Correlations between the expression of hTERT and α and β splice variants in human brain tumors

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Abstract

Background. Astrocytomas are diffusible infiltrative and aggressive brain tumors that are extensive and heterogeneous clusters of neoplastic growths in the central nervous system (CNS). Meningioma tumors are commonly benign but may demonstrate an invasive pattern with frequent recurrences. Human telomerase reverse transcriptase (hTERT) is an unfavorable prognostic factor for several types of cancers, and there are controversies about its role.

Objectives. In the present study, we investigated the relative expression of hTERT splice variants in 2 groups of brain tumors compared to non-tumor samples.

Material and methods. The mRNA of 40 brain tumor samples and 4 control samples was extracted; mRNA expression of hTERT α-deletion and β-deletion variants, as well as the wild type isoform, was quantified using quantitative reverse transcription polymerase chain reaction (RT-qPCR).

Results. The α-deletion variant was significantly expressed in primary benign meningeal tumors (p = 0.01). The results indicate a positive correlation between the relative expression of hTERT mRNA transcript and α-deletion and β-deletion variants in both groups of tumors (meningiomas and astrocytomas). A strong association between the expression of the full-length splice variant and the β-deletion variant was observed in astrocytoma tumors (p = 0.045). The most significant correlations were found between the hTERT full-length and β-deletion variants in high-grade meningiomas (p = 0.018, correlation coefficient (CC) = 0.964) and grade II astrocytomas (p = 0.015; CC = 0.580). In addition, in low grades of both types of tumors, the hTERT full-length variant and especially the α-deletion variant were the predominant isoforms. The over-expression of hTERT and β-deletion variants in high grades of these tumors was statistically significant. Our findings indicate that α-deletion and β-deletion isoforms are associated with high levels of full-length hTERT mRNA in both groups of brain tumor patients.

Conclusions. Changes in the splicing pattern of hTERT splice variants in brain tumors and their correlation with pathological alterations in cells could be applied as diagnostic or prognostic biomarkers, or possibly as targets for cancer therapy. However, the function and biological role of hTERT splice variants remain to be clarified.

Key words: meningioma, astrocytoma, human telomerase reverse transcriptase, α-deletion splice variant, β-deletion splice variant

Astrocytomas are aggressive brain tumors that are lethal in their malignant form. Most of them show high rates of invasion that lead to recurrences of the disease.[1] The World Health Organization (WHO) assigned 4 grades of astrocytoma in 2007, but the recent update of the WHO classification uses both histology and molecular genetic alteration to define central nervous system (CNS) tumor diagnoses.[2–4]

Meningiomas are commonly benign, slow-growing extra-axial tumors that originate from arachnoid cells.[5] This type of neoplasia comprises 34% of primary brain and CNS tumors. In the 2007 WHO classification of tumors of the CNS, the 3 grades of meningioma are defined by histologic criteria predicting the risk of recurrence.[6] The 2016 update of the WHO classification made notable changes, including the addition of brain invasion as a criterion for atypical meningiomas.[4] Malignant meningiomas frequently relapse after a short time, regardless of total resection. The molecular basis of this variation is unknown, but gene expression variations may predict patient outcomes more accurately than pathological measurements. It would be beneficial to identify prognostic markers for these tumors.[7]

The ends of eukaryotic chromosomes are made of tandem-repeated short sequences associated with specific proteins, called telomeres. The length of telomeres is gradually shortened with each cell division.[8] Telomere shortening can be used as a biological clock that represents the progression of a cell to the end of its replicative lifespan. Most of the cell division stops when the cells reach the threshold of senescence.[9] Experimental data demonstrates that cells that escape replicative senescence have adopted a strategy to counteract the loss of telomeric repeats.[10] Immortal cells have solved the problem of truncated telomeres using telomerase.[11] Telomerase is a ribonucleoprotein enzyme complex that synthesizes the telomeric sequence (TTAGGG) at chromosomal ends, compensating for the progressive loss of DNA that occurs during replication.[12] This enzyme is composed of 2 core subunits of an RNA (hTR) as a template for the synthesis of new telomeric repeat sequences and a catalytic subunit with reverse transcriptase activity (human telomerase reverse transcriptase [hTERT]).[13]

Telomere shortening and telomerase activity have been found to be strong markers of cellular malignancy in most human tumors, including brain tumors.[14–16] Reactivation of telomerase seems to be related to unlimited cell proliferation and cancer progression.[17] The enzymatic activity of telomerase is subject to strict regulation.[18] The transcriptional and post-transcriptional regulation of hTERT is complicated and is not fully understood.[19] Its reactivation in immortalized cells is associated with the increased growth potential needed for malignancy.[20] High hTERT expression levels directly correlate with poor clinical outcomes in most cancer types.[21] Studies on hTERT deletion splicing transcripts, rather than the overall hTERT transcripts, may improve our understanding of telomerase regulation.[22] The most widely studied variants involve splicing

at 2 main sites: the α splice site, which produces a 36-bp inframe deletion within the conserved reverse transcript motif A; and the β site, which results in a 183-bp deletion and non-sense mutation that truncates the protein.[23] A quantitative assay for each deletion transcript can accurately quantify the alternative splicing variant expression and assess its correlation with clinico-pathological parameters.[24] Splice variants that are found predominantly in tumors have clear diagnostic value and may provide potential drug targets.[25] At present, not much is known about the functionality of the hTERT β-deletion variant and it is unclear whether spliced hTERT is important in determining tumor genesis.[26,27] The α-deletion variant of hTERT with negative telomerase regulatory properties is expressed in very low amounts in cell culture systems, which is an issue that remains to be clarified.[28] There is considerable heterogeneity regarding hTERT splicing patterns among various primary and metastatic lesions.[29] Few studies have directly examined the differences in hTERT splice variants in various classes of CNS tumors, and the biological role of the isoforms has not been investigated at all. The aim of this study was to compare the expression of hTERT splice variants, including both functional and deleted variants, in 2 groups of brain tumors of different grades and in a group of healthy control samples.

Material and methods

Samples and patients

The tumor samples – 20 meningiomas and 20 astrocytomas – were taken at Isfahan Medical University Hospital (Alzahra, Iran). All the patients had been diagnosed pathologically with astrocytoma and meningioma, and clinical pathological details were determined using the 2007 WHO classification criteria. The control samples were obtained from 4 patients who had underwent resections of normal brain tissue for purposes other than intracranial malignancy treatment. To store the samples for the analysis of telomerase, the surgical specimens were immediately frozen in liquid nitrogen and stored at −80°C until use. The study was performed according to the instructions of the Ethics Committee of Isfahan University of Medical Sciences, Iran, and informed consent was obtained from the patients.

RNA extraction and cDNA synthesis

RNA was extracted from the frozen tissues and TRIzol Reagent (Invitrogen, Carlsbad, USA) was used for total RNA extraction. All the preparations and handling of RNA were carried out in a laminar flow hood under RNase-free conditions. The isolated RNA was resolved in 60 mL of RNase-free water, and the quality and quantity of the extracted RNA samples were determined

by spectrophotometry. Then, the isolated RNA (from 100 ng tissue) was transcribed to cDNA using a first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. Total RNA was used to synthesize a total volume of 20 μL cDNA, which was then stored at a temperature of –70°C.

Primers and reverse transcription polymerase chain reaction procedure

Four high-quality specific primers were designed for hTERT and α-deletion and β-deletion splice variants. *GAPDH* was considered the internal control gene (Table 1). The RNA expression of the hTERT variants was measured using quantitative reverse transcription polymerase chain reaction (RT-qPCR). A RealQ Plus 2x Master Mix Green kit (Ampliqon A/S, Odense, Denmark) containing SYBER Green dye was used. Polymerase chain reactions were set up in a total volume of 10 μL per reaction, and 1 μL of cDNA was poured into a 9 μL reaction mixture. All the reactions were done in triplicate using an ABI Step One Plus system (Applied Biosystems Corp., Foster City, USA). To normalize the data, RNA of the internal control gene (*GAPDH*) was used.

Table 1. Sequence of reverse transcription polymerase chain reaction (RT-PCR) primers used for human telomerase reverse transcriptase (hTERT) mRNA variants

Primer	Sequence	Expected amplicon size (bp)
hTERT	F: ACGGCGACATGGAGAACAA	214
	R: CACTGTCTTCCGCAAGTTCAC	
α-deletion	F: GTACTTTGTCCAGGACAGGCT	194
	R: GGAGGTCTGTCAAGGTAGAGAC	
β-deletion	F: GCTGTACTTTGTCAAGGTGGA	195
	R: ACTGGACGTAGGACTTGGCT	

Statistical analysis

The real-time PCR data was examined using the $2^{-\Delta\Delta CT}$ relative quantization method. IBM SPSS v. 20.0 software (IBM Corp., Armonk, USA) was used for the data analyses. The data distribution was determined by the one sample Kolmogorov-Smirnov test. Comparisons of transcript expression levels in the patients and healthy controls were made using the nonparametric Kruskal-Wallis test. Correlations between all the variants in different grades of samples were evaluated by the independent sample t-test. The independent sample t-test was also used to determine the expression levels in meningiomas compared to astrocytomas. Spearman's correlation coefficient (CC) was used to find correlations between the levels of expression of the different transcripts in different grades of tumors. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

Clinical characteristics of the patients

The samples consisted of 20 meningiomas (80% from female patients (F) and 20% from male patients (M)) and 20 astrocytomas (60% F and 40% M). According to 2007 WHO classification criteria, 12 of the meningioma cases were histologically diagnosed as grade I, 5 were atypical grade II and 3 were malignant (anaplastic) grade III. Among the astrocytomas, 15 cases were diagnosed as grade II, 4 cases as grade I and 1 as grade III. The clinicopathologic characteristics of the patients are listed in Table 2.

Table 2. The clinicopathologic characteristics of 40 patients affected with brain tumors

Characteristics	Meningioma (non-neuroepithelial)	Astrocytoma (neuroepithelial)
Mean of age ±SD	58.6 ±4.5	40.8 ±3.1
Gender		
M	4	8
F	16	12
Grade		
I	12	4
II	5	15
III	3	1
IV	–	–
Mean tumor size [cm] ±SD	6 ±1.6	4 ±1.2
Total	20	20

SD – standard deviation; M – male; F – female.

Comparison of expression

Quantitative reverse transcription polymerase chain reaction showed that 5 out of the 40 cases (12,5%: 1 astrocytoma and 4 meningiomas) did not express any splice variants. In the comparison of different grades of meningiomas, the grade III meningioma tumors exhibited the highest expression of full-length hTERT variant and the β-deletion variant. The α-deletion isoform was the dominant variant in grade I meningioma tumors. In grade I, II and III meningiomas, the full-length hTERT variant showed the highest level of expression. In grades I, II and III astrocytomas, the β-deletion variant exhibited the highest level of expression.

In 1 patient with a grade III astrocytoma (anaplastic oligoastrocytoma), there was no difference between the expression of full-length hTERT variant and the β-deletion variant.

Expression of the full-length human telomerase reverse transcriptase transcript

According to our results, the full-length hTERT transcript, which encodes active hTERT protein, is expressed at a high level in tumors. Human telomerase reverse transcriptase expression was significantly higher in meningiomas

(n = 16; mean = 26.1 ±3.1; p = 0.003) than in astrocytomas (n = 19; mean = 24 ±4.2; p = 0.23). In astrocytoma tumors, increased expression of the full-length hTERT variant in higher grades is associated with increased expression of the β-deletion variant. Our results showed that generally all astrocytomas and meningiomas showed full-length hTERT mRNA expression as the most predominant variant in the majority of tumor samples, but the expression of the full-length transcript in high grades of tumors was found to be higher than in low grades (Fig. 1).
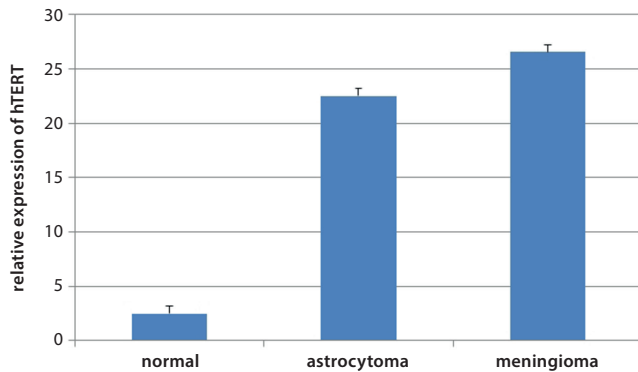

Fig. 1. The full-length human telomerase reverse transcriptase (hTERT) transcript and 2 common isoforms (α-deletion and β-deletion) were detected in patients with brain tumors and compared with healthy controls. The data revealed the functional transcript of hTERT was expressed at higher levels in the meningioma patients

Expression of other human telomerase reverse transcriptase splice variants

Our results showed diversity in the relative expression of hTERT transcript variants. The β-deletion transcript was the most abundantly expressed splice variant in astrocytoma patients. Both full-length hTERT variant and β-deletion splice variants were the most abundant transcripts in high grades of meningiomas and astrocytomas. Expression of the α-deletion variant was not significant in high grades of either astrocytomas or meningiomas compared to the β-deletion variant (Fig. 2). However, the
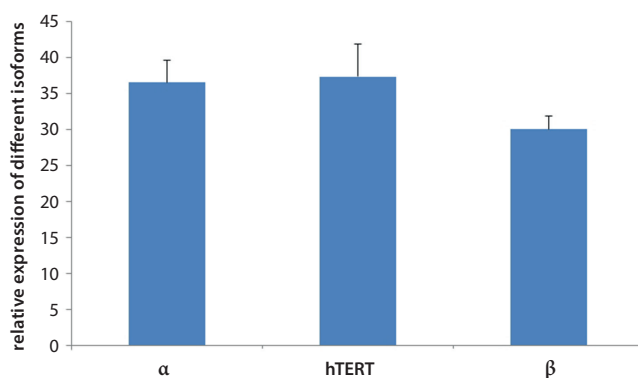

Fig. 2. The β-deletion transcript was the main transcript in the astrocytoma patients. Expression of the α-deletion variant is significantly lower than other splice variants in the astrocytoma patients

hTERT – human telomerase reverse transcriptase.

expression of this transcript was dominant in low grades of meningiomas (I and II) and grade I of astrocytomas (p = 0.01; mean = 31.1 ±4.3). No appreciable difference was observed between mRNA expression of full-length hTERT variant and the α-deletion variant in the astrocytoma samples (p = 0.97). Expression levels of the α-deletion transcript were statistically significant in meningioma patients (p = 0.01).

Correlations in expression between different isoforms

Our results indicated a positive correlation between the relative expression of hTERT mRNA transcripts and α-deletion and β-deletion variants in both meningiomas and astrocytomas (p = 0.018; CC = 0.0964). In lower grade meningiomas, a significant correlation was found between α-deletion isoforms and the β-deletion variant (p = 0.022; CC = 0.0614). A strong positive correlation between the expression of hTERT and the α-deletion variant was clearly detected in lower grade meningiomas but not in the higher grades (p = 0.031 and CC = 0.859). A positive correlation was found between the expression of α-deletion and β-deletion variants in grade I meningiomas (p = 0.022; CCs = 0.614) and astrocytomas (p = 0.016; CC = 0.910), but this correlation was not observed in the higher grades of the tumors. Expression of the β-deletion isoform showed a direct correlation with hTERT mRNA expression in grades II and III of astrocytoma (p = 0.045). We did not find any correlation between the expression of hTERT and β-deletion variants in grades I or II of meningioma. There was also no correlation between the expression of hTERT and β-deletion variants in grade I astrocytomas.

The most significant correlations were found between full-length hTERT and the β-deletion variant expression in grade III meningiomas (p = 0.018; CC = 0.964) and in grade II astrocytomas (p = 0.015; CC = 0.580) (Table 3).

In grade III meningiomas and grades II and III of astrocytoma tumors, expression of β-deletion variants was dominant, which correlated with the expression of hTERT (p = 0.018; CC = 0.964). The levels of expression of the full-length hTERT transcript significantly correlated with β-deletion variants in both meningiomas and astrocytomas.

Discussion

Amplification of the hTERT telomerase catalytic subunit is associated with a poor prognosis in intracranial and primary tumors.[30] An assessment of hTERT expression patterns, including both the wild type and the deletion variants in different CNS tumors is presented for the first time in this article. In addition, correlations between the expression of the wild type and 2 common deletion

Table 3. Correlations between the expression of human telomerase reverse transcriptase (hTERT) (full-length) and other isoforms in the 2 groups of brain tumors of different grades

Variant			Grade of meningioma				Grade of astrocytoma			
			all	I	II	III	all	I	II	III
hTERT	α	CC p-value N	0.453* 0.022 20	0.455 0.080 12	0.859* 0.031 15	0.390 0.031 3	0.655** 0.005 20	0.811* 0.048 4	0.412 0.07 15	– – 1
	β	CC p-value N	0.741** <0.001 20	0.481 0.067 12	0.313 0.304 15	0.964* 0.020 3	0.673** 0.001 20	0.496 0.019 4	0.580* 0.015 15	– – 1
Alpha	β	CC p-value N	0.605** 0.002 20	0.614* 0.022 12	0.325 0.291 15	0.133 0.430 3	0.872** <0.001 20	0.910* 0.010 4	0.815** <0.001 15	– – 1

* significant at level 0.05; ** significant at level 0.01; CC – correlation coefficient; N – number of patients.

variants in different grades of meningioma and astrocytoma have not previously been investigated.

Most previous studies investigated only telomere length or telomerase activity and did not assess differences in the expression of hTERT splice isoforms between benign and malignant brain tumors.[15,31] It has been shown that hTERT mRNA is expressed in 100% of glioblastomas, regardless of whether they show positive or negative telomerase activity.[32] Human telomerase reverse transcriptase mRNA expression has been found not only in neoplastic regions but in normal tissues as well, and the expression patterns of hTERT mRNA are consistent with increases in the severity of histopathologic changes.[33] The upregulation of hTERT mRNA in liver cancers and in the early stages of tumor progression are principally concomitant with the β-deletion variant.[11] In general, after the wild type transcript, the β isoforms are the most abundant and highly expressed.[34] Apparently, cell stress conditions cause changes in splicing variant foms.[35] In some cell lines, the loss of polymerase activity upon differentiation can illustrate a variation in the splicing patterns of the β-deletion variant.[36] In this study, we compared the expression of hTERT splice variants. In addition, we showed that the variance of expression of hTERT mRNA isoforms can have a determinant effect on the mechanisms of tumorigenesis in the human brain. Variations in hTERT expression can determine the mechanism of tumorigenesis. Our findings indicate a surprisingly high degree of variation in the proportions of the expression of hTERT splice variants in 2 groups of patients with brain tumors. High levels of the full-length hTERT transcript and 2 common deletion isoforms (α-deletion and β-deletion) were detected significantly more frequently in the patients with brain tumors compared to healthy controls. Our data revealed that the functional full-length hTERT variant was expressed at higher levels in the meningioma patients compared to the controls and astrocytoma patients (Fig. 1,2). High expression of the full-length hTERT splice variant had prognostic implications for meningioma patients: the data analysis suggested that there is an association between the quantification of hTERT expression and tumor progression. All of the meningeal tumor samples expressed hTERT mRNA, and as the degree of tumor increases, the rate of expression also increased (Table 4), which is in agreement with a study by Falchetti et al., in which hTERT expression was related to the MIB-1 and Ki-67 proliferation indexes and with the recurrence rate of high-grade meningiomas.[37]

With the progression of grade I tumor cells to higher grades, including II and III, there was an increase in the expression pattern of hTERT splicing, favoring β-deletion and full-length wild type isoforms, but the dominant form in grade II and III of meningioma and astrocytoma was the β-deletion variant (Fig. 3). This might explain the heterogeneity of neuroepithelial tumor cells.[26,33] In our study, a higher level of β-deletion isoform expression in high-grade astrocytomas was observed. The higher ratio of this isoform as compared to the full-length isoform indicates that there might be a higher proportion of nonsense mutations in malignant astrocytoma patients compared to the control samples (Fig. 1). The high expression of the β-deletion isoform in high grades of brain tumors suggests that unlimited cell proliferation and aggressiveness are dependent on the expression of the β-deletion isoform, which is consistent with the results of previous studies.[33,38]
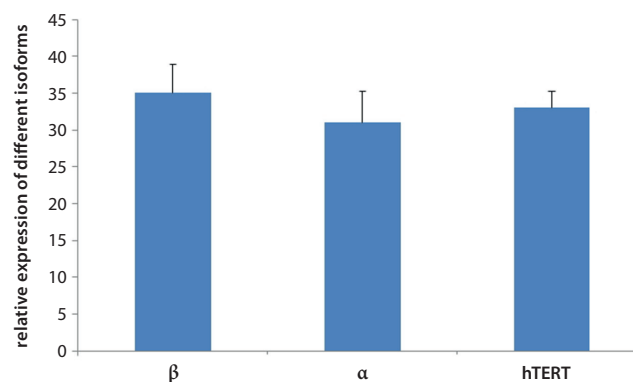

Fig. 3. The expression pattern of different splice variants in the meningioma patient showed high expression of human telomerase reverse transcriptase (hTERT) and α variants in benign tumors

Table 4. Relative expression patterns of human telomerase reverse transcriptase (hTERT) splice isoforms in different grades of brain tumors

Tumor type/ grade	hTERT	α-deletion variant	β-deletion variant
Astrocytoma			
Grade III	+++++	–	++++++
Grade II	++++	+	+++++
Grade I	+++	++++	++
Meningioma			
Grade III	++++++	+	++++
Grade II	+++++	+++	+++
Grade I	++++	+++++	+

+ level of expression; – lack of expression.

A relatively low percentage of the α-deletion splice variant, a negative inhibitor of telomerase activity, is overexpressed in both normal and benign tumor cells.[23,39] The α-deletion variant is expressed in low-grade meningioma and astrocytoma tumors that show the highest positive correlation with the expression of β-deletion variants and it is mostly absent in high-grade tumors.[26,33] The α-deletion isoform is the least abundant in meningiomas as well as astrocytomas (Fig. 2). This transcript is always detected in cells and tissues expressing hTERT.[36,40] Our data analysis showed that the differences between α-isoform expressions and hTERT transcript in low-grade meningiomas are statistically significant. In low-grade meningiomas and astrocytomas, the most abundantly expressed splice variant was the α-deletion variant, which probably has a dominant negative effect over the hTERT full-length variant (Table 4).

The fact that there is considerable heterogeneity in the expression of hTERT splice variants in brain tumors generates many questions about programs of alternative splicing and the role of each transcript in molecular changes in the field of tumorigenesis. Our results show that the β-deletion variant is more important in patients with astrocytomas, while full-length hTERT mRNA is more important in patients with meningiomas. Our findings indicate that α-deletion isoforms are associated with full-length hTERT mRNA levels in grades I and II meningiomas, in addition to grade I astrocytomas. Increased expression of the β-deletion variant was generally equal to or slightly higher than the expression of the full-length hTERT variant in grade III meningiomas and grades II and III astrocytomas. In low grades of brain tumors, the expression of the α-deletion variant was dominant while the expression of full-length hTERT variant and the β-deletion variant in high-grade tumors were statistically significant. Apparently, an ongoing process favoring the expression of the β-deletion variant in high grades, abating the expression of the functional transcript and variations in the expression pattern of hTERT splice variants with increasing tumor grade is associated with a poor prognosis.

In conclusion, changes in the splicing pattern of hTERT splice variants in brain tumors and their correlations with pathological changes in cells could be used as diagnostic or prognostic biomarkers, or as possible targets for cancer therapy. However, the function and biological role of hTERT splice variants remain to be clarified.

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