in GBM, and indicates a potential association between spontaneous DNA double-strand breaks and apoptosis via the negative correlation between γ-H2AX and cleaved caspase-3 in patients with GBM prior to treatment. This result suggests the potential of γ-H2AX as a prognostic biomarker in the routine clinical practice for GBM patients. However, further studies are necessary to broaden the understanding of cellular mechanisms in GBM, particularly the actions of apoptotic and anti-apoptotic proteins, which may affect the conclusions of the present study.

Acknowledgements

The authors would like to thank the neurosurgeon Dr Syaiful Ichwan (Dr Cipto Mangunkusumo Hospital, Jakarta, Indonesia) for kindly providing four tumor tissue specimens from patients with GBM for analysis in this study.

Funding

This study was financially supported in part by a PUTI grant from Universitas Indonesia (contract no. NKB-577/UN2. RST/HKP.05.00/2020).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CTUB contributed to the concept and design of the study, data acquisition, statistical analysis, data interpretation. was involved in drafting and revising the manuscript, and gave final approval for publication. NSH contributed to study concept and design, helped with general data analysis and gave final approval for publication. MS was responsible for the planning and execution of all research activity, including experiments and funding acquisition. EJW was responsible for all research activity, planning, implementation and the provision of research samples. ADH was involved in visualizing, drafting and revising the manuscript, and assisted in interpreting the data. All authors participated sufficiently in the study to take public responsibility for appropriate portions of the content, and agree to be accountable for all aspects of the study to ensure that questions related to the accuracy and integrity of the work are appropriately investigated and resolved. CTUB and NSH confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Faculty of Medicine, Universitas Indonesia (Jakarta, Indonesia). The patients and/or their guardians provided written informed consent related to storage and sharing of data and tissue specimens for future research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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