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AGR2 Gene Expression in Glioblastoma: A Novel Molecular Potential Target for Diagnosis and Treatment

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ABSTRACT

AIM: To assess anterior gradient protein 2 (AGR2) gene expression in patients with human glioblastoma (GBM) in comparison to levels in healthy brain tissues.

MATERIAL and METHODS: We evaluated the expression levels of AGR2 gene in 34 tissue samples: 29 of them were derived from patients with glioblastoma (GBM group) and 5 were derived from patients with mesial temporal lobe epilepsy (control group). Moreover, in order to demonstrate the AGR2 gene expression, we performed RNA isolation from tissue samples, cDNA acquisition from RNA via reverse transcription and the demonstration of gene expression via real-time polymerase chain reaction. We therefore confirmed findings of both groups.

RESULTS: The mean age of the GBM and control groups were 53.1 ± 12.82 years and 40.4 ± 10.92 years respectively. AGR2 gene expression levels of the GBM group were significantly higher than those of the control group ($p<0.01$). There were no significant differences of AGR2 gene expression levels across age groups, levels of glucose, urea, creatinine, white blood cell count (WBC), neutrophil, lymphocyte, hemoglobin, platelet, thyroid-stimulating hormone (TSH), T3 and T4 in GBM group ($p>0.05$).

CONCLUSION: AGR2 gene expression was significantly higher in patients with GBM. Thus, AGR2 gene can be considered as a potential therapeutic target.

KEYWORDS: Glioblastoma, GBM, Anterior gradient protein 2, AGR2, Gene

INTRODUCTION

Glioblastoma (GBM) is the most common malignant tumor of the central nervous system. Despite the existence of multimodal treatment options, it has poor prognosis with a 5-year survival rate of 5.5% (29). The surgical resection of GBM is not curative due to presence of the tumoral cells beyond the macroscopic view, leading to recurrence or progression, within the perilesional brain tissue (43). Although radiotherapy (RT) after surgery was standard treatment previously, addition of the temozolomide (TMZ) to RT caused better median survival (12.1 months for RT alone and 14.6 months for TMZ plus RT) (40). Despite these treatment options, the prognoses of patients are not satisfactory. Considering the complex pathogenesis of GBM, cellular pathways such as tyrosine kinase and signal transduction inhibitors or immunotherapy by the usage of monoclonal antibodies and vaccines has gained much attention (4). Thus, potential therapeutic targets for the treatment of GBM are in view.

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Anterior gradient protein 2 (AGR2) is the human orthologue of the *Xenopus laevis* protein XAG-2 (5). In *Xenopus,* AGR2 induces cement gland differentiation (1). In humans, AGR2 is a protein disulfide isomerase (10) and functions in protein binding (12). Moreover, it is strongly expressed in tissues with great secretory functions such as the lung, stomach, intestine and prostate (39). High expression of the AGR2 gene is associated with some cancers (20,35). However, very limited publications in the literature demonstrates the relationship between AGR2 gene and GBM, and all were based on cell culture. Thus, we investigated the AGR2 gene expression in the real human GBM tissues which has not been published previously, in order to enhance reliability.

MATERIAL and METHODS

This prospective study was performed in collaboration with the Department of Neurosurgery, Istanbul Haseki Training and Research Hospital; the Departments of Medical Genetics and Biochemistry of Balıkesir University Medical Faculty. We included patients with a Grade 4-World Health Organization (WHO) GBM in this study. The patients along with their relatives signed a standard consent form after debriefing of its content. The Ethical Committee of Balıkesir University Medical Faculty approved the study design with the registration number 94025189-050.03-10362 on 02/03/2018. All procedures were consistent with the Declaration of Helsinki.

Patient Population and Sample Collecting

From May 2018 to February 2021, we included 29 patients with pathologically confirmed GBM who needed surgical intervention. We excluded patients with additional malignancies, severe systemic or metabolic disease, infectious conditions and recent surgical history. Moreover, we did not include patients who refused participating in the study.

Healthy brain parenchyma tissues were used as control group to compare expression levels of *AGR2* in GBM samples. For this purpose, five patients who underwent an anterior temporal lobectomy and amygdalophippocampectomy due to mesial temporal lobe epilepsy were constituted our control group after applying the exclusion criteria.

Among the 34 tissue samples collected, 29 were derived from patients with GBM and 5 from control patient. All samples were stored in dry tubes at –80°C before RNA isolation.

RNA Isolation from Tissue Samples

RNA isolation from frozen tumor and control tissues was achieved using the TRIzol reagent (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Quality (purities and concentrations) of isolated RNA was measured using NanoDrop ND-2000c (Thermo Fisher Scientific, Inc., Wilmington, DE). All extracted RNAs were stored at –80°C till cDNA synthesis.

cDNA Synthesis and Quantitive Real-Time PCR

Reverse transcription of the RNA samples was performed using the TaqMan™ Reverse Transcription Reagents kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The relative expression levels of *AGR2* were measured through quantitative reverse transcription polymerase chain reaction (qRT-PCR) using the TAQMAN probe (AGR2 probe Hs00356521, ACTB Hs 99999903, Thermo Fisher Scientific, Waltham, MA, USA) in *Applied Biosystems™ 7500* Real-Time *PCR device.*

All reactions were performed in triplicate and data were analyzed through normalization with *B2M* (β-2 Microglobulin) housekeeping gene. The relative quantification analysis was performed using the "delta-deltaCt" method (22).

Statistical Analyses

NCSS (Number Cruncher Statistical System) (Kaysville, Utah, USA) program was employed for overall statistical analysis. We described the variables by presenting their mean, standard deviation, frequency, percentage, minimum, and maximum values. Shapiro Wilk test and box plot graphs were used to test the distribution of variables. Kruskal Wallis and Mann Whitney U tests were used for the intergroup comparisons of parameters that were non-normally-distributed. Spearman's correlation analysis was used to evaluate the relationships between variables. Statistical significance was set at $p<0.05$.

RESULTS

Demographic and Clinical Data

The GBM group comprised of 14 (48.3%) women and 15 (51.7%) men with a mean age of 53.1 ± 12.82 years ranging from 18 to 82 years. The control group consisted of 2 (40%) women and 3 (60%) men with a mean age of 40.4 ± 10.92 years ranging from 24 to 51 years.

In the GBM group, 16 (55.2%) patients experienced headaches, 7 (24.1%) experienced seizures and 6 (20.7%) experienced a paresis in the first admission. Moreover, 11 (37.9%) tumors were located in the frontal lobe, 11 (37.9%) in the temporal lobe, 6 (20.7%) in the parietal lobe and 1 (3.5%) in the occipital lobe.

In the GBM group, the mean of blood glucose level was 135.07 ± 52.49 *mg/dL* (range: 84–342 *mg/dl),* urea level was 37.66 ± 14.62 *mg/dL* (range: 12–79 *mg/dL*), creatinine level was 0.78 ± 0.18 *mg/dL* (range: 0.51–1.22). In complete blood

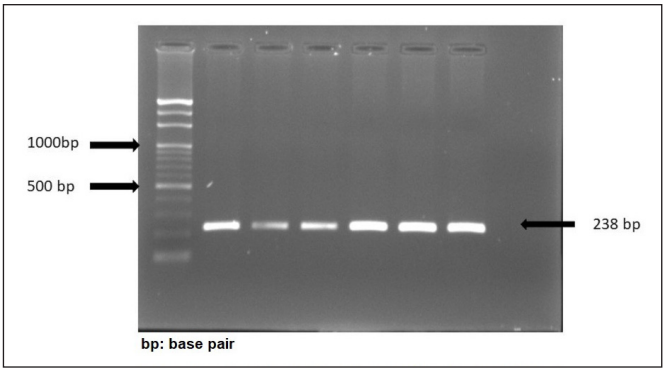

Figure 1: Polymerase chain reactions of control β2-microglobulin and synthesized cDNAs.

count, the mean WBC was 9.91 ± 10.94 x $10^3/\mu L$ (range: 4.57–59.81 × $10^3/\mu L$), neutrophil was 64.14 ± 18.63 × $10^3/\mu L$ (range: 16.6–93.0 × $10^3/\mu L$), lymphocyte was 24.69 ± 13.33 × $10^3/\mu L$ (range: 5.78–60.70 × $10^3/\mu L$), platelet was 264.25 ± 86.45 × $10^3/\mu L$ (range: 145.4–539.0 × $10^3/\mu L$), hemoglobin was 13.08 ± 2.23 *g/dL* (range: 7.69–16.90 × $10^3/\mu L$).

The mean levels of thyroid-stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) levels were 1.39 ± 1.60 *mU/L* (range: 0.46–1.42 *mU/L*), 3.04 ± 1.04 *ng/L* (range: 2.56–3.48 *ng/L*) and 1.21 ± 0.21 *ng/L* (range: 1.05–1.35 *ng/L*) respectively. There was coexistent diabetes mellitus (DM) in 6 (20.7%) patients and hypertension (HT) in 8 (27.6%) patients. Though a history of smoking was observed in 8 (27.6%) patients, no patient consumed alcohol (Table I).

The mean of the *AGR2* gene expression of the patient group (determined by accepting the mean of the control group as 1), was 13.86 ± 15.85 (range: 0.01–63.7). The GBM group expressed significantly higher mean *AGR2* gene levels than the control group ($p<0.01$) (Table II).

There was no significant difference of the AGR2 gene expression across age groups, levels of glucose, urea, creatinine, WBC, neutrophil, lymphocyte, hemoglobin, platelet, TSH, T3, and T4 in the GBM group ($p>0.05$) (Table III).

AGR2 gene expression levels were separately compared with sex, localization, coexistent DM or HT and smoking history. Each of these comparisons yielded differences in *AGR2* gene expression that was not significant ($p>0.05$) (Table IV).

DISCUSSION

Despite recent advances in neurosurgery in recent decades, the prognosis of the GBM is still poor. Despite multimodal treatments including surgery, radiotherapy and chemotherapy, the median survival of patients with GBM is less than 2 years (28). At this point, molecular investigations, focusing on various mechanisms such as angiogenesis or tumor suppressor genes of primary interest both to researchers and clinicians in this field.

GBM expresses angiogenesis, which is mostly regulated by hypoxia inducible factor-1 (HIF-1) (9). This explains why anti-angiogenic treatment has become an option for GBM owing to its considerably high expression of vascular endothelial growth factor (VEGF) and endothelial cell proliferation (24). Despite these theoretically rational anti-angiogenic treatments, there is no demonstrated agent in the treatment of GBM with promising survival owing to existing resistance mechanisms (38). Hong et al. investigated the *AGR2* expression and its relationship with hypoxia and how angiogenesis caused tumor progression in GBM cell lines (14). Firstly, they found out that AGR-2 and HIF-1α levels were elevated and induced by hypoxia. They investigated the effect of HIF-1α on the expression of AGR-2. When HIF-1α was knocked down by transfection of HIF-1α siRNA and $CoCl_2$ (a hypoxia-mimetic agent) was added into the milieu; elevated AGR2 response was not observed. As a result, they concluded that *AGR2* expression is regulated by HIF-1α. Lastly, the effects of

Table I: Clinical Data of GBM Patients

	Min-Max (median)	**Mean±sd**
Age (years)	18-82 (53)	53.1 ± 12.82
AGR2 expression levels	0.01-63.753 (8.4)	13.86 ± 15.85
Glucose (mg/dl)	84-342 (119)	135.07 ± 52.49
Urea (mg/dl)	12-79 (36)	37.66 ± 14.62
Creatinine (mg/dl)	0.51-1.22 (0.73)	0.78 ± 0.18
WBC (10^3/µl)	4.57-59.81 (8.44)	10.94 ± 9.91
Neutrophil (10^3/µl)	16.6-93 (60.45)	64.14 ± 18.63
Lymphocyte (10^3/µl)	5.78-60.7 (22.515)	24.69 ± 13.33
Haemoglobin (g/dl)	7.69-16.9 (13.13)	13.08 ± 2.23
Platelet (10^3/µl)	145.4-539 (264.2)	264.25 ± 86.45
T3 (ng/l)	2.56-3.48 (3.06)	3.04 ± 1.04
T4 (ng/l)	1.05-1.35 (1.19)	1.21 ± 0.21
TSH (mU/l)	0.46-1.42 (0.68)	1.39 ± 1.60
	n	**%**
Sex		
Female	14	48.3
Male	15	51.7
Localization		
Frontal	11	37.9
Occipital	1	3.5
Parietal	6	20.7
Temporal	11	37.9
Diabetes Mellitus		
(-)	23	79.3
(+)	6	20.7
Hypertension		
(-)	21	72.4
(+)	8	27.6
Smoking		
(-)	21	72.4
(+)	8	27.6
Alcohol consumption		
(-)	29	100
(+)	-	-

sd: *Standard deviation.*

Table II: AGR2 Gene Expressions of GBM and Control Groups

n		AGR2			
		Min-Max (Median)	**Mean ± sd**	**p**	
Group	GBM	29	0.01-63.7 (8.4)	13.86 ± 15.85	[a]***0.005*****
	Control	5	1-1 (1)	1 ± 0	

[a]*Mann Whitney U test,* ***p<0.01 **sd:** Standard deviation.*

Table III: The Comparison of *AGR2* Gene Expression and Other Parameters in GBM Group

	AGR2	
	r	**p**
Age (years)	0.032	***0.870***
Glucose (mg/dl)	-0.241	***0.209***
Urea (mg/dl)	-0.047	***0.809***
Creatinine (mg/dl)	0.205	***0.286***
WBC (10^3/µl)	-0.059	***0.760***
Neutrophil (10^3/µl)	-0.129	***0.513***
Lymphocyte (10^3/µl)	0.026	***0.894***
Haemoglobin (g/dl)	-0.029	***0.883***
Platelet (10^3/µl)	-0.017	***0.933***
TSH (mU/l)	0.111	***0.565***
T3 (ng/l)	0.259	***0.175***
T4 (ng/l)	-0.102	***0.606***

***r:** Spearman's correlation coefficient **sd:** standard deviation.*

AGR2 on the migration and formation of human umbilical vein endothelial cells were evaluated; a correlation was found with AGR2 expression for both parameters. When all findings were analyzed, they concluded that the *AGR2* is induced by hypoxia and has effects on tumor growth and angiogenesis. Thus, AGR2 could be considered as a new target for anti-angiogenic therapy. Similarly, in our study, the expression of AGR2 gene in patients with GBM was higher than that of normal glial control samples. Moreover, our findings could be more congruent as our study was the first real tumoral tissue-based study instead of cell culture.

Although the involvement of the Chemokine (C-X-C motif) Receptor 4 (CXCR4) pathway in GBM has been described (3,23); stromal cell derived factor-1 (SDF-1)-CXCR4 pathway, which has important role in the tumorigenesis of various cancers (7,41), was first investigated by Xu et al. for GBM (45). SDF-1 induced the expression of AGR2 and epithelial mesenchymal transition (EMT) markers in GBM cell lines. While the depletion of AGR2 supresses the SDF-1-induced upregulation of EMT markers; the knockdown of AGR2 led to cell cycle arrest in G0/G1, attenuated migration and invasion

Table IV: Assessment of *AGR2* Gene Expression in Different Patient Subgroups

	n	AGR2		[a]p
		Mean±sd	**Median**	
Sex				0.930
Female	14	10.52 ± 7.63	7.71	
Male	15	16.98 ± 20.67	8.54	
Localization				[b]0.917
Frontal	11	14.5 ± 18.89	5.28	
Occipital	1	10.63 ± 0	10.63	
Parietal	6	15.21 ± 13.25	12.4	
Temporal	11	12.79 ± 15.99	8.38	
DM				0.389
(-)	23	15.26 ± 17.03	8.54	
(+)	6	8.52 ± 9.38	6.98	
HT				0.696
(-)	21	14.87 ± 17.35	7.05	
(+)	8	11.23 ± 11.6	8.46	
Smoking				0.922
(-)	21	12.5 ± 13.67	8.38	
(+)	8	17.46 ± 21.25	9.89	

[a]*Mann Whitney U test,* [b]*Kruskal Wallis test,* ***DM:** Diabetes mellitus, **HT:** Hypertension, **sd:** Standard deviation.*

of cell lines. They concluded that AGR2-targeted therapy is a promising option. In our current study, the higher expression of AGR2 has been demonstrated in real human tumor samples but the further mechanisms of its pathway such as SDF-1-CXCR4 need to be investigated for better understanding and knowledge of treatment targets.

Alterations of tumor suppressor *p53* gene are linked to more progression (17), proliferation (8), invasion (6), and less apoptosis (32), in the pathogenesis of GBM. This is not linked with poor prognosis unlike other cancers (33). The interaction between *AGR2* and *p53* has been investigated by Pohler et al. (34) in Barret's epithelium which is a premalignant tissue. They reported that the Barret's epithelium overexpresses *AGR2*, causing an attenuation on tumor suppressor *p53*. Regarding the recent advances in p53 as new therapeutic targets in cancers including GBM (25), exploring the relationship between *AGR2* gene expression and *p53* mutations could be of great value in the treatment of GBM. Although our current study is the first report of overexpressed *AGR2* gene in real GBM tissues, further studies exploring other pathways are recommended.

P38 mitogen-activated protein kinase (MAPK) creates a balance between cell death and survival (36,47). The activation of p38 MAPK pathway leads to TNF-α-induced apoptosis in glioma cells (49). Moreover, p38 MAPK pathway causes autophagy inhibition and TMZ induced GBM cell death (48). Thus, various molecules such as hesperetin (18) or Sweroside (30) are considered antitumoral agents the modulation of the p38 MAPK pathway in the treatment of GBM. Although the p38 MAPK pathway and p53 are possible targets for GBM, Hrstka et al. showed that the *AGR2* oncoprotein had similar effects as p38 MAPK inhibitor and p53 (15). They concluded that the *AGR2* is a prognostic marker and molecular target for breast cancers. Considering that *AGR2* has not been explored in GBM, unlike in breast cancer, our study elucidates on the already-known mechanisms.

Although there is no clinical utility of *AGR2* as a target molecule for GBM, promising studies, many studies have contradicted this in recent years. Negi et al. investigated the effects of anti-AGR2 monoclonal antibody, mAb18A4, in the mice xenograft model for lung cancer (26). They reported that mAb18A4 inhibited lung cancer progression and metastasis with no major side effects on blood or organs. Moreover, inhibited proliferation and colony formation were demonstrated in the mAb18A4-treated cell lines to enhance p53 expression and apoptosis. In addition, this treatment resulted in reduced VEGF expression and neovascularization, thereby inhibiting AGR2-induced angiogenesis and angiogenesis-dependent metastasis. VEGF is one of the most important angiogenesis mediator of GBM and various strategies have been investigated to target VEGF or VEGF receptor-mediated angiogenesis (42), AGR2 could be considered as a novel target with its directly or angiogenesis-related involvement in GBM.

Liu et al. investigated the involvement of the microRNA-3647-5p (miRNA-3647-5p) in cervical cancer cell lines (21). They used the reverse transcription quantitative polymerase chain reaction (RT-qPCR) to modulate various genes and transcription factors. They concluded that the TP53 upregulates the miR-3647-5p and restrain the progression of cervical carcinoma via AGR2 inhibition. They also indicated this TP53/miR-3647-5p/AGR2 axis can be a new target for treatment of cervical cancer because of its apoptotic and antiproliferative effects. Considering the many GBM related miRNAs have been reported in the current literature (37) and the p53 is a well-known tumor suppressor gene, investigating the further pathways, and the role of AGR2 in these; can be beneficial for clinical usage of AGR2 as a diagnostic or therapeutic target.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an ubiquitous environmental pollutant and classified as a group 1 human carcinogen according to the International Agency for Research on Cancer (16). The aryl hydrocarbon receptor (AhR) is involved in the fulfillment of various effects of TCDD (11). Moreover, the TCDD causing attenuation of p53 response to DNA-damaging agents in hepatocellular carcinoma (HCC) cell lines was previously reported (31). In this respect Ambolet-Camoit et al. hypothesized the relation between p53 and TCDD/AhR pathways could be controlled by an AhR target gene (2); and selected the AGR2 as the novel gene because of its overexpression leads to attenuation of p53 serine phosphorylation (34). They suggested that the TCDD treatment induces the binding of the AhR to the endogenous AGR2 promoter, and concluded the AhR ligands such as TCDD might promote tumor progression via inhibition of p53 response, which is induced by genotoxicants, by the increased expression of AGR2 in HCC cell lines. Considering the environmental chemicals such as carbon tetrachloride create risk of GBM development (27), the overexpression of the AGR2 may have significant roles in the GBM formation or progression, which can ease by external factors, and the further studies are needed to understand these pathways.

Higher expression of highly upregulated in liver cancer (HULC), a long non-coding RNA (lncRNA), has been reported in glioma cells (46). Also, it is associated proliferation and colony formation capability of glioma cells. However, Zhu et al. suggested that the HULC had pro-angiogenic activity in glioma cells (50). Additionally, recent studies demonstrated the presence of an interaction between HULC and forkhead box M1 (FOXM1) in cancer development and progression (13,44). Li et al. hypothesized that HULC is involved in FOXM1/AGR2/HIF-1α regulatory axis and related with glycolysis and stemness of glioma cell lines (19). Their findings indicated that HULC promotes the FOXM1 protein by ubiquitination, resulting in upregulation of *AGR2* and HIF-1α. Knocking down HULC resulted in the inhibition of stemness and proliferation of glioma stem cells. They concluded that HULC stabilizes the FOXM1 expression and activation of FOXM1/AGR2/HIF-1α axis, thus promoting glycolysis and stemness of the glioma stem cells. Similarly, in our current issue, *AGR2* expression has been found higher than normal brain tissues. Thus, the relationship between expression levels of various oncogenes or lncRNAs with AGR2 can be investigated in further studies to understand the exact mechanism. This is useful in the treatment of glioblastoma, which is not still curable in clinical practice.

Limitations

We encountered two main limitations in this study. Firstly, the sample size was restricted for certain inferences. Secondly, the relationship between *AGR2* gene expression and glioma staging could not be demonstrated as all tumors were WHO grade 4. Further studies with wider patient groups including different grades of gliomas are highly recommended.

CONCLUSION

Our study is the first real human tissue-based study that investigates the expression of AGR2 gene in patients with GBM. Considering the insufficiency of current therapies for GBM, it is clear that the alternative treatment methods are necessary and can be achieved by further understanding of their underlying mechanisms. Molecular-based therapy gained attention in cancer research. Thus, AGR2 gene can be considered as a novel potential target for the treatment of GBM. Further studies are recommended to enhance knowledge on the mechanisms underlying AGR2 gene in the treatment of patients with GBM.

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AUTHORSHIP CONTRIBUTION

Study conception and design: HUA, SE, SK

Data collection: HUA, OT, OG

Analysis and interpretation of results: AO, AK, ASA, BG

Draft manuscript preparation: HUA, SE, SK, AO, AK

Critical revision of the article: SE, AK

All authors (HUA, SE, SK, AO, AK, ASA, OG, BG, OT) reviewed the results and approved the final version of the manuscript.

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