



Research Article

The Emphasis of Tumor Suppressor Genes and Oncogenes in Diagnosis and Prognosis of Anaplastic Brain Tumors

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Summary

The aim of the study is to determine the profiles of tumor suppressor genes and oncogenes which cause brain tumor, establishing the association between the prognosis of cancer and the quantitation of genetic and epigenetic changes, and bringing a molecular approach to definite diagnosis. For this purpose, explant cell cultures are performed from the anaplastic brain tumor tissues of the cases. The expression analysis of the tumor suppressor genes (p53, RB1, PTEN, MGMT, RUNX3, DMBT1, PIKE) and oncogenes (EGFR, PIK3CA, MDM2, Olig2, GSTT1, COX-2 and hTERT) were determined by comparing the expression of GAPDH housekeeping gene using real-time online RT-PCR. The promoter regions of all the tumor suppressor genes' hypermethylation and also methylated and unmethylated copy numbers were determined with Q-PCR by using methylation specific primer and probes and the quantitation was carried out by comparing with each other. A significant difference was determined among the oncogenes; EGFR and hTERT gene expressions in patient tumor group. hTERT gene expression showed a significant difference with tumor grades. DMBT1 gene expression showed a significant difference with tumor grades. A prominent decrease was found in the aberration of tumor suppressor gene copy number in the glioma group. Gene copy number and gene expression of GSTT1 gene showed a significant correlation. RB1 and MGMT promoter methylation showed a significant difference in tumor patient group. Over expression of PIK3CA, EGFR and COX-2 among oncogenes and loss of copy number of PTEN, RB1 and RUNX3 among tumor suppressor genes found associated with short survival.

Key words: Anaplastic Brain tumors, oncogenes, tumor suppressor genes

Anaplastik Beyin Tümörlerinin Tanı ve Prognozunda Tümör Süpresör Genlerin ve Onkogenlerin Önemi

Özet

Beyin tümörü tanısı almış olgularda; beyin tümörü fenotipine neden olan tümör süpresör ve onkogen profillerinin saptanması, ilgili genlerin genetik ve epigenetik değişimlerin kantitasyonu ile kanserin prognozu arasındaki korelasyonun belirlenmesi ve kesin tanı almamış olgularda tanıya moleküler bir yaklaşım getirilmesi amaçlanmıştır. Bu amaçla, anaplastik beyin tümörü tanısı almış olguların tümör dokusundan eksplant hücre kültürü yapılmıştır. Tümör süpresör genlerin (p53, RB1, PTEN, MGMT, RUNX3, DMBT1, PIKE) ve onkogenlerin (EGFR, PIK3CA, MDM2, Olig2, GSTT1, COX-2 ve hTERT) ekspresyon analizleri RT-PCR ile GAPDH geninin ekspresyonuna oranlanarak belirlenmiştir. Tümör süpresör genlerin promotör bölgenin metilasyonu, metilasyona spesifik primerler ve problemler

kullanılarak Q-PCR ile metile ve metile olmayan kopya sayıları belirlenip ve birbirlerine oranlanarak kantitasyonu gerçekleştirilmiştir. Tümör süpresör ve onkogenlerdeki kayıp ve kazanımlar için Q-PCR ile gen kopya sayıları elde edilerek, ortalama gen kopya sayısına oranlanarak kantitasyonları gerçekleştirilmiştir. Onkogenlerden; EGFR ve hTERT gen ekspresyonlarında olgu tümör gruplarında anlamlı fark saptanmıştır. hTERT gen ekspresyonu tümör dereceleri ile anlamlı fark göstermiştir. Tümör süpresör genlerden DMBT1 ekspresyonu tümör dereceleri ile anlamlı fark göstermiştir. Tümör süpresör gen kopya sayı aberasyonunda glioma grubunda belirgin bir azalma bulunmuştur. GSTT1 geninin, gen kopya sayısı ile gen ekspresyonu anlamlı korelasyonunda göstermiştir. RB1 ve MGMT promotör metilasyonu tümör olgu gruplarında anlamlı farklılık sergilemiştir. Onkogenlerden PIK3CA, EGFR ve COX-2' nin aşırı ekspresyonu ve tümör süpresörlerden PTEN, RB1 ve RUNX3' ün gen kopya sayılarındaki kayıplar kısa sağ kalımla ilişkili bulunmuştur.

Anahtar Kelimeler: Anaplastik beyin tümörleri, onkogenler, tümör süpresör genler

INTRODUCTION

Brain cancer constitutes approximately 1.4% of all cancer types and 2.3% of those cancers that result in death. More than 35,000 Americans (approximately 6/100,000) were diagnosed as having brain tumor in 2002. The annual rate of mortality is 13.000/year according to the data obtained from the groups meeting the classified requirements. Approximately 16.800 cases with brain tumors have been diagnosed as being malignant every year using today's advanced diagnosis methods. However, cases diagnosed as benign and are treatable interfere with the normal brain functions that are necessary for a normal life⁽¹⁾. The merciless picture prevailing for the patients, the destructive effect of even treatable low grade pediatric cancers and other benign ailments caused research on brain cancer to gain a new acceleration.

Primary brain tumors constitute a fairly heterogeneous group of all diseases. For this reason, there is an increasing need for appropriate classification criteria and molecular tumor markers to characterize the heterogeneous categories of brain tumors as clinically homogeneous subgroups⁽²⁾. This and other concepts corroborate the idea that brain tumors do not have a single cause in the etiology of this disease. The molecular characterizations and the fact that their

subtypes consist of a group of cells with similar molecular lesions may help researchers to explain tumors. Examination of genes or protein sequences may largely contribute to the classification of the tumor etiology by dividing it into more homogeneous groups according to its lesions or diagnostic significance. The major difficulty that may be encountered when interpreting this molecular information is to differentiate as to which changes are the first signs of its etiological significance and which ones are the later signs that lead to clinical and prognostic outcomes. The genetic and molecular epidemiologic methods help us obtain complex information on the molecular change of a tumor^(3,4,6).

Works towards discovery of relevant tumor markers and susceptibility genes attract neurosurgeons, oncologists, pathologists, epidemiologists and medical biologists to come together to carry out molecular epidemiologic researches on primary brain tumors and their causes. Primary brain tumors are caused by potential exogenous and endogenous events⁽⁹⁾. Although brain malignancies are destructive conditions, the works towards explaining their causes are promising and molecular approaches enable us to understand the new concepts on neuro-oncogenesis. In the future, the survival rates in patients with brain tumors are expected to increase with the use of

molecular-based treatment options in connection with specific diagnostic genetic and expression profiles⁽⁸⁾. The quantitation of tumor suppressors and oncogenes at the gene and transcription levels and demonstration of the relationship between the two as intended in the present study seems to be the first time in the literature and constitutes the specificity of the study. Moreover, the quantitation of epigenetic regulations of tumor suppressor genes as covered by the study is also for the first time. Given that the survival rate is as low as 20% for the first year following the diagnosis, it is also targeted to determine the correlation between the profiles of tumor suppressors and oncogenes, and the rate of disease-free survival to identify optimum treatment protocols to contribute to the economy by decreasing the treatment costs and to the humanistic values by increasing the patients' potential life times and quality of life.

MATERIAL AND METHODS

Subjects and Cell Lines

One hundred subjects with a diagnosis of brain tumors at the Neurosurgery Department of Ege University were included in the study. Tumor samples were taken during routine biopsies for diagnosis or during operations where samples were resected from the subjects who agreed to take part in our study and signed an informed consent form. Some of the tumor samples underwent concurrent histopathologic examination at the Pathology Department to identify the type and grade of the tumor. To ensure a controlled progress of the study, cell lines U87MG (glioblastoma, astrocytoma, grade IV), U-118MG (glioblastoma, astrocytoma), LN-18 (brain cerebrum; right temporal lobe, grade IV, glioblastoma; glioma), Daoy (brain, cerebellum desmoplastic cerebellar medulloblastoma) were used for positive control and cell lines WI-38 (normal lung fibroblast) and Hs 605.SK (normal, skin fibroblast) for negative control.

Tumor Cell Cultures

BIOAMF-1 (Biological Industries), BIOAMF-1 supplement, 2mM L-glutamine and 1% Penicillin-Streptomycin were used as culture medium for tumor tissues and control cells. They were incubated in an incubator at 37°C with 95% humidity and 5% CO₂ until adequate reproduction was obtained from the cells. Total RNA and DNA were isolated from the patients and control cells.

Total RNA Isolation

To evaluate gene expression, a total RNA isolation was conducted to extract RNA from the tumor cells and cell lines using a High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany).

Reverse Transcriptase RT-PCR

Complementary DNA (cDNA) syntheses were performed on the total RNAs obtained from tumor samples to an amount of 1-5 µg/ml using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). The expression analyses of the tumor suppressor genes and oncogenes used in our study were conducted on Real Time Online RT-PCR 2.0. The primers and probes used for expression analysis in our study are shown in the table (Table 1-2). To quantify gene expressions, a calibration curve was constructed using copy numbers 10⁸, 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10², and 10 of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Error: 0.100, Efficiency: 1.790). The expression results were proportioned to the GAPDH gene (housekeeping gene) expressions to calculate relative expression ratios. The relative ratio (RR) of gene expressions was calculated using the formula below:

$$RR = \text{gene copy numbers} / \text{GAPDH copy numbers} \times 10000$$

Gene Copy Numbers

Adhering to the kit manual, DNAs were isolated from the cells cultured for analysis of the gene copy numbers of tumor suppressor genes (patients and cell lines)

using the High Pure PCR Template Preparation Kit for Genomic DNA (Roche Diagnostics, Germany). The gene copy numbers of tumor suppressor genes and oncogenes were quantified by the real-time online PCR using primer and UPL probes. The primers and probes from which we would calculate the gene copy numbers were designed using the "ProbeFinder Software". The relative gene copy ratio of each oncogene / tumor suppressor gene was found by dividing the logarithm of the gene copy number by the logarithm of the mean gene copy number of oncogene / tumor suppressor genes.

Quantitation Analyses of Hypermethylation of Tumor Suppressor Genes

Since there was no study in the literature relating to the real-time online PCR quantitation of the tumor suppressor genes with the exception of DMBT1, we had to design the methylated and non-methylated primers and probes. To spot the methylated areas, a DNA denaturation and bisulphate conversion procedure was conducted using an EZ DNA Methylation-Gold Kit® (Zymo Research). The quantitative analysis of the alleles methylated as described above was carried out using the Taqman method on the real-time online PCR.

Statistical Analysis of Gene Quantitation Values

The expressions and copy number changes of tumor suppressor genes and oncogenes in anaplastic brain tumors and the averages, standard deviations, minimum and maximum values of promoter region methylations of tumor suppressor genes, as well as 95% confidence interval were examined using descriptive statistics. Its existence in tumor groups using one-way variance analysis, the mutual interaction of genes using correlation analysis, the comparison of gene copy numbers to gene expressions using regression analysis, the comparison of the degrees of promoter region methylations to the tumor groups

using χ^2 test, the comparison of all the parameters to the tumor patient groups using discriminant analysis and the relation between the tumor suppressor genes and oncogene expressions and survival using Kaplan-Mayer analysis. The parametric and non-parametric tests performed were analyzed on SPSS v.15.0 statistical analysis software. The level of significance was set at $p < 0.05$.

RESULTS

The subjects' demographic characteristics are given in Table 3 and their clinical characteristics in Table 4. The subjects were composed of 45 female (45.92%) and 53 male (54.08%) patients with a mean age of 49.90 ± 15.02 (ranging from 14 to 78).

Gene Expression Analyses

Oncogenes

Oncogenes were made subject to expression analyses using EGFR (epidermal growth factor receptor), PIK3CA (phosphoinositide-3 kinase, catalytic, alpha polypeptide), MDM2 (transformed 3T3 cell double minute 2, p53 binding protein), Olig2 (oligodendrocyte transcription factor 2), GSTT1 (glutathione S-transferase theta 1), COX-2 (cyclooxygenase 2) and hTERT (human telomerase reverse transcriptase) compatible primers and employing the real-time online RT-PCR using the UPL Taqman method and a quantitation of the gene expressions was made. When gene expressions of oncogenes were evaluated, no statistically significant difference was found in the comparison of PIK3CA, MDM2, GSTT1, OLIG2 and COX2 gene expressions to the tumor groups ($p > 0.05$). A statistically significant difference was found when EGFR gene expression was compared to the tumor groups ($p = 0.0034$). The EGFR gene expression in the schwannoma group shows a statistically significant difference with oligodendroglioma ($p = 0.0049$), with diffuse astrocytoma ($p = 0.0023$) and with glioblastoma ($p < 0.0001$). Similarly, a

statistically significant difference was found when the hTERT gene expression was compared to the tumor groups ($p=0.0006$). The hTERT gene expression in the pilocytic astrocytoma group shows a statistically significant difference with oligodendroglioma ($p=0.0049$), with anaplastic astrocytoma ($p<0.0001$), with oligodendroglioma ($p=0.0004$), with diffuse astrocytoma ($p=0.0028$), with glioblastoma, indifferently malignant tumor ($p=0.0017$), with carcinoma metastasis ($p=0.0017$), with meningioma metastasis ($p=0.0007$), with oligodendroglioma ($p=0.0070$) and with schwannoma ($p=0.0108$).

When the changes in oncogene expression were evaluated according to tumor grades, the hTERT gene expression was found to be the lowest at grade I and the highest at grade III (Table 5). A statistically significant difference was observed when the tumor grades were compared to the hTERT gene expression ($p=0.0339$).

Tumor suppressor genes

Tumor suppressor genes were made subject to expression analyses using primers compatible with DMBT1 (gene deleted in malignant brain tumors 1), MGMT (O-6-methylguanine-DNA methyltransferase), PIKE (phosphatidylinositol-3-kinase enhancer), (CENTG1)), PTEN (Phosphatase and tensin homolog, MMAC-1), RB1 (Retinoblastoma), RUNX3 (Runt-related transcription factor 3) and p53 compatible primers and UPL (Universal Probe Library, Roche) probes and employing the real-time online RT-PCR using the UPL Taqman method and a quantitation of the gene expressions was made. The glyceraldehyde 3-phosphate dehydrogenase gene was used as the "housekeeping" gene in tumor suppressor gene and oncogene expressions.

When DMBT1, MGMT, PIKE, PTEN, RB1, RUNX3 and p53 gene expressions were compared to the tumor groups, no statistically significant difference was

found ($p>0.05$). However, the DMBT1 tumor suppressor gene expression showed a statistically significant difference with the tumor groups of glioblastoma, pilocytic astrocytoma ($p<0.0001$) and schwannoma ($p=0.0221$) and meningioma with the pilocytic astrocytoma group ($p=0.0162$).

When the tumor suppressor genes were classified according to the tumor grades, the DMBT1 gene expression showed a statistically significant difference ($p=0.0047$). Grade I and Grade IV also showed a significant intra-group difference ($p=0.012$) (Table 6).

Changes in Gene Copy Numbers

Changes in Oncogene Copy Numbers

No significant difference was observed in the distribution of oncogene copy numbers among the tumor groups. However, a statistically significant difference ($p=0.0018$) was found between OLIG2 gene copy number Ependymoma and Pituitary adenoma ($p=0.0378$), between Ependymoma and Indifferently malignant tumor ($p=0.0092$), between Glioblastoma and Pituitary adenoma ($p=0.0334$) and between Glioblastoma and Indifferently malignant tumor ($p=0.0065$). In terms of hTERT gene copy number, a statistically significant difference was found between the groups of Ependymoma and Diffuse Astrocytoma ($p=0.0030$), between Ependymoma and Carcinoma Metastases ($p=0.0100$), between Ependymoma and Meningioma ($p<0.0001$) and between Glioblastoma and Carcinoma Metastases ($p=0.0022$).

Changes in Tumor Suppressor Gene Copy Numbers

A distinct decrease was seen in the tumor suppressor gene copy numbers in the glioblastoma group. There was a statistically significant difference for DMBT1 gene with Glioblastoma Pituitary Adenoma ($p=0.0003$) and Meningioma ($p=0.0170$). There was a significant change for PIKE gene with Glioblastoma Pituitary Adenoma ($p=0.0003$), Indifferently

Malignant tumor ($p=0.0065$), Carcinoma Metastases ($p=0.0004$), Meningioma ($p=0.0405$) and Schwannoma ($p=0.0273$); for PTEN with Carcinoma Metastases ($p=0.0014$); for RB1 with Carcinoma Metastases ($p=0.0027$); for RUNX3 with Indifferent Malignant tumor ($p=0.0010$), Carcinoma Metastases ($p=0.0002$) and negative control cell line ($p=0.0041$). A statistically significant difference was found between Pituitary Adenoma and Oligodendroglioma ($p=0.0133$) in the MGMT gene and between Carcinoma Metastases and Meningioma ($p=0.0180$) in RUNX3 gene.

Quantitation Analyses of Tumor Suppressor Gene Hypermethylation

When the methylation analyses of tumor suppressor genes are evaluated, the quantitation of methylation in the promoter region of RB1 gene shows a statistically significant difference in methyl / non-methyl ($p=0.0247$) and methyl / mixing ratios ($p=0.0134$) in tumor-bearing groups. The methylation in the promoter region of p53 gene was interestingly found to be very low in all patient groups and a statistically significant difference was found between the patient groups ($p=0.0007$). The promoter region of MGMT gene showed a different methylation pattern in tumor-bearing groups and there was a statistically significant difference between the groups ($p=0.0007$).

The quantitation of methylation of the promoter regions of tumor suppressor genes was graded as negative (-) for the values up to the ratio of Methyl / Non-Methyl became 0, those from 0 to 0.49, those between 0.50 and 0.99, those between 1.00 and 1.99 and any quantitation value equal to or more than 2 as “+”, “++”, “+++”, “++++”, respectively. When tumor-bearing groups are compared according to methylation grading as shown above, a statistically significant increase was found in the methylations of RB1 and MGMT genes ($p<0.05$).

Oncogene and Tumor Suppressor Gene Expressions and Copy Numbers and Distinctiveness of Tumor Suppressor Gene Hypermethylation

In defining tumor-bearing groups as a quadruple parameter, tumor suppressor gene and oncogene expressions, gene copy numbers and gene promoter region methylations (methyl and non-methyl) were classified as Neuroepithelial Tissue Tumors [(Astrocytic Tumors – Group I), (Oligodendroglial Tumors – Group II), (Oligoastrocytic Tumors – Group III), (Ependymal Tumors – Group IV)], Cranial and Paraspinal Nerve Tumors (Schwannoma – Group V), Tumors of Meninges (Tumors of Meningothelial Cells / Meningioma – Group VI) and Metastatic Tumors – Group VII. According to the discriminant analyses made, Group I was classified 96.2% correctly with 4 parameters and its distinctive power was statistically significant ($p=0.028$). Two cases in this group were found to be more suitable for a manifestation of Diffuse Astrocytomas within this classification. Group II was classified 100% correctly and its distinctive power was statistically significant ($p=0.004$) (Table 7).

Relationship of Oncogenes and Tumor Suppressor Genes with Survival

In oncogene expression, any value of each gene above the tumor-bearing group average was considered as positive. When the relationship between the oncogene positivity and survival was examined, an excessive expression of PIK3CA, EGFR, OLIG2 and COX-2 genes was significantly related with short survival ($p<0.05$) (Table 8).

Any gene copy number of each tumor suppressor gene below the patient group averages was considered as a copy number aberration. When the relationship between the copy number aberration and survival was examined, PTEN, RB1, RUNX3 and p53 gene aberrations were found to show a statistically significant relationship with short survival ($p<0.05$) (Table 9).

The oncogene values above the average expression values in tumor-bearing groups were categorized as positive and those below them as negative and an oncogene positivity scale was constructed by cumulatively adding positivities for each gene. When survival of the cases was analyzed using this scale, the mean survival time of cases which were not positive for any oncogene (oncogene 0) was calculated as 79.00 ± 3.54 months. The mean survival time was found as low as 6.00 ± 0.8 months in cases where all

oncogenes were positive (oncogene 7). The overall survival in these cases was found to be 24.46 ± 3.07 months. The difference between oncogene positivity and survival was found to be statistically significant (log rank=23.63; $p=0.001$) (Table 10).

A similar scale was constructed for tumor suppressor positivity. No statistically significant relationship was observed between survival and tumor suppressor positivity (log rank=9.00; $p=0.252$) (Table 11).

Table 1: Relevant primers and probes identified for tumor suppressors

Gene	Forward Primer	Reverse Primer	Probe
DMBT1	gctcaggaaaccatctatcgac	gaagcctccgcaggaatagt	ctgctcct
MGMT	gcccttagaacgctttgc	ttgtccattttccaagtacca	ccgccgcc
PIKE	aacaccatccaagactgaagg	aggatttttagttccacattttgc	cttcagcc
PTEN	ggggaagtaaggaccagagac	tccagatgattctttaacaggtagc	ttcccagt
RB1	cagaataatcacactgcagcagata	cacgcgtagttgaacctttt	caggagaa
RUNX3	tcagcaccacaagccactt	aatgggttcagttccgaggt	gcagccag
TP53	ccccagccaagaagaagaaac	aacatctcgaagcgctcac	ggatggag
GAPDH	gaaggtgaaggtcggagtc	gaagatgggtgatgggatttc	caagcttcccgttctcagcc

Table 2: Relevant primers and probes identified for oncogenes

Gene	Forward Primer	Reverse Primer	Probe
COX-2	tcacgcatcagttttcaaga	tcaccgtaaataatgatttaagtcac	gggctggg
EGFR	cagccacccatagtaccatc	aacttgggacgactatctgc	gctggatg
GSTT1	acggggacttcaccttgac	gaccttatattgcgcgtcag	tcctgctc
hTERT	gccttcaagagccacgctc	ccacgaactgtcgcatgt	ctccagcc
MDM2	ccatgatctacaggaacttgtagta	tcactcacagatgtacctgagtc	cagcagga
OLIG2	tctctctttctcctctctct	atagtcgtcgcagctttcg	ggaaggag
PIK3CA	cacgagatcctctctctgaaatc	ggtagaatttcgggatagttaca	caggagaa

Table 3: Demographic characteristics of patient groups with brain tumors

Sex	Number	Age	
		Average	Standard Deviation
F	45	50.22	14.69
M	53	49.62	15.42
Total	98	49.90	15.02

Table 4: Clinical characteristics of patients

Diagnosis	Incidence	%	Cumulative %
Anaplastic Astrocytoma	3	3.06	3.06
Anaplastic Oligoastrocytoma	2	2.04	5.10
Anaplastic Oligodendroglioma	6	6.12	11.22
Diffuse Astrocytoma	7	7.14	18.37
Ependymoma	2	2.04	20.41
Glioblastoma	41	41.84	62.24
Pituitary Adenoma	2	2.04	64.29
Indifferent Malign Tumor	2	2.04	66.33
Carcinoma Metastasis	2	2.04	68.37
Meningioma	19	19.39	87.76
Oligodendroglioma	5	5.10	92.86
Pilocytic Astrocytoma	2	2.04	94.90
Schwannoma	5	5.10	100.00
Total	98	100.00	

Table 5: Changes in oncogene expression according to tumor grades

	Grade	N	Mean	SD	95% Confidence Interval for Mean		Minimum	Maximum	p
					Lower bound	Upper bound			
PIK3CA	I	28	0.7287	0.2657	0.6256	0.8318	0	1.0097	0.4642
	II	14	0.7570	0.1986	0.6423	0.8716	0.3058	1.2304	
	III	11	0.8576	0.1307	0.7698	0.9454	0.7364	1.0895	
	IV	41	0.7944	0.2644	0.7110	0.8779	0.2519	1.9320	
	Total	94	0.7767	0.2441	0.7267	0.8267	0.0000	1.9320	
MDM2	I	28	0.8196	0.2274	0.7314	0.9077	0.0000	1.2457	0.9763
	II	14	0.8120	0.1286	0.7378	0.8863	0.6026	1.1781	
	III	11	0.7773	0.0668	0.7324	0.8222	0.7173	0.9606	
	IV	41	0.8081	0.3381	0.7014	0.9148	0.0000	1.9349	
	Total	94	0.8085	0.2591	0.7554	0.8615	0.0000	1.9349	
GSTT1	I	28	0.7078	0.4421	0.5364	0.8793	0.0000	1.3350	0.5837
	II	14	0.7013	0.4038	0.4682	0.9345	0.0000	1.2713	
	III	11	0.4976	0.4676	0.1835	0.8118	0.0000	1.1168	
	IV	41	0.6964	0.4747	0.5466	0.8462	-0.2847	1.9932	

	Total	94	0.6773	0.4520	0.5847	0.7699	-0.2847	1.9932	
EGFR	I	28	0.7516	0.6738	0.4903	1.0129	-2.3313	1.2654	0.4558
	II	14	0.8823	0.1675	0.7856	0.9790	0.6997	1.3648	
	III	11	0.8796	0.1313	0.7915	0.9678	0.7351	1.2024	
	IV	41	0.9179	0.2966	0.8242	1.0115	0.0967	2.1847	
	Total	94	0.8586	0.4249	0.7715	0.9456	-2.3313	2.1847	
OLIG2	I	28	0.2161	0.3371	0.0853	0.3468	-0.1795	0.9674	0.4922
	II	14	0.2419	0.2915	0.0736	0.4102	0.0000	0.8514	
	III	11	0.3563	0.2637	0.1791	0.5334	0.0000	0.8316	
	IV	41	0.3190	0.3468	0.2095	0.4285	-0.1352	1.3038	
	Total	94	0.2812	0.3269	0.2143	0.3482	-0.1795	1.3038	
COX2	I	28	0.4262	0.4490	0.2521	0.6003	-0.9695	1.2517	0.6003
	II	14	0.5308	0.3246	0.3434	0.7182	-0.0904	1.2342	
	III	11	0.4161	0.3072	0.2097	0.6225	-0.3170	0.7399	
	IV	41	0.2530	1.0217	-0.0695	0.5755	-4.7487	1.3510	
	Total	94	0.3651	0.7372	0.2141	0.5161	-4.7487	1.3510	
hTERT	I	28	-0.0366	0.6136	-0.2745	0.2013	-2.9655	1.0000	0.0339
	II	14	0.0519	0.1172	-0.0158	0.1196	0.0000	0.3948	
	III	11	0.3332	0.3012	0.1309	0.5356	0.0000	0.8399	
	IV	41	0.2280	0.3784	0.1086	0.3474	-0.2160	2.0198	
	Total	94	0.1353	0.4482	0.0435	0.2271	-2.9655	2.0198	

Table 6: Classification of tumor suppressor genes according to tumor grades

95 % Confidence Interval for Mean									
	Grade	N	Mean	SD	Lower bound	Upper bound	Minimum	Maximum	p
DMBT1	I	28	0,2786	0,3003	0,1622	0,3951	0,0000	0,8684	0,0047
	II	14	0,3038	0,2350	0,1681	0,4395	0,0000	0,6767	
	III	11	0,3396	0,2033	0,2030	0,4762	0,0000	0,6184	
	IV	41	0,5320	0,3482	0,4221	0,6419	0,0000	1,3489	
	Total	94	0,4000	0,3232	0,3338	0,4662	0,0000	1,3489	
MGMT	I	28	0,4976	0,4032	0,3413	0,6540	-0,0109	1,2451	0,6256
	II	14	0,6222	0,2758	0,4629	0,7814	0,0000	1,1614	
	III	11	0,7815	0,2103	0,6403	0,9228	0,4346	1,0362	
	IV	41	0,5719	0,8390	0,3071	0,8367	-3,7538	1,7028	
	Total	94	0,5818	0,6103	0,4568	0,7068	-3,7538	1,7028	
PIKE	I	28	0,1801	0,9441	-0,1860	0,5462	-3,5379	1,6776	0,5744
	II	14	0,4749	0,2499	0,3307	0,6192	0,0000	0,8770	
	III	11	0,3019	0,4605	-0,0075	0,6112	-0,7192	0,8325	
	IV	41	0,3009	0,5007	0,1429	0,4590	-1,2179	1,0698	
	Total	94	0,2910	0,6379	0,1603	0,4216	-3,5379	1,6776	
PTEN	I	28	0,8219	0,1615	0,7593	0,8845	0,2180	1,1599	0,9691
	II	14	0,8226	0,1894	0,7132	0,9319	0,5002	1,3141	
	III	11	0,7902	0,3162	0,5778	1,0026	-0,0960	1,0570	

	IV	41	0,7961	0,3231	0,6941	0,8981	0,1323	2,0836	
	Total	94	0,8070	0,2616	0,7535	0,8606	-0,0960	2,0836	
RB1	I	28	0,7494	0,2525	0,6515	0,8473	0,0000	1,2898	0,5227
	II	14	0,7829	0,1577	0,6918	0,8739	0,5429	1,2194	
	III	11	0,7473	0,1540	0,6438	0,8507	0,3740	0,9201	
	IV	41	0,8402	0,3331	0,7351	0,9454	0,1046	1,9536	
	Total	94	0,7938	0,2722	0,7380	0,8495	0,0000	1,9536	
RUNX3	I	28	0,4705	0,2878	0,3589	0,5821	-0,3168	0,9513	0,7310
	II	14	0,6274	0,2039	0,5096	0,7451	0,1103	0,9687	
	III	11	0,4718	0,3045	0,2672	0,6763	0,0334	0,9593	
	IV	41	-1,6742	13,8726	-6,0530	2,7045	-88,3080	1,2032	
	Total	94	-0,4415	9,1654	-2,3187	1,4358	-88,3080	1,2032	
TP53	I	28	0,8118	0,2993	0,6957	0,9278	0,0000	1,2271	0,4492
	II	14	0,8636	0,1695	0,7657	0,9615	0,5993	1,2669	
	III	11	0,8512	0,1543	0,7475	0,9549	0,6369	1,1623	
	IV	41	0,9131	0,2684	0,8284	0,9978	0,1863	2,0535	
	Total	94	0,8683	0,2558	0,8159	0,9207	0,0000	2,0535	

Table 7: Correlation of tumor suppressor gene and oncogene expression, gene copy number and gene promotor region methylation values with the clinical manifestations of subjects

Group		Predictive Group				Total
		Anaplastic Astrocytoma	Diffuse Astrocytoma	Glioblastoma	Pilocytic Astrocytoma	
Original	Anaplastic Astrocytoma	3				3
	Diffuse Astrocytoma		7			7
	Glioblastoma		2	39		41
	Pilocytic Astrocytoma				2	2
	Anaplastic Astrocytoma	100.0				100.0
	Diffuse Astrocytoma		100.0			100.0
%	Glioblastoma		4.9	95.1		100.0
	Pilocytic Astrocytoma				100.0	100.0

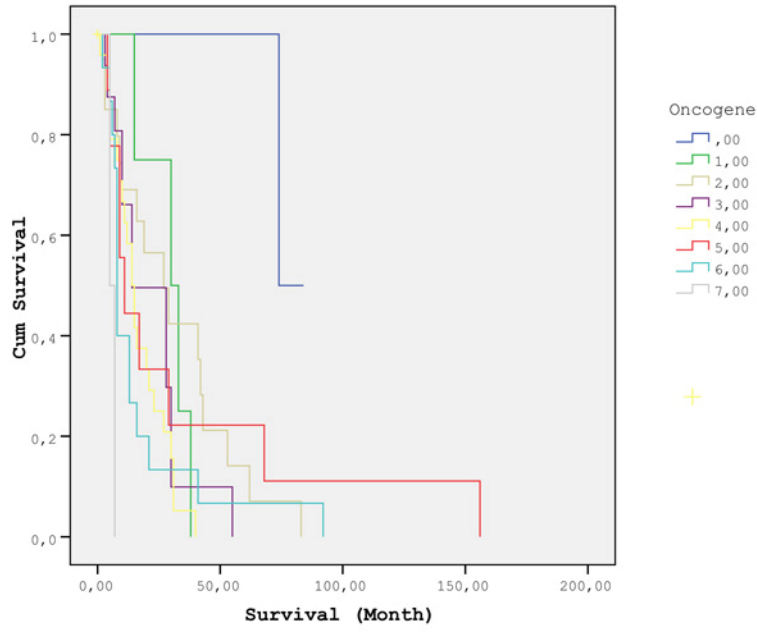


Figure 1: Oncogene expression positivity / survival curve (Log Rank=23.63, $p=0.001$)

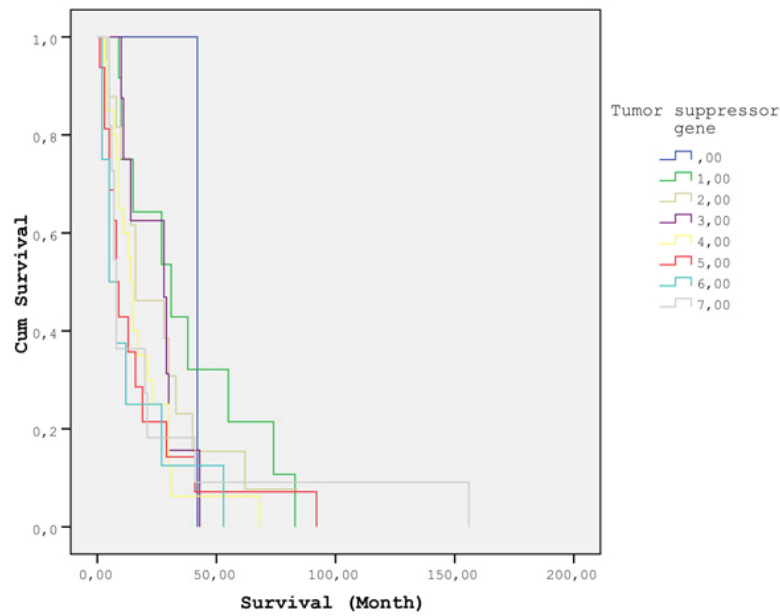


Figure 2: Tumor suppressor gene expression positivity / survival curve (Log Rank=9.01, $p=0.252$)

Table 8: Relationship between positivity of oncogene expression and estimated survival

Oncogene		Mean						Median		Log Rank	p
		Estimated	SE	95% Confidence Interval for Mean		Estimated	SE	95% Confidence Interval for Median			
				Lower Bound	Upper Bound			Lower Bound	Upper Bound		
PIK3CA	Negative	27.15	3.12	21.02	33.27	27.00	8.08	11.16	42.84	3.942	0.047
	Positive	21.25	4.46	12.50	30.00	9.00	1.27	6.52	11.48		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
MDM2	Negative	25.62	3.37	19.01	32.23	19.00	4.30	10.57	27.43	1.119	0.290
	Positive	21.86	4.31	13.40	30.31	12.00	2.31	7.46	16.54		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
GSTT1	Negative	24.97	4.80	15.57	34.38	27.00	14.15	0.00	54.73	0.160	0.689
	Positive	23.50	3.41	16.82	30.17	14.00	1.11	11.83	16.17		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
EGFR	Negative	29.75	4.02	21.87	37.62	27.00	9.71	7.97	46.03	5.212	0.022
	Positive	19.63	3.67	12.44	26.83	11.00	1.95	7.18	14.82		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
OLIG2	Negative	28.72	4.68	19.54	37.90	16.00	4.74	6.70	25.30	3.718	0.054
	Positive	18.46	2.97	12.65	24.27	11.00	2.56	5.99	16.01		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
COX-2	Negative	32.68	5.06	22.76	42.61	28.00	7.71	12.88	43.12	5.583	0.018
	Positive	20.43	3.25	14.05	26.80	13.00	1.95	9.17	16.83		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
hTERT	Negative	27.84	4.22	19.56	36.11	19.00	3.12	12.88	25.12	2.948	0.086
	Positive	18.79	4.11	10.73	26.84	9.00	1.36	6.34	11.66		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		

Table 9: Relationship between tumor suppressor gene copy number aberration with estimated survival

Copy number aberration	Mean					Median				Log Rank	p
	Estimated	SE	95% Confidence Interval for Mean		Estimated	SE	95% Confidence Interval for Median				
			Lower bound	Upper bound			Lower bound	Upper bound			
DMBT1	-	26.68	3.77	19.29	34.07	16.00	2.75	10.61	21.39	2.643	0.104
	+	16.97	4.16	8.81	25.13	10.00	3.68	2.79	17.21		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
MGMT	-	24.67	3.16	18.48	30.86	15.00	1.31	12.44	17.56	0.109	0.741
	+	15.80	3.63	8.69	22.91	10.00	1.10	7.85	12.15		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
PIKE	-	26.84	4.16	18.69	35.00	15.00	3.67	7.81	22.19	1.589	0.206
	+	19.30	3.80	11.84	26.75	14.00	3.23	7.67	20.33		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
PTEN	-	26.82	3.33	20.30	33.35	21.00	4.35	12.48	29.52	4.640	0.031
	+	19.66	4.57	10.70	28.63	9.00	1.03	6.98	11.02		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
RB1	-	25.37	3.22	19.06	31.68	15.00	1.74	11.60	18.40	5.100	0.024
	+	9.20	2.03	5.21	13.19	11.00	5.48	0.26	21.74		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
RUNX3	-	29.27	3.88	21.66	36.87	21.00	4.57	12.04	29.96	22.961	0.000
	+	9.74	1.23	7.33	12.15	9.00	1.39	6.28	11.72		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		
p53	-	19.67	3.26	13.29	26.06	10.00	1.98	6.13	13.87	8.785	0.003
	+	33.61	5.03	23.75	43.48	30.00	1.64	26.79	33.21		
	Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64		

Table 10: Relationship between oncogene expression positivity and estimated survival

Oncogene	Mean				Median			
	Estimated	SE	95% Confidence Interval for Mean		Estimated	SE	95% Confidence Interval for Median	
			Lower bound	Upper bound			Lower bound	Upper bound
0	79.00	3.54	72.07	85.93	74.00	.	.	.
1	29.00	4.95	19.30	38.70	30.00	9.00	12.36	47.64
2	30.87	5.85	19.40	42.34	27.00	8.67	10.01	43.99
3	21.64	4.33	13.16	30.12	14.00	6.82	0.63	27.37
4	16.67	2.21	12.34	20.99	14.00	1.84	10.40	17.60
5	34.22	16.61	1.66	66.79	11.00	2.98	5.16	16.84
6	17.07	5.87	5.55	28.58	8.00	0.38	7.26	8.74
7	6.00	0.58	4.87	7.13	5.00	.	.	.
Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64

Table 11: Relation between tumor suppressor gene expression positivity and estimated survival

Tumor suppressor gene	Mean				Median			
	Estimated	SE	95% Confidence Interval for Mean		Estimated	SE	95% Confidence Interval for Median	
			Lower bound	Upper bound			Lower bound	Upper bound
0	42.00	0.00	42.00	42.00	42.00	.	.	.
1	37.02	8.49	20.38	53.67	31.00	11.75	7.96	54.04
2	27.39	6.01	15.60	39.18	16.00	8.03	0.26	31.74
3	24.69	4.39	16.09	33.28	28.00	8.95	10.45	45.55
4	18.59	3.69	11.35	25.82	14.00	1.49	11.08	16.92
5	18.14	6.25	5.89	30.40	8.00	1.27	5.51	10.49
6	14.25	6.24	2.02	26.48	5.00	2.83	0.00	10.54
7	25.82	13.43	0.00	52.14	8.00	0.80	6.44	9.56
Overall	24.46	3.07	18.45	30.47	15.00	1.35	12.36	17.64

DISCUSSION

Accumulation of genetic and epigenetic changes causes extensive changes occurring in the expressions of those genes involved in an oncogenesis and this situation plays a leading role in having such oncogenesis. Many studies have been carried out about genetic mutations underlying brain tumors and their potential signaling pathways. While some of these are employed in early tumor formation, some others are thought to play a role in late or end stages. The genetic and epigenetic changes underlying the tumorigenesis of brain tumors are still not very clear. Diagnosis at an early stage, good performance status, improved contrast in preoperative imaging, surgical resection and detection of heterozygosity loss are all important^(19,21,22). In our study, we investigated oncogene and tumor suppressor gene expressions, promoter region methylations of tumor suppressor genes and the gene copy anomalies of these genes, which we think influence the tumorigenesis of brain tumors.

An abnormal activation of PI3K/AKT signaling has been found in most of human glioblastomas and it contributes to various biological characteristics such as control of cell cycle, resistance to apoptosis, increased cell migration and invasion, and abnormal neoangiogenesis^(5,10,12). Although it is known that PIK3CA gene mutations are important in various cancers involving malignant gliomas, the study of Knobbe and colleagues where they demonstrated amplification and excessive expression of PIK3CA gene in glioblastomas is one of the limited numbers of studies focusing on expressions in cancers^(13,17). When PIK3CA gene expression was compared to the tumor groups in our study, no statistically significant difference was found ($p>0,05$) and the mean PIK3CA gene copy number was found to be 0.9565 ± 0.1522 in brain tumor-bearing groups, producing no statistically significant difference between the groups.

MDM2 protein, mRNA and gene have high expressions in various types of carcinomas^(7,11,14,15,18,20). When the tumor-bearing groups were compared within themselves in terms of MDM2 gene expression in our study, MDM2 gene expression in each case group was close to the overall average and no significant difference was found. When assessed in terms of gene copy numbers, the mean MDM2 copy number was 0.8579 ± 0.4363 in the glioblastoma group which is the group with the lowest copy number change among the patient groups. There are various studies in the literature on the amplification of MDM2 gene region and our study is one of the few studies where the patient group does not show the amplification of this region.

Excessive activation of epidermal growth factor receptor (EGFR) is known to be an important step in the pathogenesis and development of many cancer forms with epithelial origin. Reports regarding EGFR family members in brain tumors are rare. The EGFR signaling pathway is thought to play an important role in the pathogenesis of brain tumor as well as in early tumor formation, supporting of tumor growth, incitement of infiltration and mediating in resistance to treatment. When EGFR gene expression was compared to the tumor groups in this study, a statistically significant difference ($p=0.0034$) was found. EGFR gene expression in the Schwannoma group shows statistically significant differences with Oligodendroglioma ($p=0.0049$), with Diffuse Astrocytoma ($p=0.0023$) and with Glioblastoma ($p<0.0001$). In the meta-analysis made by Rorive and colleagues on the studies in Medline using the key words human, glioma and astrocytoma, they showed that EGFR expression in low-grade tumors was suppressed as compared to high-grade glial tumors⁽¹⁶⁾. The tumor grades and EGFR expression did not demonstrate any statistically significant relationship in our tumor-bearing group. However, as the tumor grade increased, the

mean EGFR gene expression also increased. This behavior of EGFR gene, which is an oncogene, was considered consistent with the oncogene profile.

A statistically significant difference ($p=0.0006$) was found when the gene expression of hTERT, one of the genes for which significant results were obtained, was compared to the tumor groups. The hTERT gene expression in the Pilocytic Astrocytoma group shows a statistically significant difference with Oligodendroglioma ($p=0.0049$), with Anaplastic Astrocytoma ($p<0.0001$), with Oligodendroglioma ($p=0.0004$), with Diffuse Astrocytoma ($p=0.0028$), with Glioblastoma and Indifferent Malignant Tumor ($p=0.0017$), with Carcinoma Metastasis ($p=0.0017$), with Meningioma Metastasis ($p=0.0007$), with Oligodendroglioma ($p=0.007$) and with Schwannoma ($p=0.0108$).

When suppressor gene expressions were evaluated, DMBT1, MGMT, PIKE, PTEN, RB1, RUNX3 and p53 gene expressions did not show a statistically significant difference in the tumor groups. However, DMBT1 tumor suppressor gene expression showed a statistically significant difference with the tumor groups of glioblastoma, pilocytic astrocytoma ($p<0.0001$) and schwannoma ($p=0.0221$) and Meningioma with the pilocytic astrocytoma group ($p=0.0162$). When copy numbers of tumor suppressor genes are investigated, a distinct decrease was seen in the glioblastoma group and it showed a statistically significant difference ($p<0.05$) with the hypophysis adenoma and meningioma groups whose DMBT1 copy numbers were close to normal. The tumor suppressor gene copy number of PTEN, which is located close to DMBT1 gene, had again a loss in the glioblastoma group and it had a statistically significant difference ($p=0.0014$) with carcinoma metastasis which had a normal copy number.

Methylation of the promoter regions of tumor suppressor genes were evaluated quantitatively and when the rate of methylation was examined in the tumor-bearing groups, the highest rate of methylation was found in RUNX3 gene and the second highest in RB1 gene. The rate 0.9072 in RUNX3 gene means that approximately one of every two alleles was methylated.

The quantitation values were scored to determine the methylation in the cases. When methylation rates are compared in the tumor cell groups according to the scoring, a statistically significant increase was found in the methylation of RB1 and MGMT genes ($p<0.05$). The quantitation of methylation in the promotor region of RB1 gene shows statistically significant differences in methyl / non-methyl ($p=0.0247$) and methyl / mixing ratios ($p=0.0134$) in tumor-bearing groups. The methylation in the promotor region of p53 gene was interestingly found to be very low in all patient groups and a statistically significant difference was found between the patient groups ($p=0.0007$). The promotor region of MGMT gene showed a different methylation pattern in tumor-bearing groups and there was a statistically significant difference between the groups ($p=0.0007$).

According to the results of survival, when the relationship between the oncogene positivity and survival was examined, an excessive expression of PIK3CA, EGFR, OLIG2 and COX-2 genes was significantly related with short survival ($p<0.05$) and when the relation between the copy number aberration and survival was examined, PTEN, RB1, RUNX3 and p5 gene aberrations were found to show a statistically significant relationship with short survival ($p<0.05$).

These “more rational” treatments for brain tumors had limited success until now and the reason for this may be that molecular medicine is newly starting to be used for therapeutic benefit. Translational research

is critical for the future of neuro-oncology. Illumination of signaling pathways which are activated by the causes of genetic changes is now being transferred to glial tumor treatment strategies. In any event, heterogeneity of brain tumors is an important problem for any molecular therapy. Therefore, a broadened molecular sub-classification should be developed for brain tumors. This can be achieved using genetic approaches such as gene profiling and new biochemical and clinical markers. Future treatment strategies for malignant glial tumors will develop a good relationship between broad-spectrum medication regimes, developments in molecular profiling of tumors and protocol design. The potential benefits of these strategies will present an important improvement in prognosis for patients with malignant glial brain tumors.

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