

Significance of TERT and ATRX mutations in glioma

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Abstract. Mutations of telomerase reverse transcriptase (TERT) and the α thalassemia/mental retardation syndrome X-linked (ATRX) genes have been the subject of numerous studies on the classification and prognosis of glioma. However, the association between TERT and ATRX in World Health Organization (WHO) grade II to IV glioma remains unclear. The present study utilized Sanger sequencing and immunohistochemical methods to detect the expression of the TERT promoter region, ATRX mutations and proliferation marker protein Ki-67 (Ki-67) protein expression in 179 cases of glioma. The current study analyzed these variables and their association with clinicopathological characteristics to further basic research and provide a theoretical basis for the clinical diagnosis and treatment of this type of tumor (1). The results demonstrated that TERT promoter mutations were negatively associated with ATRX. Additionally, Ki-67 protein expression in TERT wild-type samples was higher compared with samples with ATRX deletion. Overall, the results demonstrated, for the first time to the best of the authors' knowledge, that TERT promoter mutations are negatively associated with ATRX expression in WHO grade II to IV gliomas. These findings provide a theoretical basis for further basic research and may improve clinical diagnosis and treatment of glioma in the future.

Introduction

Mutations in telomerase reverse transcriptase (TERT) and the α thalassemia/mental retardation syndrome X-linked (ATRX) gene are the subject of numerous studies on the classification and prognosis of glioma (1). A telomere is a nucleoprotein complex containing hundreds of nucleotide repeats that exists

at the end of all eukaryotic chromosomes and gradually shortens with each round of mitosis (2). Telomerase is a reverse transcriptase that uses its own RNA as a template to add nucleotides to telomeres (3). Cancer cells characteristically acquire the infinite capability to divide by maintaining telomere length through the sustained expression of telomerase, or in the absence of telomerase, by an alternative lengthening of telomeres (ALT) mechanism (3). Previous studies have demonstrated that mutations in the core region of the TERT promoter in glioma and various other tumors lead to increased telomerase activity (2,4-7). In addition, various tumors with alternative telomeres exhibit ATRX gene mutations, with an incidence of ~75% in grade II-III glioma (2). Therefore, these mutations may be important to glioma formation and development.

The increasing number of reports on the mechanism of telomere maintenance in glioma has been crucial for disease treatment and patient prognosis (4,5,7-15). However, to the best of the authors' knowledge, no studies have assessed the distribution and importance of ATRX alterations and TERT promoter mutations in glioma. The aim of the present study was therefore to assess ATRX and TERT promoter mutations in glioma and to analyze their role in the proliferative activity and WHO pathological grades of gliomas, thus furthering research and providing a theoretical basis for the clinical diagnosis, and treatment of glioma.

Materials and methods

General information. Tumor tissues were obtained from August 2016 to June, 2017 at Tianjin Huanhu Hospital (Tianjin, China). To ensure the accuracy of pathological diagnosis, the human glioma genome database in China, (Chinese Glioma Genome Atlas, CGGA, <http://www.cgga.org.cn/>), was used, and >80% of the tissue was tumor tissue. All of the tissues were diagnosed by an experienced pathologist. The present study was approved by the Medical Ethics Committee of Tianjin Huanshu Hospital and all patients provided signed informed consent. The inclusion criteria were as follows: i) Specimens must be pathologically diagnosed with grade II-IV glioma using the 2016 version of the World Health Organization (WHO) central nervous system tumor classification (16); and ii) The pathology library had complete and adequate paraffin specimens. Samples were excluded if tissues were of poor quality and if they were classified as WHO grade I (low grade) glioma. Following

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screening, 179 specimens were obtained, which included 38 WHO grade II glioma samples, 43 grade III samples and 98 grade IV samples (108 males and 71 females; age range, 8-84 years; median age, 48.5 years).

Immunohistochemistry. ATRX immunohistochemistry of polyclonal rabbit antibody (dilution 1:400; product code HPA001906; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was performed using an automated immunostainer (Leica Bond III; Leica Microsystems GmbH, Wetzlar, Germany) and standard protocols (10). Evaluation was performed by two observers simultaneously on a multi-headed microscope and scoring was done in consensus. Only nuclear staining was considered for evaluation. Cases with more than 10% positive tumor cells were scored as positive. Endothelial cells, cortical neurons and infiltrating inflammatory cells were generally positive and served as internal positive controls. Cases with negative tumor cells in which vessel cells and neurons were not stained were not evaluated and not considered for further statistical evaluation. In cases with inhomogeneous immunoreaction, areas with highest staining were scored. Regarding the analysis of Ki-67, the following methods were used in this study: Immunohistochemical staining was conducted and the proportion of the malignant cells staining positive for the nuclear antigen Ki-67 was evaluated in a quantitative and visual way using light microscopes (magnification, x400). The Ki-67 percentage score is defined as the percentage of positively stained tumor cells among the total number of malignant cells assessed. Withal only the positivity is of interest independent of the intensity of coloration. To ensure quality assurance of the staining, positive control tissues are completed. A Ki-67 cut-off point of 27.2% was defined mean of Ki-67 expression (Table I) The complete specimen is investigated and checked for immunostaining tumor cell nuclei. Scoring is conducted considering the whole tumor section and not only limited to the hot spots of the carcinoma or to the most evident positive parts within the invasive segment or the front of necrosis.

Sanger sequencing. A pyrosequencing assay was designed to examine the two reported mutation hot spots in the TERT promoter. They result in a cytidine to thymidine transition at position 1,295,228 or 1,295,250 on the reverse strand of chromosome 5. These mutations are termed C228T and C250T, respectively. The following primer pair for polymerase chain reaction (PCR) was designed to amplify a region including both sites; forward, GTCCTGCCCCCTT CACCTT (biotinylated at the 5' end) and reverse, GCACCT CGCGGTAGTGG (biotinylated at the 5' end). The primers for pyrosequencing were designed immediately upstream of the hotspot as follows: TGTAACACGACGGCCAGT (biotinylated at the 5' end), CAGGAAACAGCTATGACC (biotinylated at the 5' end). The mutation hotspots at codon 132 of IDH1 and codon 140 of IDH2 were also screened using pyrosequencing. The following oligonucleotide primers were used for amplification; IDH1 forward, ATGAGAAGAGGG TTGAGGAGTT (biotinylated at the 5' end) and reverse, GCA AAATCACATTATTGCCAAC (biotinylated at the 5' end); IDH2 forward, GCTGCAGTGGGACCACTATT (biotinylated at the 5' end) and reverse, CTAGGCGAGGAGCTCCAGT

Table I. Associations between various factors and TERT status.

| Variable | Total number | TERT mutations | | P-value |
|----------------------|--------------|----------------|----|---------|
| | | Mut | WT | |
| Number | 179 | 97 | 82 | |
| Age (years) | | | | |
| ≤48.5 | 71 | 46 | 25 | 0.038 |
| >48.5 | 108 | 72 | 36 | |
| Sex | | | | |
| Male | 108 | 58 | 50 | 0.253 |
| Female | 71 | 31 | 40 | |
| WHO grade | | | | |
| II | 38 | 17 | 21 | 0.015 |
| III | 43 | 26 | 17 | |
| IV | 98 | 54 | 44 | |
| IDH | | | | |
| Mut | 73 | 35 | 38 | 0.066 |
| WT | 106 | 62 | 44 | |
| ATRX | | | | |
| Expression | 128 | 86 | 37 | 0.001 |
| Loss | 50 | 11 | 39 | |
| Ki-67 expression (%) | | | | |
| ≤27.12 ^a | 88 | 45 | 43 | 0.167 |
| >27.12 | 91 | 53 | 38 | |
| Recurrence (n) | | | | |
| Recurrence | 8 | 5 | 3 | 0.442 |
| Non-recurrence | 180 | 93 | 87 | |

^aMean of Ki-67 expression is 27.12%. TERT, telomerase reverse transcriptase; WHO, world health organization; IDH, isocitrate dehydrogenase; ATRX, α thalassemia/mental retardation syndrome X-linked; Mut, mutation; WT, wild-type.

(biotinylated at the 5' end). The primers for pyrosequencing were designed immediately upstream of the hotspot as follows: For IDH1, IDH2: TGTAACACGACGGCCAGT (biotinylated at the 5' end), CAGGAAACAGCTATGACC (biotinylated at the 5' end). The pyrosequencing assays were designed to detect all known mutations at IDH1 R132 or IDH2 R140. Templates for Sanger sequencing were prepared by amplifying genomic DNA with the same primer pair as for pyrosequencing without biotinylation of the reverse primer. Cycle sequencing was carried out using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol with the forward PCR primer as a sequencing primer.

Statistical methods. SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA) was used for data analysis. The association between the indexes was statistically analyzed using logistic regression, the Fisher exact test and Logistic Regression. One-way analysis of variance was used to analyze Ki-67 expression data with Fisher's Least Significant

Table II. Logistic regression analysis of factors associated with TERT.

| Factor | B | S.E. | df | Sig. | Exp (B) | 95% CI of EXP (B) | |
|-----------|--------|-------|----|-------|---------|-------------------|-------------|
| | | | | | | Minimum | Upper limit |
| Age | 0.049 | 0.024 | 1 | 0.038 | 1.050 | 1.003 | 1.100 |
| WHO grade | | | 2 | 0.015 | | | |
| WHO II | 2.008 | 0.883 | 1 | 0.023 | 7.447 | 1.319 | 42.034 |
| WHO III | 2.106 | 0.827 | 1 | 0.011 | 8.211 | 1.623 | 41.542 |
| ATRX | -3.007 | 0.927 | 1 | 0.001 | 0.049 | 0.008 | 0.304 |

TERT, telomerase reverse transcriptase; WHO, world health organization; ATRX, α thalassemia/mental retardation syndrome X-linked; B, regression coefficient; S.E., standard error; Sig., significance; df, degree of freedom; exp, odd ratio.

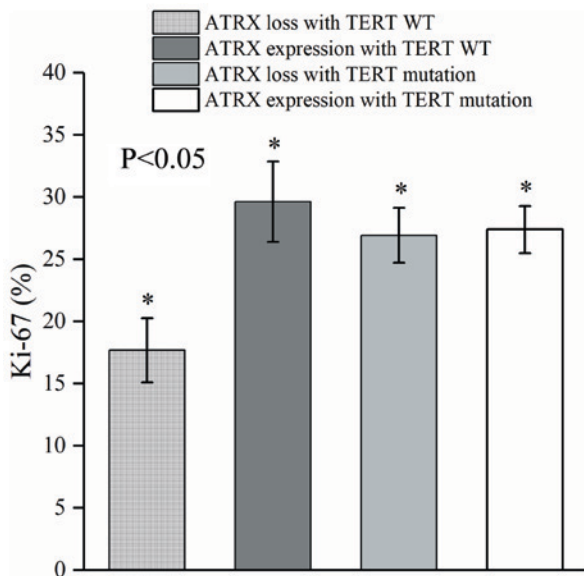


Figure 1. Difference between ATRX mutants and wild-type populations. In the wild-type TERT group, Ki-67 protein was least abundant in ATRX-deficient patients. In the four groups, Ki-67 expression was highest in patients without ATRX deletion. * $P<0.05$. TERT, telomerase reverse transcriptase; ATRX, α thalassemia/mental retardation syndrome X-linked; WT, wild-type; Ki-67, proliferation marker protein Ki-67.

Difference post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TERT promoter mutations are positively associated with age and WHO grade, but negatively associated with ATRX mutations. In the present study, the Logistic Regression was utilized to assess the effects of age, sex, WHO classification, IDH mutation, ATRX loss and Ki-67 protein expression on TERT promoter mutations (Table I). The results demonstrated that sex, IDH mutation and Ki-67 protein expression did not significantly affect TERT promoter mutations. However, age, WHO grade and ATRX loss were significantly associated. Logistic regression was then utilized to assess age, WHO grade and ATRX loss in TERT promoter mutations. The three variables included in the model (age, WHO grade and ATRX loss) were all determined to be associated with

TERT mutation status (Table II). The probability of mutation in the TERT promoter in ATRX wild-type samples was 20.40 (1/0.049) times higher than that in ATRX mutants. Furthermore, the TERT mutation probability increased by 4.9% for each additional year of age (Table II). In addition, TERT promoter mutations were associated with WHO grade. Using WHO grade IV as a reference variable, the odds ratio of TERT promoter mutation was 7.447 and 8.211 for WHO grades II and III, respectively (Table II).

ATRX mutations are positively associated with age and IDH mutations. The Logistic Regression was also used to assess the effects of age, sex, WHO classification, IDH mutation, TERT mutation and Ki-67 protein expression on ATRX status (Table III). The results indicated that sex, WHO classification and Ki-67 expression did not significantly affect ATRX status. However, age and IDH mutation were determined to be significantly associated. The associations between age, IDH mutation and ATRX loss was further assessed using logistic regression. The two variables included in the model (age and IDH mutation) were determined to be statistically significant (Table IV). The probability of ATRX increased by 8.8% for each additional year of age. Additionally, the probability of ATRX mutation in IDH mutant samples was 14.03 times higher than that in IDH wild-type samples.

TERT wild-type and ATRX wild-type tumors exhibit high Ki-67 protein expression; however the wild-type TERT promoter with ATRX mutations exhibits low Ki-67 protein expression. Karsy *et al* (17) have identified ATRX and TERT mutations in anaplastic gliomas. To assess the association of ATRX and TERT mutations with WHO grade II-IV gliomas, tissues in these genes were collected (Table V). In addition, ATRX mutations were added to TERT promoter mutations to observe their association with Ki-67 protein expression (Fig. 1). Difference of measurement data was compared with single factor analysis of variance ($P=0.018$). The results demonstrated that TERT promoter mutations were significantly different in ATRX mutant and wild-type populations. In the TERT wild-type group, Ki-67 protein was least abundant in patients that were ATRX-deficient, and Ki-67 expression was highest in patients with ATRX expression (Fig. 2).

Table III. The association between various factors and ATRX status.

| Variable | Total number | Loss | Expression | P-value |
|------------------------|--------------|------|------------|---------|
| Number | 179 | 50 | 123 | |
| Age | | | | |
| ≤48.5 | 71 | 39 | 32 | 0.005 |
| >48.5 | 108 | 17 | 91 | |
| Sex | | | | |
| Male | 108 | 31 | 77 | 0.739 |
| Female | 71 | 25 | 46 | |
| WHO grade | | | | |
| II | 38 | 21 | 17 | 0.507 |
| III | 43 | 24 | 19 | |
| IV | 98 | 71 | 27 | |
| IDH | | | | |
| Mut | 73 | 34 | 39 | 0.003 |
| WT | 106 | 22 | 84 | |
| TERT | | | | |
| Mut | 97 | 11 | 86 | 0.001 |
| WT | 82 | 45 | 37 | |
| Ki-67 expression index | | | | |
| ≤27.12 | 87 | 36 | 51 | 0.131 |
| >27.12 | 91 | 20 | 71 | |
| Recurrence (n) | | | | |
| Recurrence | 8 | 4 | 4 | 0.135 |
| Non-recurrence | 171 | 52 | 119 | |

ATRX, α thalassemia/mental retardation syndrome X-linked; WHO, world health organization; IDH, isocitrate dehydrogenase; TERT, telomerase reverse transcriptase; Ki-67, proliferation marker protein Ki-67.

Table IV. Logistic regression analysis of various factors with ATRX.

| | B | S.E. | df | Sig. | Exp (B) | 95% CI of EXP (B) | |
|-----|-------|-------|----|-------|---------|-------------------|------------|
| | | | | | | Minimum | Upperlimit |
| Age | 0.085 | 0.030 | 1 | 0.005 | 1.088 | 1.026 | 1.154 |
| IDH | 2.641 | 0.888 | 1 | 0.003 | 14.033 | 2.464 | 79.927 |

ATRX, α thalassemia/mental retardation syndrome X-linked; IDH, isocitrate dehydrogenase; B, regression coefficient; S.E., standard error; Sig, significance; df, degree of freedom; exp, odd ratio.

Discussion

Telomeres consist of hundreds of nucleotide repeats that are present at the end of all eukaryotic chromosomes as a nucleoprotein complex (18). These gradually shorten in length following each round of mitosis, which eventually leads to aging (19). The gradual depletion of telomeres has been defined as one of the signs of biological senescence (20). Telomerase is a reverse transcriptase that uses its own RNA as a template to add nucleotides to telomeres (6). Cancer cells characteristically acquire the infinite capability to divide by maintaining telomere length

through the sustained expression of telomerase or via the ALT mechanism (3). The human TERT gene encodes a telomerase catalytic subunit, which maintains telomeres by increasing their length (21). TERT is located on chromosome 5p15.33 and the promoter region of this gene is considered the most important regulatory element for its expression (22). At present, there are known inhibitors of TERT transcription, including cellular tumor antigen p53 (p53), which downregulates TERT transcription in a E3 ubiquitin-protein ligase SP1-dependent manner (23). Therefore, the deletion of p53 can activate the ALT pathway (24). TERT mutations are most commonly observed in

Table V. The association between ATRX and TERT status.

| WHO grade | ATRX loss/ retention (%) | TERT loss/WT (%) | ATRX loss and TERT WT | ATRX loss and TERT loss | ATRX retention and TERT WT | ATRX retention and TERT loss |
|-----------|-----------------------------|------------------|--------------------------|----------------------------|-------------------------------|---------------------------------|
| II | 21/17 (44.74) | 21/17 (44.74) | 17 | 0 | 4 | 17 |
| III | 22/19 (44.18) | 17/26 (60.47) | 12 | 3 | 5 | 23 |
| IV | 68/27 (27.55) | 44/54 (55.10) | 16 | 8 | 28 | 46 |

ATRX, α thalassemia/mental retardation syndrome X-linked; TERT, telomerase reverse transcriptase; WHO, world health organization; WT, wild-type.

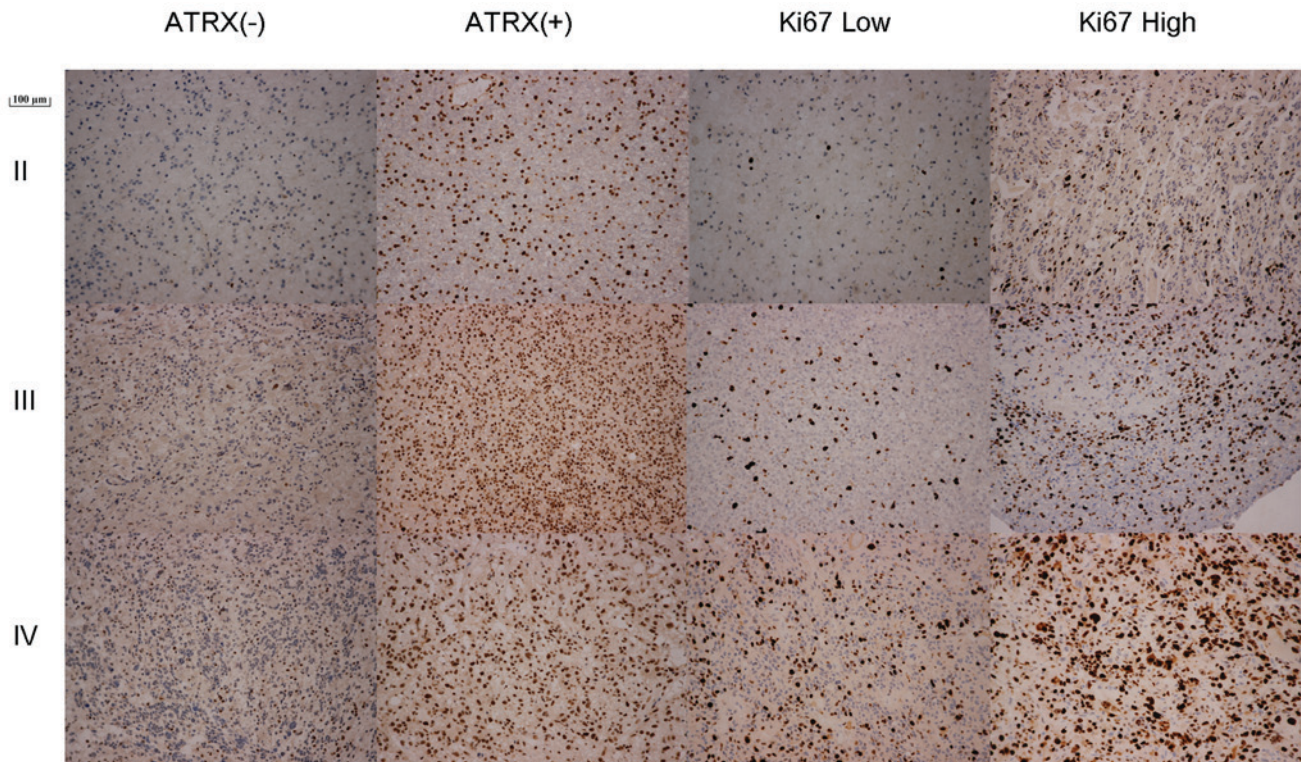


Figure 2. ATRX and Ki-67 staining in II-IV grade glioma (magnification, x400). The dark brown stain indicates ATRX wild-type (+) and the dark blue stain indicate ATRX mutant-type (-). ATRX, α thalassemia/mental retardation syndrome X-linked; Ki-67, proliferation marker protein Ki-67.

medulloblastomas and gliomas, with TERT promoter mutations being identified in ~80% of all primary glioblastoma (7). The results of the present study indicated that TERT promoter mutations were 44.74% (17/38) in WHO grade II gliomas, 60.47% (18/43) in WHO grade III gliomas and 55.10% (42/98) in WHO grade IV gliomas, which are lower than those reported in previous literature (6,25). However, the data of the present study also revealed that TERT promoter mutation was associated with WHO classification, indicating that TERT promoter detection may be used for glioma prognosis, prediction and molecular typing.

ATRX is located on the Xq21.1 chromosome and encodes a 280 kDa nucleoprotein, which is involved in numerous cellular functions, including DNA recombination, repair and transcriptional regulation (26). Human ATRX mutations lead to the development of thalassemia, mental retardation, α -thalassemia X-linked mental retardation and other genetic conditions (27). ATRX mutations are present in at

least 15 types of human tumors, including neuroblastoma, osteosarcoma and pancreatic neuroendocrine tumors (28). However, the role of ATRX in tumorigenesis remains to be elucidated. The ATRX protein may serve an important epigenetic role as it can be deposited on heterochromatin and telomeric DNA (29). In numerous different types of tumor, ALT activation is associated with the loss of function of the ATP-dependent helicase ATRX or its interacting partner (histone H3.3) (30). Similar to TERT promoter mutations, ATRX mutations are also associated with mutations in the tumor suppressor gene p53 (31); ATRX regulates the upstream pathways of cancer by modulating p53 (32). In the current study, the probability of mutations in the TERT promoter region in ATRX mutants samples was less than that in ATRX wild-type which the Exp (B) is 0.049 (Table II). Due to the association between ATRX deletion and ALT phenotype, another mechanism of telomere retention in tumor cells involves TERT (29). TERT promoter

point mutations result in an increase of telomerase expression (33). Certain studies have demonstrated that TERT mutations are present in gliomas (2,4-7,17,33). Therefore, the present study hypothesized that alterations in ATRX and mutations in the TERT promoter region of glioma tissues may be mutually exclusive due to functional redundancy. Furthermore, a previous study demonstrated that ATRX and TERT mutations are mutually exclusive in anaplastic gliomas (7). Following this, the current study tested glioma tissues for mutations within these genes. In tissues with ATRX loss and wild-type TERT, 17 cases of WHO grade II, 12 cases of WHO grade III and 16 cases of WHO grade IV gliomas were identified (Table V). Although previous studies have demonstrated that ATRX loss and TERT promoter mutations in glioma may be mutually exclusive due to functional redundancy (17). Our results, which were 3 and 8 parents with grade III and IV gliomas, respectively, who had both mutations (Table V), suggest that contrary to previous conclusions in WHO grade II-IV glioma. However, it was also suggested that either mutation may contribute to the formation of glioma.

In glioma, molecular markers have independent prognostic significance (34). ATRX mutations are associated with alternative lengthening of telomeres in gliomas and tumor progression (15). The present study revealed that ATRX mutations were closely associated with IDH mutations in the present study. ATRX serves an important role in the regulation of chromatin remodeling (35). It is associated with IDH mutations and it may exhibit different epigenetic features (36). It is closely associated with gliomas of the CpG island methylation phenotype (36). The genetic differences between ATRX expression and non-transcription factors may be the basis of different clinical course in glioma.

The status of IDH in glioma may be predicted by ATRX sequencing (10). ATRX mutations/ALT phenotypes in gliomas can be observed by immunohistochemical staining (29). ATRX mutations are also associated with alternative lengthening of telomeres in gliomas and tumor progression; however, wild-type ATRX does not necessarily exclude the presence of IDH mutations (11,17,37). In the current study, almost all WHO II glioma were not associated with ATRX deletion. In one study on glioma, none of the 12 patients with IDH mutations exhibited ATRX deletions (38). Therefore, the current study hypothesizes that IDH and ATRX should be analyzed simultaneously in these tumors to reliably classify gliomas.

The results of the current study determined that mutations in the TERT promoter and ATRX gene were associated with age. This may be due to patients with low-grade glioma exhibiting high levels of DNA methylation, resulting in increased TERT expression (39). The prolongation and maintenance of telomere length in the tumor cells of young patients also may be more dependent on ALT mechanisms and the epigenetic regulation of telomerase (40). In addition, low-grade glioma cells originate from cells that maintain a strong ability to differentiate (41). These telomerases are in an activated state, such that mutation of the TERT promoter or ATRX is not required to achieve telomerase activation (3).

According to the WHO classification of tumors of the 2016 World Health Organization Classification of Tumors of the

Central Nervous System, in small specimens, such as those obtained from a stereotactic biopsy, a single case of mitosis may suggest significant proliferative activity. Ki-67 labelling may be helpful to illustrate this (16). Therefore, Ki-67 was used to evaluate proliferation in the present study. The Ki-67 protein, which encodes two isoforms with molecular weights of 345 and 395 kDa, was originally discovered by Scholzen and Gerdes in 2000 (42). The expression of the Ki-67 protein is associated with the proliferative activity of the internal cell population in malignant tumors and is a marker of tumor invasiveness (43). The role of Ki-67 in the determination of prognosis has been previously studied, such that it is considered a potential marker for tumors of the central nervous system (44). The present study did not identify a statistically significant association between ATRX mutations and Ki-67 expression in the TERT promoter mutation group. However, when ATRX mutations were added to the TERT promoter mutations, it was demonstrated that TERT promoter mutations were significantly different between ATRX mutants and wild-type populations (Fig. 2). Difference of measurement data was compared with single factor analysis of variance ($P=0.018$). In the wild-type TERT group, Ki-67 was least abundant in the ATRX-deficient patients. Furthermore, Ki-67 expression was highest in the four groups without ATRX deletion. Due to the fact that the Ki-67 promoter possesses three Sp1 binding sites and that p53 inhibits Sp1 transcription (45), p53 may inhibit Ki-67 expression via a p53-Sp1-dependent pathway. One possibility is that the p53-binding motif affects the transcriptional inhibition of the Ki67 promoter (42). P53 may also change the interaction at the Sp1 binding site of the Ki67 promoter (42). ATRX mutations are associated with mutations in the tumor suppressor gene p53 (38). Therefore, the results of the present study demonstrated that the expression of Ki-67 was lowest in the wild-type TERT/ATRX deletion group. Ki-67 expression reflects the rate of tumor proliferation (16,42). Many factors are adjusted in tumor proliferation, including the second mitochondria-derived activator of caspases (Smac) (46), DNA replication licensing factor MCM7 (47), p53 (48), B-cell lymphoma 2 (49) proliferating cell nuclear antigen (50) and cluster of differentiation 105 (49). The highest expression of Ki-67 was observed in wild-type TERT/ATRX-deficient patients, which may indicate an association with the aforementioned regulatory mechanisms. Further studies are required to demonstrate these connections. To the best of the authors' knowledge, the present study assessed ATRX and the TERT promoter mutations together for the first time, dividing them into four subtypes based on two markers. The molecular classification of these mutations may better predict patient prognosis and guide clinical treatment strategies in the future.

However, the present study was limited as statistical methods were only utilized to determine the association among TERT, ATRX and other associated indicators, with the aim of solving clinical problems, ranging from tumor proliferation, recurrence and metastasis. The current study only assesses significant statistical information with regard to stage II to IV glioma tumors and TERT and ATRX analysis. Further experiments are therefore required to assess its internal associations, such as signal transduction pathway, interactions between proteins and epigenetic differences.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL and XZ contributed to the writing of this manuscript, while MS, YH, XY and YF contributed the conception design and editing of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Tianjin Huanshu Hospital and all patients provided written informed consent.

Patient consent for publication

Study participants provided their consent for the publication of any data/associated images.

Competing interests

The authors declare that there have no competing interests.

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