Investigating the regulatory role of miR-663a in the tissue of patients with glioblastoma multiforme cancer compared to healthy tissue

Roghayeh roshani [1]

PhD student, Department of genetic, Faculty of biological sciences, Islamic Azad University, North Tehran branch, Tehran, Iran.

Fatemeh Ashrafi [2*]

Assistant Professor, Department of Microbiology, Faculty of biological sciences, Islamic Azad University, North Tehran branch, Tehran, Iran.

Elham moslemi[2]

Assistant Professor, Department of Biology, East Tehran Branch, Islamic Azad University, Tehran, Iran.

Hamid Raza khaledi[4]

Assistant Professor, Department of agriculture, yadegar-e-imam Khomeini (RHA) shahre rey branch, Islamic Azad University, Tehran, Iran.

* Name and address of Corresponding Author

E mail: F_ashrafi@iau-tnb.ac.ir

Abstract

Introduction

A new group of regulatory biomarkers, which are about 22 to 24 nucleotides long, are micro RNAs. Investigating the dysregulation of miRNA regulation in cancers has recently become a focus of research. These small structures can act as oncogenes and tumor suppressors. In the present study, the relationship between the expression level of miR-663a in the tissues of patients with glioblastoma multiforme and that of healthy individuals was investigated. Moreover, the correlation between the expression level of miR-663a and age, gender and tumor grade was done.

Materials and Methods

In this study, which was conducted on 50 patients with glioblastoma multiforme cancer and 50 patients as a control group, the genomic content of miR-663a was measured using Real-Time PCR technique. The biomarker value of miR-663a was evaluated using the ROC curve.

Results

The research results show that the expression level of miR-663a is higher in patient samples compared to healthy ones (Pv=0.0001). The increase in the concentration of miR-663a in serum samples in patients has a significant relationship with the final grade of disease progression of grade I and IV (Pv=0.0173). The curve analysis and the determination of the 6.145 cut-off level was found that miR-663a with the specificity of 0.78 and sensitivity of 1 can detect glioblastoma multiforme malignancy in the patients' serum samples compared to healthy individuals.

Discussion and Conclusions

In this study, the possible oncogenic role of miR-663a has been identified and it is possible to check the progress of glioblastoma cancer in the final stages by measuring its level in the serum of patients.

Keywords: Cancer, real-time-PCR, miR-663a, glioblastoma multiforme

Tob Regul Sci. ™ 2023;9(1): 562-572

DOI: doi.org/10.18001/TRS.9.1.42

Introduction

Glioblastoma multiforme (GBM) is the most frequent and invasive primary malignant tumor of the central nervous system that occurs in the brain or spinal cord. The tumor originates from astrocyte cells [1]. Sometimes, breast or lung tumors metastasize to the brain and cause cancer, but the origin of GBM tumor is in the brain itself [2]. In the WHO classification, astrocytoma is classified into four groups, which a grade four astrocytoma is glioblastoma multiforme [3]. The tumor prevalence is 2 to 3 people per hundred thousand people [4]. Glioblastoma accounts for 20% of all intracranial tumors and 60% of astrocytic tumors and it is more common in people over 35 years old and rare in people under 20 years old [5]. Several genetic factors in the creation of cancer have been significantly studied, among which long non-coding RNAs (lnc-RNAs) can be mentioned. The size of these non-coding molecules is less than 200 nucleotides. They do not have the ability to code proteins but they play an essential role in many biological processes. Moreover, their involvement in some diseases such as cancer [6], cardiovascular and diabetes [7] has been proven. DNA methylation, acetylation of histones and micro RNAs, as epigenetic mechanisms, play an important role in regulating gene expression [8]. In fact, micro RNAs are regulatory molecules with a length of 19-25 nucleotides that are the products of sh RNA or Pre-miR. These molecules play a vital role in biological reactions [9]. MiR-663a is a non-coding micro RNA in the form of steam loop and its location is on chromosome 20 (20p11.1). MiR-663a is involved in various biological activities such as autoimmune diseases, inflammatory responses in gastric and lung cancer. Furthermore, its activity as an oncogene in nasopharyngeal carcinoma and breast cancer has been proven [10]. Recently, studies have investigated the tumor suppressor role of miR-663 in glioblastoma [8, 11]. TGF-β1 regulates three important oncogenes including surviving, EGFR and MMP9 in glioblastoma. TGF-β induces ZEB1-dependent epithelial-mesenchymal transition (EMT) in glioblastoma, thereby promoting tumor

invasion [12]. The results indicate that AKT1 is a direct target of miR-633. Also, the expression of miR-633 is correlated with the negative level of AKT1 mRNA in CD+4T cells. Not only blocking AKT1 can directly downregulate mTOR, but also overexpression of AKT1 has the same effect. All results show that AKT1 regulates miR-633T, and in turn, activates the AKT/mTOR pathway [13]. In this regard, considering the importance and also the limited information in the field of this disease as well as the high costs of treatment to identify an appropriate biomarker, in this study we investigate the expression of miR-633 in glioblastoma multiforme cancer cells.

Materials and Methods

Sample preparation:

50 tissue samples with glioblastoma cancer in different stages of the disease along with 50 samples of healthy people were collected from Imam Khomeini and Shohada Tajrish hospitals in 1998 and 1997 in the age range of 26 to 77 years. It should be noted that 50 samples of people who referred with similar symptoms but were diagnosed as healthy or non-cancerous after examination were selected as the control group. The age range of the patient samples was between 26 and 77 years with an average of 49 years and a standard deviation of 0.3694. Demographic information of patients was collected through face-to-face interviews and medical record review. All subjects participated in this study with informed written consent, and all of them were assured that they will not suffer any financial or personal loss during the study. The process of its ethical steps was approved by the Ethics Committee of Azad University of North Tehran Branch with the code of ethics IR.IAU.TNB.REC.1400.051.

Finally, the collected samples were kept at -80°C until use.

RNA extraction:

In order to investigate the expression of the miR-663a gene, RNA extraction was performed using the AZMA RNrich tissue kit (ARE1101-1) with the relevant protocol. Also, 2% agarose gel was used to investigate the quality of extracted RNA, and to ensure the quantity, concentration, and OD of the samples, it was taken using NanoDrop (Nanodrop Technologies, Wilmington, DE, USA) and the purity and quality of RNA (the optimal ratio of OD 260/280 between 1.8-2) was considered.

cDNA synthesis:

One microgram of extracted total RNA was used as template for making cDNA. It should be noted that the cDNAs were synthesized using the cDNA synthesis kit (AESS 1211-2). In order to inactivate the RT enzyme, the reaction mixture in the reverse transcription stage was placed at 37°C for 15 minutes and at 85°C for 5 seconds and stored at -20°C.

Also, the sequence of primers for miR-663a and ACTB was sub-designed: the sequence of forward primers: TTGTTCCTTCCGGCGTCC and GTGCAGGGTCCGAGGT: Reverse, and also the ACTB gene as an internal control with the sequence of forward primers: ATGTACGTTGCTATCCAGGC and AGTCCATCACGATGCCAG Reverse: with a fragment length of 70 nucleotides were designed.

Relative real-time polymerase chain reaction

Real-Time PCR reaction was performed by SYBER Green method and using Real-Time PCR System StepOne (Applied Biosystems, Singapore). The real-time PCR reaction of the samples was carried out during 40 cycles and each cycle had 3 steps: activation step was performed at 95°C for 30 seconds, and then denaturation at 95°C for 5 seconds separated the two strands. Also, the final phase of primer annealing and extension was performed at 58°C for 30 seconds. To perform Real-Time PCR, Precision TM 2X and q PCR Master mix (Amplicon Denmark) were used. This test was performed for miR-663a and ACTB gene according to the protocol obtained from the qPCR test on patient and normal samples and the linear graph of the gene and sample multiplication curves was drawn by measuring the changes in the amount of fluorescence by the device.

It is necessary to explain that in the above method ACTB gene was used as an internal control and normal tissue samples were used for the purpose of calibration, which reduced the occurrence of errors in the research analysis and false negative cases were ignored in the test due to the presence of the internal control.

Statistical analysis

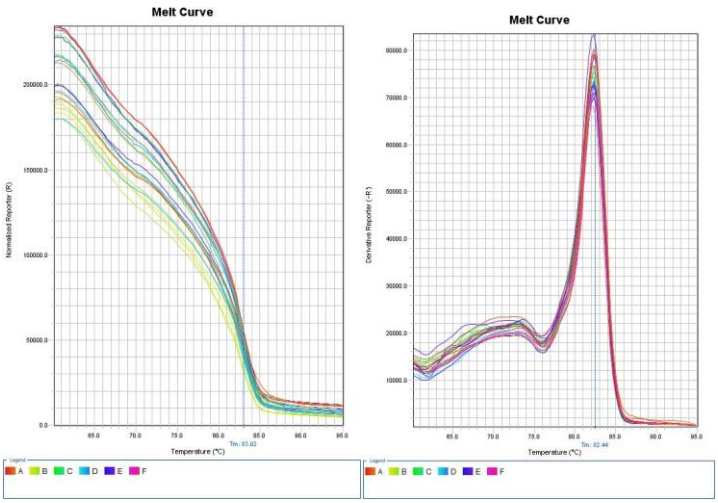
During amplification by Real-Time qRT-PCR method, raw data was obtained as CT using Real-Time PCR System StepOne (ABI, SINGAPORE). After performing the replication cycles, the raw threshold data (CT) for each of the mentioned genes was quantitatively examined for the relative expression of the target genes using the threshold cycle difference method (-ΔΔct). In this research, the GAPDH gene was used as a reference gene to calculate relative gene expression using Relative Quantification (RQ). The presented results are the result of two repetitions. The analysis of the expression of the studied genes and the analysis of CTs were done using GraphPad Prism 8. One-way ANOVA and t-test were used to analyze parametric data, and for data that did not follow Gaussian distribution, Mann-Whitney test and non-parametric Kolmogorov-Smirnov, Shapiro-Wilk, and Pearson tests were used to compare two groups of data. D'Agostino's test was also used to compare more than two non-parametric data groups. A significance level of $P \leq 0.05$ was considered, and in glioblastoma, the amount of changes in the expression of target genes was normalized with the expression of the internal control gene, and the expression level of the genes in the control or calibrator sample was quantitatively evaluated.

Results

	GBM	Normal	Total	Mir-663a mean RQ	P value

Melting curve diagram:

In the next step, by the StepOne Real-Time PCR Systems-Applied Biosystems device, the specificity of the primers and the fluorescence color (SYBER Green) and ensuring the amplification of specific fragments and the absence of non-specific fragments and primer dimers in the PCR product, examined by the melting curve diagram for miR- 663a and ACTB gene were drawn separately (Figure 1). Also, the correct binding of primers and their specificity to genes were confirmed. The results show that the PCR product obtained for the target gene is specifically amplified.



B A

Figure 1. The melting curve of 663a gene (B) in patient samples and the melting curve of ACTB gene (A) in healthy samples.

In the present study, the level of MicroRNA expression in the tissue of patients with glioblastoma multiforme and healthy individuals was studied. The analysis of the gene expression level on the tissues of the samples in comparison with the natural tissues was investigated using Quantitative relative real-time PCR technique. The patients in this study included 29 men and 21 women with an age range of 26-77 years. The clinical characteristics of the patients are presented in Table 1.

A. Age					0.7498
Number of person	50	50	100		
<50 years	16	20	36	5.162	1.586
>50 years	34	30	64	3.305	1.710
B. Mir-663a expression					
Mir- 663a mean RQ	3.004	1.427			
Max Mir-663a RQ	6.078	1.870			
Min Mir- 663a RQ	0.598	1.219			
C. Gender					0.6400
Male	29	30	59	2.792	0.884
Female	21	20	41	3.214	0.731
		50	100		
E. Grade					0.0320
I	15	-	15	0.541	2.714
II	11	-	11	1.203	2.310
III	13	-	13	0.775	3.287
IV	11	-	11	0.641	3.481

Table 1.Clinical characteristics of the subjects studied

Gene expression analysis results:

In the gene expression analysis, the data showed that the expression of miR-663a in the tumor tissue of the patients compared to the healthy subjects was not significantly related in the gender of the patients (P value = 0.6400) (Fig. 2).

Also, there was no significant correlation between age index and gene expression in the studied subjects (P value <0.7498) (Fig. 3).

The grade index in the examined patients shows that the expression of mir-663a in grades I and II of the disease is insignificant (P value <0.61) compared to grades I and III (P value <0.4321), but in the comparison of grades I and IV is significant with a significance level of P value < 0.0173 (Fig. 4).

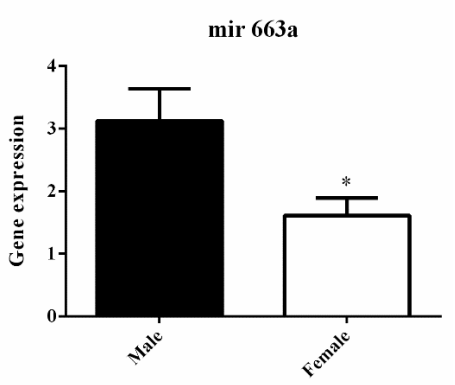


Figure 2. Investigating the expression of mir-663a in the samples of patients with glioblastoma cancer in men and women

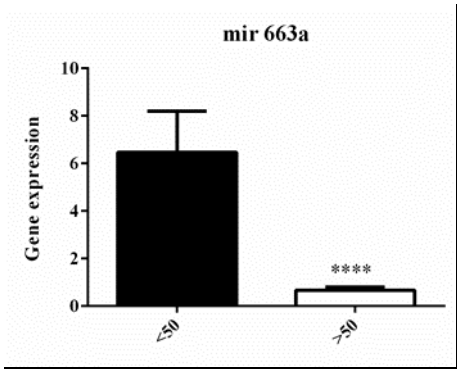


Figure 3. Examining the expression of mir-663a in the samples of patients with glioblastoma cancer in two age groups, more than 50 years and less than 50 years.

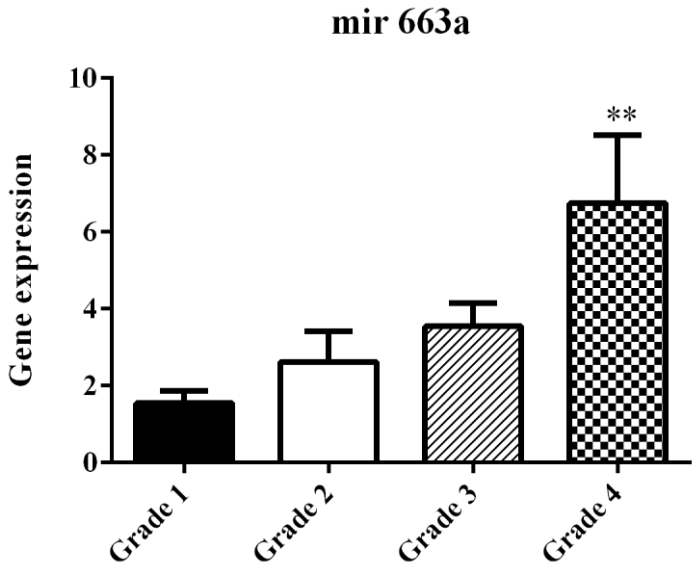


Figure 4. Investigating the expression of mir-663a in the samples of patients with glioblastoma cancer in grade I, grade II, grade III and grade IV

Also, the ability to evaluate the biomarker of this studied miRNA was done using the ROC curve. As shown in Figure 5, the area under the curve for miR-663a is 0.8860, p<0.0001 and CI=0.83. Also, its sensitivity and specificity were 0.78 and 1, respectively.

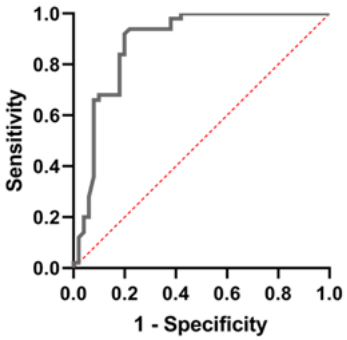


Figure 5. ROC curve analysis for mir-663a in 50 glioblastoma cancer patients and 50 control samples

The area under the ROC curve (AUC) indicates the accuracy in differentiating glioblastoma cancer patients from the control group in terms of sensitivity and specificity. The operator-receiver ROC (characteristic) curve analysis showed that the area under the curve for miR 663a is 0.8860 and it can well differentiate between patients and control subjects. By analyzing the curve and preparing the off-cut value for this biomarker, it was determined that this value for healthy

people can detect patients with a specificity of 0.78 and a sensitivity of 1, which can be known as a suitable biomarker due to its high sensitivity.

Discussion

In recent years, many studies have been conducted on the role of different microRNAs in the creation of tumor. It can be clearly stated that the role of these factors is important in the creation and development of cancer cells.

In this regard, this study compared the expression profile of miRNA named miR-663a between glioblastoma multiforme patient samples and healthy individuals matched in terms of age, gender and disease grade, and by using the Real Time PCR technique; a more integrated molecular network was created to identify interactions. Potential miRNA target involved in glioblastoma carcinogenesis was performed. The statistical results show that the expression in grades 1 and 4 is significant and insignificant in the age and gender indicators of the studied subjects. Researchers reported that downregulation of miR-663a is associated with NSCLC progression. MiR-663a suppresses proliferation and invasion by targeting the AP-1 JunD component in NSCLC cells. A decrease in miR-663a was observed in 42 of 62 lung cancer tissues compared to healthy adjacent tissues [14]. Research also shows that miR-663a plays a role in the development and progression of human cancers [15-17]. On the other hand, miR-663 has a high expression in lung cancer and helps the proliferation of lung cancer cells directly or indirectly through the regulation of TGF-β1, p53, Bax and Fas [18]. In contrast, recently, two studies demonstrated a tumor suppressor role of miR-663 in glioblastoma. Researchers report that miR-663 causes mitotic growth arrest in human gastric cancer cells [19]. Evidence shows that TGF-β1 is a direct target gene of miR-663, and miR-663 negatively regulates TGF-β1 protein expression in glioblastoma cell lines A172 and U87. It was reported that TGF-β1 is associated with various human cancers including glioblastoma [20]. In this regard, the expression of mir-663a in this study was significant in grade 4. Also, in some studies, the expression of miR-663 in lung cancer has been measured by reverse transcription-quantitative polymerase chain reaction method, and the results show that this molecule has low expression in tumor cells [21]. Along with these results, the expression of miR-663a in the present study did not change much compared to the indicators of gender and age and is meaningless.

Conclusions

According to the results obtained from the present study, it can be concluded that miR-663a is produced by glioblastoma tumor tissue. Also, its serum level is significantly higher in patients in grades I and IV compared to healthy people, thus it helps to differentiate between these two groups and it can also be said that the high expression level of miR-663a in the serum is directly related to the grade of tumor progression. As a result, measuring the level of miR-663a in serum

can be used as a non-invasive method in the early diagnosis of patients involved in the malignancy of glioblastoma multiforme.

Acknowledgment

The author thank Mohamad Javad Salehi and Institute of Engineering Education and Research(IEER) for Providing expenses and Dr.Alireza Ashofteh and Dr.Alireza Taherizadeh and Dr.Maryam Abbasi for remarkable contribution of this research.

Refrence

1. Young JS, Chmura SJ, Wainwright DA, Yamini B, Peters KB, Lukas RV.J et al. Management of glioblastoma in elderly patients.2017;380:250-255.
2. de Paula LB, Primo FL, Tedesco AC. Nanomedicine associated with photodynamic therapy for glioblastoma treatment. Biophysical Reviews. 2017:1-3.
3. van den Bent MJ, Smits M, Kros JM, Chang SM. Clin Oncol J. Diffuse Infiltrating Oligodendroglioma and Astrocytoma.2017;35(21):2394-2401.
4. Broestl L, Rubin JB, Dahiya S Fetal microchimerism in human brain tumors.Brain Pathol. 2017 .
5. Kleihues P, Burger PC, Scheithauer BW. Histological typing of tumours of the central nervous system, 2nd Ed. International Histological classification of tumours, vol 21. Berlin, Springer-Verlag, 1993.
6. Xing C, Sun SG, Yue ZQ, Bai F. Role of lncRNA LUCAT1 in cancer. Biomedicine & Pharmacotherapy. 2021 Feb 1;134:111158.
7. Strachan T, Read AP. Human Molecular Genetics. GS Garland Science:Taylor & Francis Group; 2004.
8. Nørøxe DS, Poulsen HS, Lassen U. Hallmarks of glioblastoma: a systematic review. ESMO open. 2016 Dec 1;1(6):e000144.
9. Jendrzejewski J, He H, Radomska HS, Li W, Tomsic J, Liyanarachchi S, et al. The polymorphism rs944289 predisposes to papillary thyroid carcinoma through a large intergenic noncoding RNA gene of tumor suppressor type. Proc Natl Acad Sci USA 2012; 109: 8646–51.
10. Pasmant E, Sabbagh A, Vidaud M, Bieche I. ANRIL, a long noncoding RNA, is an unexpected major hotspot in GWAS. FASEB J 2011; 25:444–48. 7
11. Cheetham SW, Gruhl F, Mattick JS and Dinger ME. Long noncoding RNAs and the genetics of cancer. Br J Cancer 2013; 108: 2419–25.
12. Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. CA Cancer J Clin 2010; 60(3): 166-93.

13. Motamed N, Jahanafrooz Z. microRNA-Based Anticancer Therapies. sjimu. 2014; 22 (4) :91-101.
14. Zhang Y, Xu X, Zhang M, Wang X, Bai X, Li H, Kan L, Zhou Y, Niu H, He P. MicroRNA-663a is downregulated in non-small cell lung cancer and inhibits proliferation and invasion by targeting JunD. BMC cancer. 2016 Dec;16:1-0.
15. Lehmann U, Hasemeier B, Römermann D, Müller M, Länger F and Kreipe H: Epigenetic inactivation of microRNA genes in mammary carcinoma. Verh Dtsch Ges Pathol 91: 214-220, 2007 (In German).
16. Tili E, Michaille JJ, Adair B, Alder H, Limagne E, Taccioli C, Ferracin M, Delmas D, Latruffe N and Croce CM: Resveratrol decreases the levels of miR-155 by upregulating miR-663, a microRNA targeting JunB and JunD. Carcinogenesis 31:1561-1566, 2010.
17. Zhang Y, Zhou X, Xu X, Zhang M, Wang X, Bai X, Li H, Kan L, Zhou Y, Niu H, et al: Waltonitone induces apoptosis through mir-663-induced Bcl-2 downregulation in non-small cell lung cancer. Tumour Biol 36: 871-876, 2015.
18. Liu ZY, Zhang GL, Wang MM, Xiong YN and Cui HQ: MicroRNA-663 targets TGFB1 and regulates lung cancer prolif- eration. Asian Pac J Cancer Prev 12: 2819-2823, 2011.
19. Pan J, Hu H, Zhou Z, Sun L, Peng L, Yu L, Sun L, Liu J, Yang Z and Ran Y: Tumor-suppressive mir-663 gene induces mitotic catastrophe growth arrest in human gastric cancer cells. Oncol Rep 24: 105-112, 2010.
20. Scarpa S, Coppa A, Ragano-Caracciolo M, Mincione G, Giuffrida A, Modesti A and Colletta G: Transforming growth factor beta regulates differentiation and proliferation of human neuroblastoma. Exp Cell Res 229: 147-154, 1996.
21. Wang G, Chen L, Jian W, Fang L. Low Expression of miR-663a Indicates Poor Prognosis and Promotes Cell Proliferation, Migration, and Invasion in Breast Cancer. Oncology Research and Treatment. 2021;44(3):119-27.