Expression profiles of PIK3CA, RUNX3, COX-2 and DMBT1 genes in brain tumor prognosis: a preliminary study

Beyin tümörü prognozunda PIK3CA, RUNX3, COX-2 ve DMBT1 genlerinin ekspresyon profilleri: ön çalışma

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Abstract

The two main subgroups of the important genes functioning in the development of cancer including brain tumors are tumor suppressor genes and oncogenes. The aim of this pilot study was to determine the expression profiles of tumor suppressor genes; RUNX3 (Runt-related transcription factor 3), DMBT1 (deleted in malignant brain tumors 1), and oncogenes PIK3CA (phosphoinositide-3-kinase, catalytic, alpha polypeptide) and COX–2 (Cyclooxygenase-2) in brain tumors. Explant cell cultures were performed using brain tumor tissues from the 14 cases. Total RNA was isolated from the cultured cells. Relative ratio of expression profiles of the tumor suppressor genes and oncogenes were determined by using real-time online RT-PCR. U-87MG cell line was used as positive control. The mean relative ratios of DMBT1, RUNX3, COX-2 and PIK3CA genes were found; 72.2, 59.3, 0.119 and 48.5; respectively. COX-2 over-expression correlated with a significantly reduced survival (Log- Rank, p= 0.049). Suppression of DMBT1 and RUNX3 is associated with decreased survive, however no significant difference was determined between expressions of PIK3CA, DMBT1 and RUNX3 genes and overall survival. High COX-2 expression is found to be associated with clinically more aggressive brain tumors. Our preliminary data suggests that COX-2 gene may be used as a therapeutic target.

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Özet

Beyin tümörleri de dahil olmak üzere kanser gelişiminde önemli rol oynayan genlerin iki ana alt grubu onkogenler ve tümör baskılayıcı genlerdir. Bu ön çalışmanın amacı, tümör baskılayıcı genlerden RUNX3 (Runt-related transcription factor 3), DMBT1 (Deleted in Malignant Brain Tumors 1) ve onkogenlerden PIK3CA (Fosfoinosid-3-kinaz, katalitik, alfa polipeptid), COX–2 (Siklooksigenaz-2) genlerinin beyin tümörlerinde ifade düzeyini saptamaktır. On dört olgunun beyin tümörü dokularından hücre kültürü ortamında çoğalması sağlandı. Kültüre edilmiş hücrelerden total RNA'lar izole edildi. Gerçek zamanlı RT-PCR metodu kullanılarak onkogen ve tümör baskılayıcı genlerin ekspresyon profillerinin bağıl oranları saptandı. U87MG hücre hattı pozitif kontrol olarak kullanıldı. DMBT1, RUNX3, COX-2 ve PIK3CA genlerinin bağıl oranları ortalaması sırasıyla 72.2, 59.3, 0.119 ve 48.5 bulundu. COX-2 aşırı ekspresyonu, anlamlı olarak azalmış sağkalım ile korelasyon gösterdi (Log-Rank, p= 0.049). DMBT1 ve RUNX3 genlerinin baskılanması azalmış sağkalımla ilişkili olduğu saptandı ancak PIK3CA, DMBT1 ve RUNX3 ekspresyonları arasında ve sağkalım üzerinde anlamlı bir farklılık saptanmadı. Yüksek COX-2 ekspresyonu klinik olarak fazla agresif beyin tümörleri ile ilişkili olduğu gösterildi. Elde ettiğimiz ön deney sonuçlarımız, COX-2 geninin terapötik hedef olarak kullanılabilir olabileceğini önermektedir.

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Anahtar sözcükler: Beyin tümörü, gen ekspresyonu, U-87MG

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Introduction

Brain tumors exhibit the most dramatic prognosis in all cancer types. There are more than 100 types of primary brain tumors which present a broad range of biological and clinical manifestations. Hence, neurooncologists and pathologists have major difficulty in the diagnosis and treatment of the brain tumors [1]. Diffuse and high grade glial tumors are the most common tumors of the central nervous system and the mortality rate reaches fairly high levels in the first year following the initial diagnosis. The major subtypes are; diffuse astrocytoma, oligodendroglioma, ependymoma and high grade glial tumors (Glioblastoma, anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic ependymoma) [2,3]. The developments in cancer research and underlying molecular mechanisms over the last years have enabled improvements in the diagnosis and in the treatment of cancer, and this has been followed by more effective and less toxic treatment schedules. Cancer which can be described as gene malfunction is classified according to the genetic changes underlying the pathogenesis of tumor and malign behavior. One of the main subgroups of the critical genes functioning in the development of different cancer types including brain tumors are tumor suppressor genes and oncogenes [4]. Tumor suppressor genes control cell proliferation and tumor development. Oncogenes are generally mutated form of protooncogenes that cause the transformation of normal cells into cancerous tumor cells. They stimulate cell growth and over-expression of these genes cause growing out of control.

Runt-related transcription factor 3 (RUNX3) is a candidate tumor suppressor gene, a Runt domain transcription factor involved in TGF-β signaling, localized in 1p36, a region commonly deleted in various human tumors such as stomach, bile duct, and pancreas [5,6]. Deleted in malignant brain tumour-1 (DMBT-1), is a candidate tumor suppressor gene that is located at chromosome 10q 25.3--26.1. In brain, lung, gastrointestinal tumors, homozygous deletions and lack of mRNA expression in DMBT1 gene are frequently observed [7-10]. One isoform of cyclooxygenase (COX), the crucial rate-limiting enzyme in the synthesis of prostaglandins (PGs) from arachidonic acid, Cyclooxygenase-2 (COX-2), is an inducible enzyme which is regulated by various factors including cytokines, growth factors and inflammatory signals in many types of cells. Increased expression of COX-2 is known in a variety of tumors, such as brain, head and neck, breast, cervix, prostate, bladder, liver, pancreas, skin, lung, colon, rectum and oesophagus [11-13]. Phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA), encodes the p110 alpha catalytic subunit of phosphatidylinositol 3-kinase (PI3-kinase). It has been found to be oncogenic and implicated in cervical cancers [13].

The major objectives of our study were: a) determination of expression profiles of RUNX3, DMBT1, COX-2 and PIK3CA genes that cause brain tumor phenotype, b) possible contribution of RUNX3, DMBT1, COX-2 and PIK3CA gene expression profile to the follow-up therapy and understanding the efficiency of novel therapy protocols in the early phases, c) classification of patients in respect of demographic and pathological findings according to the response to the therapy, d) determining concerned gene profiles in cases with diffuse and high grade glial tumor and establish a correlation between the data and prognosis, and provide benefit to the standard treatment schedules in optimum level, e) the determination of correlation between the cancer prognosis and genetic variation of the related genes, f) performing a molecular approach to the diagnosis of the cases pre-diagnosed.

Materials and Methods

Subjects

Subjects (six female, eight male) were recruited into the study that have been diagnosed as diffuse and high grade glial tumors and taken the decision of surgical operation in the Ege University Faculty of Medicine Department of Neurosurgery between 2005 and 2007 years. Cases signed a written informed consent statement approved by local ethics committee. The mean age was 44.50±13.58. Clinical and demographic features of patients who had brain tumor are given in Table 1.

Explant cell cultures of the tissues that were obtained during surgical operation were cultured in BIOAMF-1 (Biological Industries, Israel) supplemented with 10 000 U/ml penicillin, 10 mg/ml streptomycin, 2 mM L-glutamine at 37^0C under a humidified 95% air 5% CO_2 atmosphere U-87 MG brain tumor cell line was used as a positive control and gene expression of the cell line was compared with positive control. The general features of U-87 MG are shown in Table 2.

Table 1. Demographic and clinical features of cases

Case	Gender	Tumor Type	Grade	Age	Outcome	Oversurvival (months)
1	M	Pituitary adenoma	I	25	Alive	11
2	M	Meningioma	I	46	Alive	13
3	M	Pilocytic astrocytoma	I	21	Alive	14
4	F	Meningioma	I	46	Alive	25
5	F	Pilocytic astrocytoma	I	37	Alive	26
6	F	Ependymoma	II	41	Alive	24
7	M	Ependymoma	II	65	Alive	25
8	F	Ependymoma	II	36	Alive	26
9	M	Glioblastoma multiforme	IV	46	Excitus	3
10	F	Glioblastoma multiforme	IV	46	Excitus	12
11	M	Glioblastoma multiforme	IV	72	Alive	12
12	M	Glioblastoma multiforme	IV	44	Alive	20
13	M	Glioblastoma multiforme	IV	56	Alive	20
14	F	Metastasis	IV	42	Alive	20

Table 2. Features of U-87 MG cell line (American Type Culture Collection)

Age:	44 years
Gender:	Female
Ethnicity:	Caucasian
Organ:	Brain
Disease:	Glioblastoma; astrocytoma
Tumor stage:	Grade III
Morphology:	Epithelial
Antigen Expression:	Blood Type A, Rh+
Cytogenetic Analysis:	This is a hypodiploid human cell line with the modal chromosome number of 44 occurring in 48% of cells. The rate of higher ploidy was 5,9%. Twelve markers were common to all cells, including der(1)t(1;3) (p22;q21), der(16)t(1;16) (p22;p12), del(9) (p13) and nine others. The marker der(1) had two copies in most cells. There was only one copy of normal X. N1, N6 and N9 were not found.
Isoenzymes:	AK-1, 1; ES-D, 1; G6PD, B; GLO-I, 1; Me-2, 1; PGM1, 2; PGM3, 1

Table 3. The sequences of primers and probe

Gene	Forward Primer	Reverse Primer	Probe (roche)
COX-2	tcacgcatcagtttttcaaga	tcaccgtaaatatgatttaagtccac	probe #23 (cat. no. 04686977001)
DMBT1	gctcaggaaaccatctatcgac	gaagcctccgcaggaatagt	probe #68 (cat. no. 04688678001)
PIK3CA	cacgagatcctctctctgaaatc	ggtagaatttcggggatagttaca	probe #15 (cat. no. 04685148001)
RUNX3	tcagcaccacaagccactt	aatgggttcagttccgaggt	probe #71 (cat. no. 04688945001)
GAPDH	gaaggtgaaggtcggagtc	gaagatggtgatgggatttc	FAM-caagcttcccgttctcagcc-TAMRA

Isolation of total RNA and cDNA synthesis

Fifty microliters of total RNA was isolated from explant cell cultures of cases that have brain tumors by using High Pure RNA Isolation Kit (Roche, Germany). Reverse transcription procedure was performed for cDNA synthesis by using Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) according to the manufacturers' instructions.

Relative quantification of PIK3CA, RUNX3, COX-2 and DMBT1

Real-time quantitative RT-PCR analyses of genes PIK3CA, RUNX3, COX-2 and DMBT1 were performed with LightCycler instrument. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH "housekeeping" gene) was chosen as an internal standard to control for variability in amplification. The sequences of primers and probes used are shown in Table 3.

PCR was performed by using TaqMan Master Kit (Roche Diagnostics, Germany) according to the instructions of the manufacturer. Target probe of studied genes was labeled at the 5' end with the reporter dye molecule 6-carboxyfluorescein (FAM). The GAPDH target probe was labeled with 6- carboxyfluorescein. Both probes were labeled with the quencher fluor 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. To quantify genes' mRNA from cell culture, we constructed a calibration curve (Error: 0.100 Efficency: 1,790) using copy number (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10) of GAPDH. Relative ratio (RR) of gene expressions was calculated using the formula:RR= Copy number of gene/Copy number of GAPDH x 10000.

Statistical Analysis

Patient follow-up was 2 years. Differences between groups and comparing continuous variables were performed by using Mann-Whitney test. Linear regression analysis was used to determine the associations between relative ratio of gene expression and age. The Kaplan-Meier method was used for survival analysis. A p value of <0.05 was considered statistically significant.

Results

In this study, we evaluated the expression levels of DMBT1, RUNX3, COX-2 and PIK3CA genes that play a role in tumor progression in 14 brain tumor patients. Cell culture from brain tumor tissues was successfully done from all cases. Two cases were (WHO) grade I meningiomas, 1 was (WHO) grade I pituitary adenoma, and 1 was (WHO) grade I pilocytic astrocytoma, 3 WHO grade II ependymomas, 1 WHO grade II astrocytoma, 5 GBM, WHO grade IV, and one case WHO grade IV metastasis [primer lung cancer, (Table 1)]. All the gene expressions of brain tumor groups were given in Table 4.

The mean relative ratios of DMBT1, RUNX3, COX-2 and PIK3CA genes were found; 72.2, 59.3, 0.119 and 48.5; respectively. There was no significant association between tumor grades, age and gene expressions. When compared the gene expression in U-87 MG cell line, mean gene expression showed significant differences in COX-2 ($p<0.001$), DMBT1 ($p=0.022$) and PIK3CA ($p=0.036$) in Grade I, COX-2 ($p<0.001$) and PIK3CA ($p=0.031$) in Grade II and COX-2 ($p<0.001$) in Grade IV.

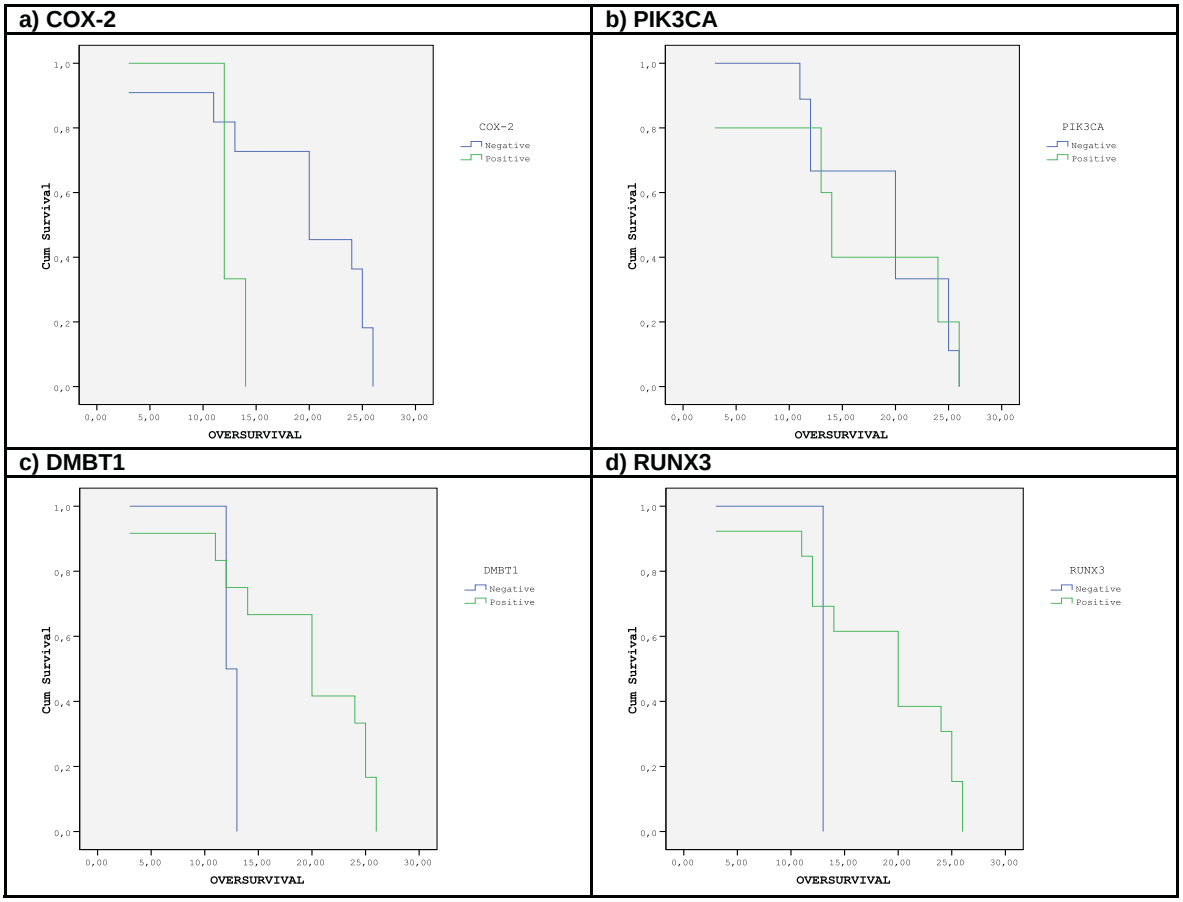

Figure 1. Survival curve obtained by the Kaplan-Meier method for expressions of tumor suppressor and oncogenes. a) COX-2, b) PIK3CA, c) DMBT1 and d) RUNX3.

Table 4. Expression of tumor suppressor genes and oncogenes

Case	Gene Expression					Relative Ratio			
	GAPDH	COX-2	DMBT1	PIK3CA	RUNX3	COX-2	DMBT1	PIK3CA	RUNX3
1	$3.57x10^{6}$	$6.93x10^{-6}$	$5.90x10^{-6}$	$1.35x10^{4}$	$1.48x10^{1}$	$1.94x10^{-8}$	$1.65x10^{-8}$	$3.78x10^{1}$	$4.15x10^{-2}$
2	$1.33x10^{6}$	$5.90x10^{-6}$	$1.24x10^{-2}$	$1.01x10^{4}$	$1.70x10^{1}$	$4.44x10^{-8}$	$9.32x10^{-5}$	$7.59x10^{1}$	$1.28x10^{-1}$
3	$2.37x10^{6}$	$1.72x10^{2}$	$1.32x10^{2}$	$1.65x10^{4}$	$1.63x10^{2}$	$7.26x10^{-1}$	$5.57x10^{-1}$	$6.96x10^{1}$	$6.88x10^{-1}$
4	$2.53x10^{5}$	$5.90x10^{-6}$	$4.34x10^{1}$	$1.42x10^{2}$	$2.23x10^{0}$	$2.33x10^{-7}$	$1.72x10^{0}$	$5.61x10^{0}$	$8.81x10^{-2}$
5	$9.03x10^{5}$	$1.80x10^{-2}$	$8.83x10^{1}$	$1.12x10^{3}$	$5.00x10^{1}$	$1.99x10^{-4}$	$9.78x10^{-1}$	$1.24x10^{1}$	$5.54x10^{-1}$
6	$1.58x10^{6}$	$9.84x10^{0}$	$8.71x10^{-2}$	$1.10x10^{4}$	$1.93x10^{2}$	$6.23x10^{-2}$	$5.51x10^{-4}$	$6.96x10^{1}$	$1.22x10^{0}$
7	$1.66x10^{5}$	$5.90x10^{-6}$	$5.90x10^{-6}$	$5.15x10^{1}$	$8.76x10^{1}$	$3.55x10^{-7}$	$3.55x10^{-7}$	$3.10x10^{0}$	$5.28x10^{0}$
8	$1.57x10^{7}$	$1.56x10^{2}$	$2.64x10^{4}$	$9.31x10^{4}$	$7.38x10^{4}$	$9.94x10^{-2}$	$1.68x10^{1}$	$5.93x10^{1}$	$4.70x10^{1}$
9	$5.39x10^{5}$	$5.90x10^{-6}$	$2.72x10^{4}$	$1.32x10^{4}$	$4.16x10^{4}$	$1.09x10^{-7}$	$5.05x10^{2}$	$2.45x10^{2}$	$7.72x10^{2}$
10	$3.73x10^{6}$	$2.31x10^{2}$	$1.65x10^{2}$	$1.30x10^{4}$	$4.03x10^{1}$	$6.19x10^{-1}$	$4.42x10^{-1}$	$3.49x10^{1}$	$1.08x10^{-1}$
11	$2.22x10^{5}$	$3.56x10^{0}$	$1.06x10^{4}$	$8.04x10^{1}$	$4.60x10^{1}$	$1.60x10^{-1}$	$4.77x10^{2}$	$3.62x10^{0}$	$2.07x10^{0}$
12	$3.49x10^{5}$	$0.00x10^{0}$	$2.06x10^{2}$	$1.68x10^{2}$	$3.91x10^{-1}$	$0.00x10^{0}$	$5.90x10^{0}$	$4.81x10^{0}$	$1.12x10^{-2}$
13	$4.77x10^{6}$	$7.03x10^{-5}$	$1.07x10^{3}$	$2.17x10^{4}$	$3.46x10^{2}$	$1.47x10^{-7}$	$2.24x10^{0}$	$4.55x10^{1}$	$7.25x10^{-1}$
14	$7.13x10^{5}$	$5.90x10^{-6}$	$1.75x10^{1}$	$8.50x10^{2}$	$6.42x10^{0}$	$8.27x10^{-8}$	$2.45x10^{-1}$	$1.19x10^{1}$	$9.00x10^{-2}$
Median	$1,12x10^{6}$	$3,86x10^{-5}$	$1,10x10^{2}$	$1,06x10^{4}$	$4,80x10^{1}$	$2,94x10^{-7}$	$7,68x10^{-1}$	$3,64x10^{1}$	$6,21x10^{-1}$
U-87MG	$4.88x10^{7}$	$7.40x10^{6}$	$2.22x10^{4}$	$8.75x10^{5}$	$1.66x10^{1}$	$1.52x10^{3}$	$4.55x10^{0}$	$1.79x10^{2}$	$3.40x10^{-3}$

Discussion

In brain tumors, especially in GBM, expression of COX-2 was positive up to 80% [14]. High COX-2 expression significantly correlated with poor prognosis and increase of tumor grade demonstrated a significant correlation with a high percentage of COX-2 expression [12]. High expression of COX-2 strongly correlated with poor survival in our study whereas some other studies found no correlation [13,14]. PIK3CA gene amplification and over-expression also showed no correlation in glioblastoma and medulloblastoma [15,16]. DMBT1 mRNA expression was detected in small subset of glioblastomas [17]. Overall, only two exitus cases were recorded within the study group. The oncogenes; COX-2 and PIK3CA showed significantly less expression in U-87MG cell line that was used for positive control. The cases with relative ratio of COX-2 and PIK3CA higher than mean relative ratio were assumed as positive and the cases with relative ratio of DMBT1 and RUNX3 lower than mean relative ratio were negative. When the effects of gene expressions were investigated on survive, for COX-2, 11 cases determined as negative and 3 cases positive. Estimated mean survivals of negative and positive cases were 19.36 and 12.67 months, respectively (Figure 1a). Kaplan-Meier survival analysis demonstrated a significantly reduced survival of COX-2 positive cases (Log-Rank c2=3.883, p= 0.049). For PIK3CA, 9 cases were negative and estimated mean survival was 19 months and estimated mean survival of 5 positive cases was 16 month (Figure 1b). Among tumor suppressor genes; for DMBT1, it is 18.83 months for 2 negative cases and 12.50 months for 12 positive cases (Figure 1c) and for RUNX3, a negative case was determined as 13.00 months and 13 cases were positive with 18.30 months (Figure 1d). A significant difference between expressions PIK3CA, DMBT1and RUNX3 and oversurvival was not determined.

Tumor suppressor genes showed significantly higher expression in our study group compare to that in U-87MG cell line. Determination of tumor suppressor genes and oncogene expressions may be used as a biomarker for prognosis of the cases.

These findings showed that high COX-2 expression is associated with clinically aggressive brain tumors. Our data suggest that inhibition of COX-2 gene expression may be used as a therapeutic target. Surgery and current treatment options have proven to be inadequate in treating and controlling brain tumors, the search for novel targets and mechanism-based agents for prevention and treatment of this disease has become a priority. However, the present study is a preliminary study, and further experiments are required to demonstrate the potential of the gene expressions in brain tumor prognosis with more cases.

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