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Comparison of TERRA expression in human brain tumors

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Summary. Telomerase activation has been found in a variety of human cancers, including brain tumors to prevent the telomere from progressive shortening, but telomeres are transcribed into a non-coding RNA called telomeric repeat-containing RNA or TERRA which acts as a natural inhibitor of telomerase activity. Considering meningioma and astrocytoma are the most common tumors of the CNS, the aim of this study was to evaluate TERRA expression level in meningioma, astrocytoma tumors and nontumor (NT) controls. Furthermore, expression levels of TERRA were compared between different grades of meningioma and astrocytoma. Additionally, we analyzed the correlation of TERRA expression and improvement outcome. The total RNA of 51 brain tumor samples and 4 samples as nontumor (NT) controls was extracted and SYBR Green real-time reverse transcription–polymerase chain reaction assays for quantitation of total TERRA levels were developed. Tumor samples from 25 patients with meningiomas and 26 patients with astrocytoma were assessed. We demonstrated the correlation between total TERRA levels of expression with different grades of brain tumors and improvement outcome. According to our study, TERRA may be a prognostic marker in meningioma and astrocytoma tumors.

Key words: expression, TERRA, meningioma, astrocytoma

«Confronto dell'espressione dell'RNA non codificante denominato TERRA nei tumori cerebrali umani»

Riassunto. L'attivazione della telomerasi è stata riscontrata in una varietà di tumori maligni umani, inclusi i tumori del cervello al fine di proteggere il telomero dall'accorciamento progressivo; ma i telomeri sono trascritti all'interno di un RNA non codificante chiamato RNA contenente ripetizione telomerica o TERRA che agisce come inibitore naturale dell'attività telomerica. Dal momento che il meningioma e l'astrocitoma sono tra i tumori più frequenti del sistema nervoso centrale, lo scopo di questi studi è stato di valutare i livelli di espressione di TERRA comparando i differenti gradi di meningioma e astrocitoma. Inoltre abbiamo analizzato la correlazione tra l'espressione di TERRA ed un miglioramento nell'esito della malattia. L'RNA totale di 51 campioni di tumore cerebrale e 4 campioni senza tumore di controllo (NT) è stato estratto e la valutazione tramite "SYBR green real-time reverse transcription-PCR" è stata eseguita per la quantificazione dei livelli totali di TERRA che si sono sviluppati. Sono stati valutati campioni di tumore provenienti da 25 pazienti con meningioma e 26 con astrocitoma. Abbiamo dimostrato la correlazione tra i livelli di espressione totale di TERRA, i differenti gradi di tumore cerebrale ed il miglioramento nell'esito della malattia. Sulla base del nostro studio, TERRA può essere considerato un marker di prognosi in caso di meningioma ed astrocitoma.

Parole chiave: espressione, TERRA, meningioma, astrocitoma

Introduction

Telomeres are long repetitive DNA sequences at both ends of linear chromosomes which act to maintain chromosomal stability, protect chromosome ends against recombination, fusion and DNA degradation, as well as avoiding replicative senescence (1, 2). In vertebrates, telomeres are constituted of tandem repeat of hexanucleotide sequences, TTAGGG (2, 3). At each cycle of cell division, telomeres lose approximately 50–100 base pairs of telomeric repeat as a consequence of the inability of DNA polymerases to replicate the 5´ end of linear DNA (4, 5). One way of preventing telomere loss is to activate a telomere maintenance mechanism (TMM) that leads to immortalization and has been described as a critical hallmark of cancer cells. Two types of TMM exist in tumor cells (3, 6, 7). Telomerase activity (TA) is the main mechanism in 85% of human cancers (TelTMM) (6). Telomerase activity is not detected in approximately 10% to 15% of human cancers and a subset of these tumors use alternative lengthening of telomeres (ALT) for maintaining telomere length (3, 7). Some tumor cells use a combination of the two telomere maintenance mechanisms (3). The ALT phenotype has rarely been identified in epithelial malignancies though it is relatively common in subtypes of sarcomas and astrocytomas. However, the prevalence of ALT has not been thoroughly assessed across all cancer types. Significantly, in the most cases ALTTMM is not observed in benign neoplasms or normal tissues. In recent analysis, telomeric repeat-containing RNA (TERRA), a class of large noncoding RNA transcripts (TERRA or TelRNA), has been recognized in animals and fungi (3, 8). TERRA is transcribed at chromosome ends from the subtelomere towards the end of the chromosomes by RNA polymerase II (9). By in silico analysis more than 20 TERRA transcripts have been predicted and are determined as telomeric transcriptome (8). RNA surveillance factors regulate TERRA transcription (10, 11). It seems that reduction of TERRA expression correlates to telomerase activity (TA) which indicates that TERRA is a natural ligand of telomerase and may inhibit telomerase (9, 10, 12). In tumors that use TA as TMM such as advanced stages of larynx, colon, and lymph node tumors, TERRA expression proves to be down-regulated compared with normal tissues (13). Tumor characterization based on the TMM type will become important in the future and help in the prognosis of some tumor types, thus having therapeutic consequences (14, 15). For instance, in this area telomerase and alternative lengthening of telomere activators and inhibitors may become important as chemopreventive or chemotherapeutic agents (16). Meningioma and astrocytoma are the most common tumors of the CNS, classified into different grades according to the WHO, and each grade shows a different level of mortality (17-19).

Given the crucial need to find a prognostic marker in astrocytomas (as a more aggressive tumor) and meningiomas (as a less aggressive), we evaluated TERRA expression level in meningioma and astrocytoma tumor tissue samples by using quantitative real-time RT-PCR. We also compared expression levels of TERRA between different grades of meningioma, astrocytoma and nontumor (NT) controls. Additionally, we assessed the correlation of TERRA expression with improvement outcome.

Materials and Methods

Patients and samples

51 patients underwent surgery for brain tumor (meningioma in 25 and astrocytoma in 26). Also, clinical brain autopsy was performed to collect tissue samples from 4 dead bodies serving as nontumor (NT) controls. Tissue samples were resected at the Alzahra hospital of Isfahan, Iran, between 2012 and 2014. The tumor tissue was promptly frozen in liquid nitrogen in the operation room and stored at –80°C. These samples were graded on the basis of the World Health Organization (WHO) classification (20). The study was performed at the Isfahan University of Medical Sciences, according to the instructions of the local Ethics Committee and with patients' informed consent. We analyzed the long-term follow-up until November 2014, it being available for 48 out of the 51 patients.

RNA extraction and cDNA synthesis

RNA extraction from tissue samples was performed using TRIZOL reagent (Invitrogen, Carlsbad,

CA), according to the manufacturer's instructions. The purity of the extracted RNA was determined by photometric measurement such as NanoDrop. The ratio of extinctions at 260 nm/280 nm was between 1.7 and 2.2. In turn, the quality of the RNA was examined by denaturing agarose gel analysis with a green viewer. The ratio of intensities of the 28S/18S rRNA signal was calculated and values between 1.5 and 2.5 being tolerated.

For removal of genomic DNA from RNA samples, DNase I treatment was carried out. Complementary DNA (cDNA) was synthesized with a "RevertAid™ H Minus First Strand" cDNA synthesis kit (Fermentas, Vilnius, Lithuania), from two micrograms of RNA with random hexanucleotide primers. All procedures were performed according to the manufacturer's protocol.

Primers and RT-PCR Procedure

Transcript levels of total TERRA were measured after cDNA synthesis by real-time PCR on a StepOnePlus™ system with standard SYBR Green Master Mix (Applied Biosystems). Total TERRA amplification was performed with telomere-specific (T) primers. Primers were obtained from previous studies (3, 21, 22). Tel1b 5´-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GGT -3´ and Tel2b 5´-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT -3´ were used for TERRA amplification (3, 22). 36B4u 5´- CAG CAA GTG GGA AGG TGT AAT CC -3´ and 36B4d 5´- CCC ATT CTA TCA TCA ACG GGT ACA A -3´ were used for 36B4 gene amplification as an endogenous control (3, 21). Reactions were set up in triplicate. Relative quantities (RQ) were determined in relation to levels of 36B4. Expression levels were assessed using the comparative CT method. 10 µl amplification mixture contained 5 µl of RT2 SYBR Green/nonROX qPCR master mix (SA Biosciences, CN.PA-012-12), 0.4 µl gene specific, 10 µM primer pairs (forward and reverse primers), 2 µl template cDNA (250 ng/µl) and 7.6 µl ddH_2O. Thermal cycling parameters entailed an initial step of 6 min at 95°C for activation of Hot Start Taq DNA polymerase, and then 40 cycles at 95°C for 15 s and 60°C for 1 min for the melting and combined annealing and extension phases of the PCR.

Statistical analysis

All measurements were done in at least triplicate and REST software 2008 (Relative expression software tool v2.0.7) was used for the calculation of relative expression. Relative TERRA levels were normalized to 36B4. The comparative Ct (ΔΔCt) method was used to calculate fold changes in gene expression. Statistical analysis to compare the relative expression of genes in both astrocytoma and meningioma tumors was performed by SPSS software for Windows software (version 19) using central and distribution indexes, and the independent two-tailed t-test was used for comparison of TERRA expression between astrocytoma, meningioma and nontumor (NT) controls. We also analyzed improvement outcome by logistic regression and cross-tab.

Results

Clinical-pathologic variables

We examined 51 brain tumor samples, including 25 men and 26 women. The mean age of 18 men and 8 women patients was 46 years (range, 5-82 years) in the case of astrocytoma, while that of the remaining 7 men and 18 women patients was 55.84 years (range, 34-81 years) for meningioma. The patients' other clinical-pathologic variables are listed in Table 1.

Analysis of RNA expression

Calculation of the relative expression between astrocytoma and meningioma tumors shows lower expression in astrocytoma than meningioma samples. This up-regulation was 2.628-fold, though independent two-tailed t-test showed that the difference in TERRA expression between meningiomas and astrocytomas was not significant (Fig. 1). Again, high grades (III and IV) of astrocytoma tumors had a lower ΔCt mean than low grades (II), and down-regulation for TERRA was -4.377-fold (p-value: 0.036). (Fig. 1).

Table 1. Clinicopathologic characteristics of 51 patients with brain tumor.

Character	Meningioma	Astrocytoma
Mean age	55.84	46
Std. Deviation of age	12.375	19.144
Sex		
Male	8	18
Female	17	8
Clinical follow-up available	22	26
Favorable outcome	21	14
Deceased	1	8
Grade		
Grade I	20	-
Grade II	4	6
Grade III	1	2
Grade IV	-	18
Total	25	26

We compared the expression of TERRA between different grades of brain tumors. Analysis of the relative expression between a low grade (I) and high grades (II and III) of meningioma tumors showed a down-regulation in high grades versus low grade that was -4.760 (p-value: 0.031) (Fig. 1) .

Comparison of RNA expression for this gene between low grades of astrocytoma and grade I of meningioma tumors showed that a higher level of expression in low grade meningioma tumors (1.152-fold), but this difference was non-significant. Again, we did not find any significant variation in TERRA expression in our comparison between high grades of meningioma (II and III) and astrocytoma (III and IV) (1.059-fold higher in meningioma than astrocytoma). Additionally, TERRA expression in astrocytoma proved 4.969-fold less than levels of TERRA expression in nontumor (NT) controls (p-value: 0.029) (Fig. 1) and its expression in meningioma was 1.887-fold less than total TERRA expression in nontumor (NT) controls, the difference again being non-significant. We also compared TERRA expression in different grades of astrocytoma and meningioma to the nontumor (NT) controls. Whereas low grade astrocytoma (II) had 1.889-fold down-regulation in comparison to nontumor (NT) controls (though we found no significant variation), high grades of astrocytoma (III and IV) showed 8.271-fold decreased expression compared to nontumor (NT) controls (p-value: 0.016) (Fig. 1). Expression of TERRA in low grade meningioma (I) was 1.488-fold less than nontumor (NT) controls (which is non-significant), whereas high grade

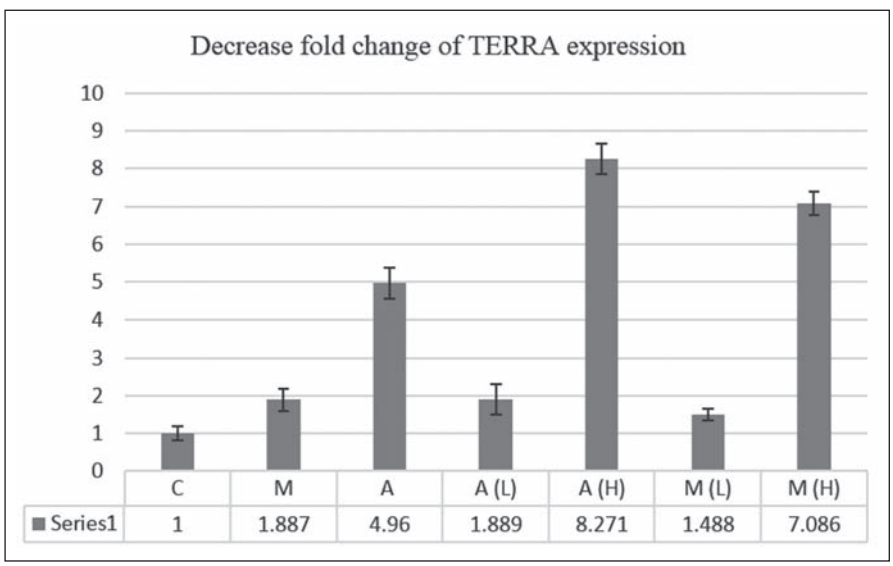

Figure 1. Comparison of the relative TERRA expression between nontumor (NT) controls, astrocytoma and meningioma tumors.
C: nontumor (NT) controls; M: Meningioma; A: Astrocytoma; A (L): low grade (II) of astrocytoma; A (H) high grades (III and IV) of astrocytoma; M (L): low grade (I) of meningioma and M (H): high grades (II, III) of meningioma tumor.

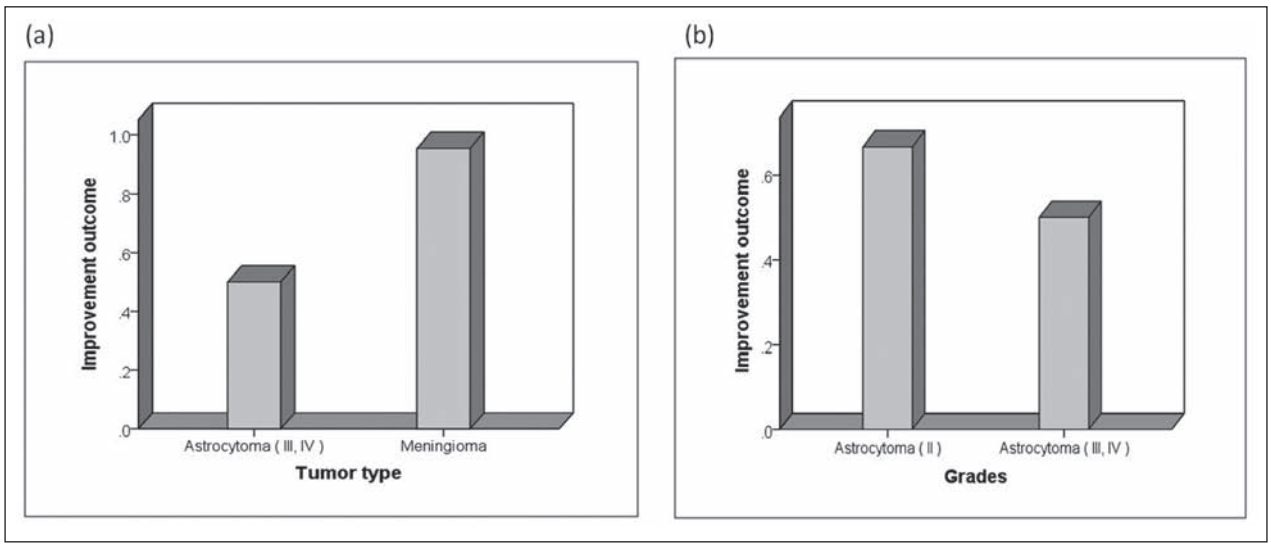

Figure 2. Comparison of the improvement outcome between (a) meningioma and high grades of astrocytoma (III and IV), (b) different grades of astrocytoma tumor.

meningioma showed a 7.089-fold down-regulation in comparison to nontumor (NT) controls (p-value: 0.021) (Fig. 1).

Furthermore, improvement outcome was assessed according to the neurologist's opinion based upon several factors that influence outcome such as the "patient Karnofsky rating", which can be used to compare the effectiveness of different therapies. According to the Karnofsky score range (0-100), 100 is "perfect" health and 0 is death. This allows physicians to evaluate patients' ability to improve their cancer outcome. According to the results of logistic regression, the rate in high grades of astrocytoma (III and IV) was 21-fold lower than meningioma (p-value 6 $\times 10^{-3}$) (Fig. 2 and Table 2).

Table 2. Patient's improvement outcome. According to the results of logistic regression, its rate in high grades of astrocytoma (III and IV) is 21-fold lower than meningioma

Improvement	Sig.	Odds ratio	95% Confidence Interval for odds ratio	
			Lower Bound	Upper Bound
Astrocytoma (III, IV)	.006	21.000	2.352	187.493
Meningioma*	.	.	.	.

* Meningiomas is intended as a reference

Likewise, comparison of the improvement in different grades of astrocytoma showed a 2-fold increase in grade II compared with high grades of astrocytoma (III and IV), but this was not significant (Fig. 3). As well, appraisal of the relationship between grades of meningioma and astrocytoma in terms of sex and age did not produce a significant p-value.

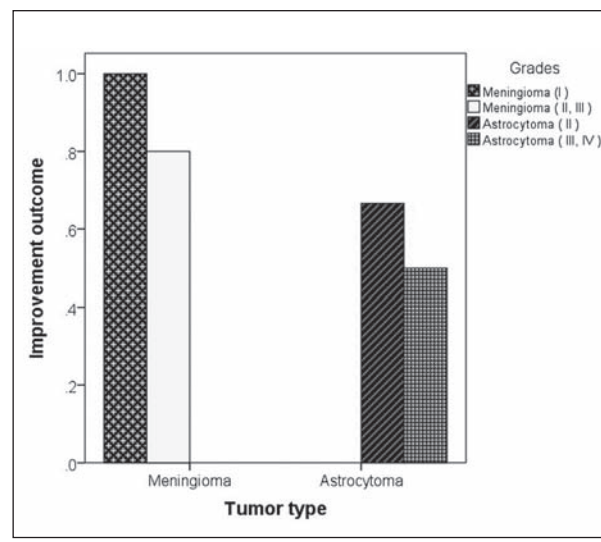

Figure 3. Comparison of the improvement outcome between different grades of astrocytoma and meningioma.

Discussion

The telomeric repeat-containing RNA (TERRA) assay is a relatively new assay for TMM assessment that may determine whether a tumor is telomerase positive or ALTTMM positive (15). TERRA expression levels are evaluated by PCR-based methods. In our study, TERRA expression levels were assessed upon different grades of astrocytoma and meningioma for the first time by the telomeric repeat-specific PCR assay which has been used in similar studies (21, 22).

Although, there are not many studies on TERRA expression in brain tumors, we know that TERRA expression reflects Telomere maintenance mechanisms (TMMs) (15) and the regulation of the telomeric state plays a greater role in disease etiology than previously predicted (23). We have also found that a decrease in total TERRA expression is significantly related to an increase in different tumor grades of both meningioma and astrocytoma, as obtained from current histopathology scoring systems. TERRA expression is inversely related to tumor grade in meningioma and astrocytoma. Indeed, the more aggressive phenotypes in higher tumor grades, by histopathological scoring, display greater reduction in gene expression. It is likely that TERRA inhibits telomerase activity (13, 24). This gene is inversely correlated with increasing telomerase activity. Although a reduction in TERRA expression is seen to have started in low grades of meningioma and astrocytoma, high grades of these brain tumors show strong down-regulation. Conversely, in agreement with our previous result, tumors that have initiated telomerase activity show high concentrations of TERRA expression (3, 25). According to another study, ALTTMM positive tumors show high concentrations of TERRA expression and increased TERRA levels can be a marker of ALTTMM (3).

In addition, the TERRA expression level is associated with improved outcome. Whereas up-regulation of TERRA in meningioma is accompanied with more improvement, down-regulation of TERRA in astrocytoma is associated with less improvement. Again, in low-grade astrocytoma (II), over-expression of TERRA is accompanied by better improvement than in higher grades of the tumor (III and IV). The main aims of treatment and follow-up are to increase survival while maximizing a patient's functional capability and quality of life and TERRA expression correlates with overall survival (3). Sampl *et al.* (2012) analyzed a small glioblastoma cohort (28 cases) and found that a combination of assays can result in a correlation with survival while at least two assays are required to identify the TMM category (3). The same study found that TERRA expression together with TA in astrocytoma may be prognostic for survival (3). In addition, they claim that detection of low TERRA levels carries a worse prognosis. On the contrary high TERRA levels could forecast a better outcome. These results are consistent with our results.

The ability to determine the TMM from blood samples is an attractive approach in clinical analysis, and may be useful for early tumor detection. This evidence is urgently calls for clinical trials. Using a combination of these methods for TMM identification to predict the patient's clinical condition may enhance sensitivity and specificity. Screening of patients according to TMM type will point the way to the best possible treatment (15). In conclusion, TERRA could be a suitable candidate for clinical prognosis. TERRA research is still in the early stages. Focus on TERRA research to find appropriate therapy will further develop in the future.

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