RESEARCH ARTICLE

Relative Expression of cMyc mRNA in Human Glioma Cells and its Relationship with the Degree of Malignancy

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Abstract

Pluripotency of cMyc genes may be one factor for the high glial cell differentiation in glioma thus it can become an alternative therapeutic target. The objective of the study is to analyze the cMyc mRNA expression and its relationship with the degrees of glioma malignancy. This is a cross-sectional study from 20 glioma samples with different degree of malignancy from Dr. Cipto Mangunkusumo General Hospital, Jakarta during the period of October 2014 until February 2015. The mRNA obtained from glioma samples are converted to cDNA and then amplified. Relative quantification of cMyc mRNA expression is measured by calculating the cycle threshold values of Real Time RT PCR and normalized towards 18s rRNA to predict the relationship between the expression of cMyc and the degree of malignancy. The cMyc expression is increased in accordance with the tumor grade. The cMyc expressions in high grade glioma are 17424.23 folds higher when calibrated to the normal cell, whereas the genes in lower grade tumors are expressed with the rate of 6167.35. Although the values obtained are not statistically significant, this research has strengthened molecular diagnosis, specifically pluripotency as the factor that gives a greater prognostic relevance than the histopathologic diagnosis. As a conclusion, there is a clinical tendency where the relative mRNA expression was higher in in glioma cMyc high degrees compared with low-grade glioma, however it is not statistically significant.
Keywords: *cMyc,;high grade glioma; low grade glioma.*

Ekspresi Relatif mRNA cMyc Sel Glioma Manusia dan Hubungannya dengan Derajat Keganasan

Abstrak

Pluripotensi mRNA cMyc dapat menjadi salah satu faktor tingginya diferensiasi sel glial pada glioma sehingga dapat menjadi target terapi alternatif. Tujuan penelitian untuk menganalisa ekspresi mRNA cMyc dan dihubungkan dengan tingkat keganasan glioma. Penelitian dengan desain cross sectional dilakukan terhadap 20 sampel glioma dengan derajat keganasan berbeda yang berasal dari RSUPN Dr. Cipto Mangunkusumo selama Oktober 2014 sampai Februari 2015. mRNA ditransformasi menjadi cDNA dan diamplifikasi menggunakan Accupower-Two-Step RT-PCR with SYBR Green. Kuantifikasi relatif mRNA cMyc ditentukan dengan menghitung nilai cycle threshold pada RT PCR ang dinormalisasi dengan rRNA 18S untuk melihat hubungan antara ekspresi cMyc dan derajat keganasan glioma. Ekspresi cMyc ternyata lebih tinggi seiring dengan meningkatnya tingkat keganasan. Ekspresi cMyc pada glioma derajat tinggi senilai 17424,23 kali lebih tinggi dibandingkan dengan ekspresi pada sel otak normal, sedangkan glioma derajat rendah mengalami ekspresi gen cMyc senilai 6167,30. Meskipun nilai yang diperoleh tidak signifikan secara statistik, penelitian ini telah menunjukkan bahwa diagnosis molekuler, terutama pluripotensi, dapat menjadi faktor penentu prognosis glioma selain ditentukan dengan derajat keganasan melalui pemeriksaan histopatologis. Terdapat kecenderungan secara klinis dimana ekspresi relatif mRNA cMyc lebih tinggi pada glioma derajat tinggi dibandingkan dengan glioma derajat rendah, namun nilainya tidak signifikan secara statistik.
Kata kunci: *cMyc; glioma derajat tinggi; glioma derajat rendah.*

Introduction

Gliomas are primary brain tumors that develop from the glial cell. They are highly pluoripotent, thus may grow their own vasculature. With an incidence of 3–5 per 100.000 people in each year, it is considered to be the most common primary brain tumor. Despite it may occur in all ages, gliomas are most prevalent in adults over 45 years of age.[1-3]

According to WHO glioma may be differentiated into a low and high degree of invasion. Patients with highly invasive glioma are usually referred of having glioblastoma. Glioblastoma cases may be primary or secondary in term of etiology, by which primary glioblastoma occurs *de novo* whereas the secondary glioblastoma is originated from the low degrees glioma that developed into glioblastoma. Patients with this malignancy have the worst prognosis with high recurrence despite receiving conventional treatments. Such inconvenience is believed to be the result of the existence of cancers' stem cells, which have such high capability of self renewal, pluripotency, and tumorigenicity.[3]

Treatments for this type of primary brain tumour have not been satisfying. Previous researchers have found that a group of glioma patients of similar degree of malignancy may respond differently to radiotherapy treatments.[5] Some high grades glioma respond well to the treatments while others still show recurrences and resistance. Moreover numbers of them might have responded better than the lower grade glioma patients. On the other hand the activity of transcription factors such the Sox2 and Nanog, which are the biological markers of pluripotency to stem cells, are found to be higher on the high grade glioma.[6] These findings lead to the possibilities that there may have been another molecular biology factors causing the different behaviours of glioma, not merely its degree of malignancy based on histopathological studies. This research is conducted to analyze the correlation of c-Myc expression as another pluripotency marker towards the different degrees of glioma malignancy.

Methods

This research used cross sectional study done in Biochemistry and Molecular Biology Laboratory, Faculty of Medicine Universitas Indonesia from October 2014 until February 2015, using subjects of 20 glioma patients from the Neurology Department of Dr. Cipto Mangunkusumo National Hospital, selected previously by the postgraduate researchers based on consecutive method which fit the criteria. A normal brain cell was used as control and was obtained from normal tissues of a glioma patient. Samples were derived from previously extracted brain cells that had been frozen down. The inclusion criteria were patients form Neurology Department of RSCM and other hospitals in Jakarta that were diagnosed with glioma of grade I-IV (WHO classifications) using histological examinations and had undergone tumour surgery. The exclusion criteria was the laboratory results from anatomical pathology did not show glioma according to WHO classifications (grade I-IV). The degrees of glioma became the independent variable being measured, whereas the gene expression on PCR as the dependent one, with no confounding variable. This research had been approved by the Ethics Committee of the Faculty of Medicine Universitas Indonesia under an addendum of the research protocol of No. 207/H2.F1/ETIK/2014.

Analyzing Degree of Invasion

To obtain the data, first we analyzed the degree of invasion determined by the histopathological features through hematoxylin and eosin (HE) staining. According to the WHO classification glioma is differentiated into four grades. Grade I is the pilomyxoid astrocytoma (PMA), determined by the extrusive myxoid matrix and angiocentric arrangements of the tumor cells. Grade II gliomas are identified with diffused astrocytoma. Grade III is called the anaplastic (malignant) astrocytoma and lastly is the grade IV, which is called multiforme glioblastoma by which the infiltration is extensive.[4]

RNA Isolation

The RNA was isolated using Geneaid kit, which was done by the postgraduate researcher. As much as 500 μL solution was added to 50-100 mg glioma tissues to be homogenized. The cell homogenate was then stored at -80°C for 1 month before performing RNA extraction or directly isolated. The first step of the isolation is adding 100 μL chloroform to the cell homogenate, which was then mixed by shaking it back and forth for 15 seconds and incubated for 2-15 minutes at room temperature. To separate the solution into 3 phases centrifugation was done at 12.000g for 15 minutes at 4°C to fulfill the protein isolation. As much as 250 μL isoproponal was added at the clear phase for RNA precipitations to occur. It was then mixed by shaking the tube in an upside-down manner and incubated for 5-10 minutes at room temperature. Afterwards another centrifugation

was done at 12.000g for 10 minutes at 4°C. RNA palette was washed with 75% ethanol, followed by another centrifugation of 7.500g for 5 minutes. The supernatants were disposed and the RNA pallete was left dry by leaving the microtest tube open. The RNA pallete was then re-suspended in 50 µL of nuclease free water and incubated at 55°-60°C for 10-15 minutes. The RNA samples were then stored in a deep freezer - 80°C until further analysis.

cDNA Synthesis

The next step was to perform cDNA synthesis using AccuPower Cyclescript RT Premix (Bioneer®). As much as 1 µL of total RNA was added as the RNA template and 20 µL reaction volume was made with distilled water. The lyophilized transparent pellet was dissolved by vortexing and briefly spun down until dissolved completely. cDNA synthesis reaction was performed with single temperature reaction. The mix tube was placed in PCR machine programmed as 37~55°C for 60 minutes and 95°C for 5 minutes. cDNA was then stored at -20°C until further analysis.

Analysis of c-Myc Pluripotency

Finally the pluripotency of c-Myc was anlyzed by amplifying the cDNA using Accupower −Two-Step RT-PCR with SYBR Green (Bioneer®).

The primers used for c-Myc were 5'-AGAAT-GCTGTCCTCGCTGTT-3' (forward primer) and 5'-TTTCTTGCAGGCTTTGGTCT-3' (reverse primer), whereas 18S rRNA primers (as internal control) were 5'-AAACGGCTACCACATCCAAG-3' (forward primer) and 5'-CCTCCAATGGATCCTCGTTA-3' (reverse primer). For the experimental protocol, PCR (40 cycles) was run for 10 minutes in 95°C; 30 seconds in 59°C (for 18S primers) or in 57 ^{o}C (for cMyc primers), 30 seconds under 72°C. Melting curve was then analyzed for 1 minute in 95°C temperature, 1 minute in 55°C and 10 seconds at 55°C.

By using the RT PCR, the amount of amplification needed was used to determine cMyc gene expressions quantitatively. The rRNA 18S gene was used as an external standard. The amplification of 18S rRNA gene was done under a similar condition with cMyc. As a negative control the RNase free water was used to replace RNA in order to cross out any false positive results. The efficient value and cycle threshold (CT) was attained as a result of the RT PCR. The gene expression analysis was measured using the relative quantification hence the value of the relative mRNA concentration was retrieved using the Livak method.

Statistical Analysis

The parametric T-independent test analysis was used if the relative expression of each degree of malignancy normal using the log data transformation. The parametric measurement is used when there are two variables that are statistically independent. In this case, T-independent test may determine the statistical difference of relative expression of c-Myc gene on each degree of malignancy.

Results

The cMyc mRNA expression levels were examined from 20 human glioma with qPCR that has been normalized to 18S rRNA as a reference gene and calibrated with one normal human brain cell. Ten samples were classified as low grade glioma whereas the other 10 samples were high grade glioma. The PCR gives out cycle threshold (Ct) values for the mRNA within each sample and were used to determine the relative cMyc expression when calculated using Livak method. Beforehand the primers' suitability and specificity has been confirmed by the one-peak presentation in melting curve analysis. The result I of Ct value is shown in Table 1, where there is a tendency that the relative expressions of cMyc mRNA in high grade glioma samples is higher than the expression rates in low grade glioma. However, certain samples of high degree malignancy presents with a lower rate than the average low degree malignancy. On the other hand the low grade glioma group shows a higher rate of expression when compared to the average high grade glioma but is still lower than the highest expression rate in high grade glioma group.

Table 1. Comparison of mRNA Expression of cMyc in Glioma Tumor Tissue by qRT-PCR Analysis

High Degree Malignancy		Low Degree Malignancy	
Pathology	**Relative Expression**	**Pathology**	**Relative Expression**
Glioblastoma grade IV	10.06	Oligodendroglioma grade II	36232.58
Glioblastoma grade IV	7.16	Glioma grade II	14.37
Glioblastoma grade IV	137112.45	Oligoastrocytoma grade II	6.15
Glioblastoma grade IV	27.28	Xantroastrocytoma grade II	24833.5
Astrocytoma grade III	586.10	Astrocytoma fibrillary grade II	564.18
Anaplastic astrocytoma grade III	18242.30	Oligoastrocytoma grade II	5.39
Astrocytoma grade III	18242.30	Astrocytoma fibrillary grade II	10.59
Astrocytoma fibrillary grade II	1.01	Ganglioma grade II	0.33
Glioblastoma grade III	10.23	Astrocytoma fibrillary grade II	1.8
Astrocytoma polycistic grade III	3.45	Astrocytoma fibrillary grade II	4.59

Figure 1 represents the ratio of cMyc mRNA for each degree of malignancy and the expressions in normal cell. It shows that there is a vast discrepancy between the rate of cMyc expression in high grade and lower grade glioma where there has been 17424.23 times of expression rate in the high degree malignancy and 6167.35 times in low degree malignancy. To obtain the best normality during statistical analysis the data is transformed with log transformation. When applied to the T-independent test analysis, the p value acquired is 0.613.

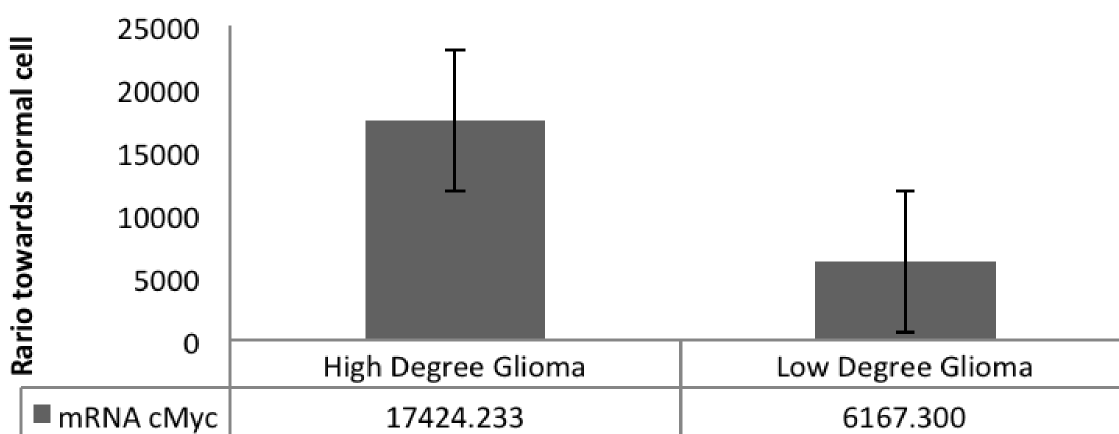

Figure 1. Relative Expression of cMyc mRNA (ratio) of the High Degree and Low Degree Malignancy of Glioma towards the Expression in Normal Brain Cell

Discussion

The mRNA expression level of cMyc were examined in 20 samples of human glioma cells and 1 sample of normal human brain cell with qPCR that has been normalized to 18S rRNA, a reference gene that resides in all cells and the expression is constant. In this experiment we only obtain 20 samples for the total samples used instead of 20 samples for each group because of the scarcity of glioma tissues available. Ten samples were classified as low grade glioma whereas the other 10 samples were high grade glioma. Moreover only 1 sample of normal brain cell is obtained because it is almost statistically impossible to attain the sample in a large amount. Cobb's[11] research had only used 5 samples of normal human brain cell as well in his research because not all glioma surgeries can invade the normal cells.

The high grade glioma shows 17424.23 times of expression than in normal brain cells and the rate of the low grade glioma is 6167.35 times. This shows the rate of cMyc mRNA expression is in fact clinically higher in accordance to the degree of malignancy. A research conducted by Guo et al[12] also confirms that the mRNA oct4 expression, a gene with pluoripotent capability, on human glioma cell goes in accordance to its degree of malignancy where high grade tumor have tendencies to have a higher mRNA gene expressions. Hardiany et al[13] in Bicochemistry & Molecular Biology at Faculty of Medicine Universitas Indonesia confirms that there is a high expression of MnSOD mRNA on high grade glioma has been maintained by the hypoxic environment. This is due to the high Reactive oxidative species produced during this condition and makes it possible for its sensitivity towards oxygen-requiring radiotherapies to be reduced.

Not every sample represents similar pattern to the average value between the two groups. For instance certain low grade glioma sample, a WHO grade II oligodendroglioma with a rate expression 36232.58 for one, has a higher rate cMyc mRNA expression than the relative expression of the high grade glioma but is still lower than the highest rate in the high degree malignancy. On the other hand, two samples of WHO grade III astrocytoma with a rate expression of 1.01 and 3.45 are relatively lower than the majority of the expressions in low grade glioma but are still higher than the lowest value in the low malignancy group. This versatile data may have contributed to the statistically insignificant result once applied to the T-independent test ($p>0.05$) despite the clinical tendency of a higher cMyc mRNA in accordance with degree of malignancy in this experiment. Among of the possible explanations of this variant and insignificant statistical result are the multiple pluripotent markers that influence differentiation of the tumor cells, an evidence for another better prognosis for the high degree malignancy, or merely an uneven distribution of malignancy within a tissue.

Pluripotency, the ability of genes as transcription factor to promote cell differentiation, may have been related greatly to the activity of cMyc expression. Because these genes have high ablilty to promote amplification of cancer cells, this might explain why certain low grade glioma has a higher rate of expression than the high grade glioma. There are several literatures suggest the high grade glioma, specifically WHO grade IV Glioblastoma, may be developed primarily or a result of a secondary development from the low grade glioma.[4] The result where certain low glioma cells have a higher rate of expression may be explained by this principle, by which they may be have the potentials to develop into the higher grade glioma. However, further cohort research is needed to prove that assumption.

Certain high grade glioma samples show lower rate of expression than the average rate on low grade glioma but are still higher than the lowest value in low degree malignancy. This may be an indicator that certain high grade gliomas might have good prognosis towards therapy. Aman et al[5] study on the variant responses of glioma patients towards radiotherapy had also proved likewise, where a few of the the high grade glioma patients gave better response towards therapy. Moreover, further cohort study is required to analyze the relationship between this pluripotency marker and radiotherapy response.

Conclusion

There is a clinical tendency where the c-Myc expression is higher in high degree glioma compared to low degree malignancy, however it is not statistically significant. Future research is recommended to conduct additional tests; pluripotency of transcription factors (Nanog, Sox 2, etc), hypoxia, and on cancer cells to determine another factors for the degree of malignancy of glioma other than histopathological method. Cohort study is needed to find the correlation between responds towards therapy and the survival rate of the patient.

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