Differential expression of p42.3 in low- and high-grade gliomas

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Abstract

Background: Malignant gliomas are the most common form of primary malignant brain tumor. It has recently been suggested that genetic changes are involved in the progression of malignant gliomas. In previous studies, a novel gene, p42.3, was characterized as a tumor-specific gene that encodes a mitosis phase–dependent expression protein which is expressed in gastric cancer, but not in matched normal tissues.

Methods: In a series of 200 human brain gliomas and 13 normal tissues, we performed RT-PCR and mRNA in situ hybridization for analysis of p42.3 gene expression in gliomas, including astrocytoma (grade 2), oligoastrocytomas (grade 2), anaplastic oligoastrocytomas (grade 3), glioblastomas (grade 4) and normal tissues. Also, the mRNA expression was detected in gliomas by in situ hybridization. After producing polyclonal antibody to p42.3, we further tested p42.3 protein expression in astrocytomas and glioblastomas by immunohistochemistry and Western blot analysis.

Results: Our results demonstrated that overexpression of the p42.3 gene is detected in gliomas, but not in normal brain tissues. Importantly, p42.3 mRNA expression is correlated with the pathological features of gliomas. In addition, p42.3 protein is expressed in both the cytoplasm and the nucleus in astrocytomas, whereas this protein appeared in the cytoplasm in glioblastomas.

Conclusions: These results indicate that p42.3 might be involved in carcinogenesis as a potential molecular marker for malignant gliomas.

Keywords: Brain, Expression, Glioma, p42.3
normal mucosa [11]. In the present study, we were able to predict the \( p42.3 \) gene expression profile in brain and liver tissues by digital Northern analysis. We confirmed gene expression in five kinds of tumor cell lines and fetal tissues, including those from the brain and liver. \( p42.3 \) gene expression was studied in clinical specimens of gliomas and in normal human brain tissues for further analysis of the correlation between \( p42.3 \) gene expression and malignant progression. Overall, our data support the hypothesis that the \( p42.3 \) gene may be involved in tumorigenesis and might be a potential molecular marker for distinguishing different grades of malignant gliomas.

**Methods**

**Ethics statement**

All patients provided their written informed consent to participate in the study, and the clinical study was approved by the Institutional Review Board of Beijing Tiantan Hospital.

**Tissue samples**

In this study, we used 162 paraffin-embedded samples, 51 nitrogen-frozen samples of human brain gliomas which that had been resected surgically and 13 normal tissues derived from traumatic decompression operations. Histopathologic analyses were independently performed by two pathologists.

**RT-PCR**

Total RNA was extracted using TRIzol reagent. cDNA was synthesized from total RNA according to the manufacturer’s instruction manual (Promega, Madison, WI, USA). RT-PCR for analysis of the \( p42.3 \) gene expression was performed using two pairs of primers (5′-TGG CAT CTT TAC TGG ACT GG-3′ and 5′-TGG CAC CTC GTG GAT AGA GC-3′; 5′-CCA GCG GCT ACC TGA CCT TC-3′ and 5′-CAG CTC CTT CAT CTG CTT CAG-3′). The RT-PCR conditions were as follows: sufficient denaturing at 95°C for 50 seconds, denaturing at 94°C for 50 seconds, annealing at 55°C for 50 seconds, elongation at 72°C for 50 seconds (30 cycles) and a final cycle at 72°C for 10 minutes, \( \beta \)-actin served as the internal positive control. The RT-PCR products were examined by gel electrophoresis.

**mRNA in situ hybridization**

The RT-PCR fragment of the \( p42.3 \) gene was cloned into the pGEM-T easy vector system (Promega) that contained SP6 and T7 promoters. After sequencing, to confirm the orientation of the insert, the plasmids were linearized by either ApaI or SacI enzyme digestion. Digoxigenin (DIG)-labeled, anti-sense- or sense-strand RNA probes were prepared by in vitro transcription with T7 RNA polymerase and SP6 RNA polymerase according to the manufacturer’s instruction manual (Roche). A tissue array was incubated overnight at 43°C in prehybridization buffer containing 5 ng/μl of a sense or antisense DIG-labeled RNA probe. Detection of hybridization was undertaken using a DIG nucleic acid detection kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer’s instructions. Hybridization with sense-strand RNA probes was used as a negative control.

**Antibody generation and identification**

Three peptides were selected on the basis of analysis using DNASTAR software (DNASTAR, Madison, WI, USA) and were synthesized by Shenggong Biological Company (Shenggong, China). We then immunized rabbits using these synthesized peptides to generate polyclonal antibodies. Preimmune and immune sera were harvested. The fragment of cDNA including CDS (coding sequence) was amplified by nested PCR and cloned into prokaryotic expression vector PET-28a. The \( p42.3 \) protein was expressed by *Escherichia coli* BL21 and purified by nickel nitroltriacetic acid (Ni-NTA) agarose. The purified \( p42.3 \) protein was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and Western blot analysis was performed to identify the specificity of polyclonal antibodies. Polyclonal antibody to the \( p42.3 \) protein was isolated using purified protein and Sepharose 4B according to the manufacturer’s instructions (Borunlaite Science&Technology Company, Beijing, China).

**Western blot analysis**

For protein analysis, tissues were lysed in protein lysis buffer. Protein (100 μg) was separated by 12% Tris-glycylamide polyacrylamide gel and transferred to a polyvinylidene fluoride membrane by electrophoretic blotting at 90 V for 2 hours. The membrane was blocked with 5% nonfat dry milk in 0.1% phosphate-buffered saline with Tween 20 (PBST) and was incubated with a 1:100 dilution of polyclonal antibody at 4°C overnight. The blot was then washed in PBST and incubated for 2 hours with horseradish peroxidase–conjugated secondary antibody goat anti-rabbit immunoglobulin G (IgG) (1:1,000) and washed in PBST. The membrane was then developed using an electrochemiluminescence Western blotting detection reagent system (Amersham, Little Chalfont, UK) according to the manufacturer’s instructions.

**Immunohistochemistry**

Immunohistochemistry was performed as follows. Briefly, sections were deparaffinized by incubation at 70°C for 2 hours. Next, the slides were subjected to antigen retrieval by autoclave heating in an ethylenediaminetetraacetic acid buffer for 2.5 minutes and left to cool down to room temperature. After that, slides were then incubated in 3%
H$_2$O$_2$ to block endogenous peroxidase after rehydration in ethanol gradient, and then they were incubated in 10% nonfat dry milk to reduce nonspecific binding. Afterward, the slides were sequentially incubated with purified anti-p42.3 antibody (1:50), biotinylated goat anti-mouse IgG and avidin-biotin complex were added for 30 minutes at room temperature and the slides were developed with diaminobenzidine (Dako, Carpinteria, CA, USA).

### Statistical methods

A $\chi^2$ test (SPSS 10.0 for Windows, Chicago, IL, USA) was performed to analyze the expression of p42.3 in grade 2 gliomas, and grade 4 GBMs were examined by RT-PCR (Table 1). Logistic regression analysis (SAS 8.01 software; SAS Institute, Cary, NC, USA) was carried out to assess the frequency of p42.3 gene expression in gliomas identified by mRNA in situ hybridization (ISH) (Tables 2 and 3). Statistical significance was set at $P < 0.05$.

### Results

**Prediction of p42.3 gene expression by digital Northern analysis**

We knew little about the novel gene p42.3 after cloning its full-length cDNA. However, bioinformatics proved to be a very useful tool in helping us to find clues for a successive study; therefore, we predicted the expression profile by digital Northern analysis of the National Center for Biotechnology Information (NCBI) reference sequence. The results showed that the p42.3 gene is highly expressed in several cancer tissues, such as prostate adenocarcinoma, brain GBM, breast carcinoma and colon adenocarcinoma, as well as in embryonic kidney tissue (Figure 1).

**mRNA expression of the p42.3 gene is associated with degree of malignancy in gliomas**

Digital Northern analysis showed that the p42.3 gene is expressed in brain GBMs and confirmed that p42.3 is expressed in human fetal brain tissue as well. Therefore, we performed RT-PCR to examine its expression in gliomas.

### Table 1 Frequency of p42.3 gene expression in gliomas identified by RT-PCR

| Histological specimens | Grade | Total cases, N | p42.3 expression, n | Percent positive |
|------------------------|-------|----------------|---------------------|-----------------|
|                        |       |                | Positive | Negative |                 |
| Astrocytoma            | 2     | 11             | 1        | 10       | 9.1%             |
| OA                     | 2     | 10             | 1        | 9        | 10%              |
| Anaplastic OA          | 3     | 6              | 0        | 6        | 0                |
| GBM                    | 4     | 11             | 5        | 6        | 45.5%            |
| Normal tissue          | 9     |                | 0        | 9        | 0                |

*GBM, Glioblastoma multiforme; OA, Oligoastrocytoma.

### Table 2 Frequency of p42.3 gene expression in gliomas identified by mRNA in situ hybridization

| Tumor type (histological grade) | p42.3 expression (%) | Sex | Age <50 yr, n | Age ≥50 yr, n | Total (N) |
|---------------------------------|----------------------|-----|---------------|---------------|-----------|
| Astrocytoma (grade 2)           | Positive (15.9%)     | Male| 5             | 1             | 6         |
|                                 |                      | Female| 4           | 0             | 4         |
|                                 | Negative (84.1%)     | Male| 30           | 4             | 34        |
|                                 |                      | Female| 18           | 1             | 19        |
| OD (grade 2)                    | Positive (9.52%)     | Male| 1             | 0             | 1         |
|                                 |                      | Female| 1           | 0             | 1         |
|                                 | Negative (80.48%)    | Male| 10           | 4             | 14        |
|                                 |                      | Female| 4           | 1             | 5         |
| OA (grade 2)                    | Positive (12.5%)     | Male| 1             | 0             | 1         |
|                                 |                      | Female| 1           | 0             | 1         |
|                                 | Negative (87.5%)     | Male| 4             | 5             | 9         |
|                                 |                      | Female| 4           | 1             | 5         |
| Anaplastic OA (grade 3)         | Positive (30.0%)     | Male| 0             | 1             | 1         |
|                                 |                      | Female| 2           | 0             | 2         |
|                                 | Negative (70.0%)     | Male| 5             | 0             | 5         |
|                                 |                      | Female| 2           | 0             | 2         |
| GBM (grade 4)                   | Positive (36.5%)     | Male| 9             | 5             | 14        |
|                                 |                      | Female| 4           | 1             | 5         |
|                                 | Negative (63.5%)     | Male| 13            | 10            | 23        |
|                                 |                      | Female| 6           | 4             | 10        |

*GBM, Glioblastoma multiforme; OA, Oligoastrocytoma; OD, Oligodendroglioma.*
including astrocytomas (grade 2), OAs (grade 2), anaplastic OAs (grade 3), GBMs (grade 4) and corresponding normal tissues. The results showed that the positive frequency of this gene expression was similar between astrocytomas (1 of 11, 9.1%) and OAs (1 of 10, 10%); however, we found that p42.3 gene was expressed in 5 (45.5%) of 11 GBMs (Figure 2A and B) and was not expressed in 6 anaplastic OA samples. Moreover, this gene was undetectable in nine normal brain tissue samples (Figure 2C and Table 2). The prostate cell line PC3 served as a positive control. Using mRNA in situ hybridization with a variety of tissues, we examined p42.3 gene expression in brain gliomas. Table 2 shows that the expression of this gene was positive in 10 (15.9%) of 63 astrocytomas, 2 (12.5%) of 16 OAs, 3 (30%) of 10 anaplastic OAs, 2 (9.52%) of 21 ODs and 19 (36.5%) of 52 GBMs. In contrast, the p42.3 gene was not expressed in four normal brain tissues (Figure 2C and Table 2). More importantly, the statistical significance of expression between grade 2 and grade 4 gliomas was 0.038 (P < 0.05) (Table 1), and the expression of the p42.3 gene was significantly different between low-grade (grade 2) gliomas and high-grade (grades 3 and 4) gliomas (P < 0.01), but there was no statistically significant difference associated with age or sex (Table 3).

### Table 3 Analysis of correlation between p42.3 gene expression and pathological grade

| Characteristics | OR    | 95% CI          | P value |
|-----------------|-------|-----------------|---------|
| Sex             |       |                 | 0.4366  |
| Female          | Ref   |                 |         |
| Male            | 1.384 | 0.610 to 3.137  | 0.5411  |
| Age             |       |                 |         |
| <50 yr          | Ref   |                 |         |
| ≥50 yr          | 0.751 | 0.299 to 1.884  |         |
| Grade           |       |                 |         |
| 2               | Ref   |                 |         |
| 3 and 4         | 3.089 | 1.430 to 6.675  | 0.0041* |

*CI, Confidence interval; OR, Odds ratio; Ref, Reference value.
*Statistically significant.

Polyclonal antibody binding of p42.3 protein

We produced polyclonal antibody to the p42.3 protein to analyze the corresponding protein expression and generated six sets of rabbit antiserum against three synthetic peptide fragments of the p42.3 protein. The p42.3 protein was expressed in *E. coli* and purified by Ni-NTA agarose (Figure 3A). Western blot analysis showed that two of the six sets could specifically bind intact protein expressed in *E. coli* (Figure 3B).

Frequent expression of p42.3 protein in glioblastoma multiforme

Western blot analysis was carried out to examine the p42.3 protein expression in gliomas and normal brain tissues. The glioma specimens included eight GBM samples (grade 4), four OA samples (grade 2), three anaplastic OA samples (grade 3), and three normal tissue samples. The results showed that p42.3 protein was frequently expressed in all eight GBM samples and detectable in one OA sample and one anaplastic OA sample. p42.3 protein was not expressed in the...
**Figure 2** Examination of p42.3 in brain gliomas. (A) and (B) p42.3 expression was confirmed in five glioblastoma multiforme (GBM) samples, one astrocytoma sample and one oligoastrocytoma (OA) sample by RT-PCR. (C) p42.3 was not expressed in normal brain tissue. The PC3 cell line served as the positive control [1]. (D) mRNA in situ hybridization analysis showed that p42.3 was significantly overexpressed in anaplastic OA (b) and GBM (c), but not in normal brain tissue (a).

**Figure 3** Generation and identification of the p42.3 antibody. (A) p42.3 was expressed in *Escherichia coli* and purified by nickel nitrilotriacetic acid agarose. (B) Western blot analysis showed that two sets of rabbit antiserum could specifically bind the purified protein of p42.3. (C) Mass spectrometry analysis confirmed that the sequence of purified protein corresponded to that of p42.3. (D) Mascot database (Matrix Science, Boston, MA, USA) search results.
p42.3 protein is differentially located between astrocytomas and glioblastomas

We further performed immunohistochemistry on ten astrocytoma samples and ten GBM samples to test p42.3 expression. Our results show that this protein was expressed in both astrocytomas (tumor grades 5 ≥++, 5+) and GBMs (tumor grades 4 ≥++, 6+). Moreover, the location of p42.3 expression is different in these two glioma types. p42.3 protein was tested in both the cytoplasm and nucleus in nine of ten astrocytoma samples (Figure 5A and B), especially the protein highly expressed in the nucleus. In contrast, this protein was strongly expressed in the cytoplasm in only nine of ten GBM samples, and there was little expression of p42.3 protein in the nucleus (Figure 5C and D).

Discussion

In order to find out whether key genes are involved in both cell-cycle regulation and carcinogenesis, we screened out a cDNA fragment between the G1 and S phases of the cell cycle in a tumor cell line, BGC823, by mRNA differential
display and then cloned the full-length cDNA, which was designated p42.3 [GenBank:DQ150361]. The p42.3 gene is located on human chromosome 9q34.3 and is composed of five introns and six exons. It encodes a 389–amino acid (42.3 kDa) protein with functional motifs. In our previous study of gastric tumor cell lines and gastric cancer, we showed that the p42.3 gene might be involved in cell-cycle regulation and might play a role in gastric cancer development [11].

A homolog of p42.3 is present in the mouse, in which it is expressed in the early neuroectoderm of the embryo [12]. The high conservation of the p42.3 gene in mammals also suggests that this gene might serve important roles in cell biology. Digital Northern analysis of the NCBI reference sequence showed that the p42.3 gene is expressed in GBMs and other malignancies, which gave us important information about this novel gene. Our examination validated that the p42.3 gene was indeed expressed in various cancer cell lines and fetal tissues, including the brain.

Gliomas are the most frequently encountered human brain tumors, and the prognosis for glioma patients worsens with increasing tumor grade. Patients with high-grade gliomas (for example, anaplastic astrocytomas and GBMs) have a median survival time of 9 months, and only 5% to 10% of patients can survive as long as 2 years [13,14]. Accumulation of multiple genetic abnormalities lead to human malignant gliomas. Inactivation of the p16-cdk4-pRb pathway has been implicated in the progression to grade 3 astrocytoma [15,16]. Secondary GBMs have a high incidence of TP53 mutations (>65%), the majority of which are already present in low-grade or anaplastic astrocytomas [17]. LOH10 is the most frequent genetic alteration in GBMs, occurring in 60% to 80% of cases [18–20]. Deletion of PTEN [21,22] and over-expression of EGFR have also been frequently found in GBMs [23,24]. These previous studies have elucidated the partial molecular mechanisms of malignant progression of gliomas, although the precise molecular events involved in the process are not yet known. Therefore, finding critical genes involved in the progression of malignancy might effectively confer an improved prognosis for patients with high-grade gliomas by application of targeted therapies.

The expression profile of the p42.3 gene was the primary focus of our research in this study, in which we analyzed clinical tumor samples taken from patients with gliomas in China. The p42.3 gene was confirmed to be expressed in these glioma samples, but not in normal human brain tissues, at both the mRNA and protein levels. More importantly, the percentage of p42.3 mRNA expression increased in tandem with the progression of glioma malignancy, with statistically significant differences found between grade 2 (benign) tumors and grades 3 and 4 (malignant) tumors. This evidence is important evidence supporting the hypothesis that the p42.3 gene might not only be involved in carcinogenesis but also play a role in the progression of glioma malignancy. Moreover, the different locations of p42.3 between astrocytoma and GBM cells suggests that this protein has different roles in high- and low-grade gliomas. Further study is needed to clarify these detailed functions in this process.

Interestingly, we found the same expression pattern in gliomas: The p42.3 gene was expressed in glioma tumor tissue samples, but not in corresponding normal tissue, in our Chinese patient population. It is generally believed that a fetal protein, highly expressed at fetal stages and quickly shut down after birth, could recur in different tumor stages. Such molecules are likely to be a tumor marker and might act as an oncogene involved in carcinogenesis [25,26]. We found that the expression profile of the p42.3 gene was in agreement with the criteria mentioned above.

Conclusion
In our present study, we establish the expression pattern of a novel gene, p42.3, which suggests that this gene is associated with glioma malignancy. Moreover, we found that p42.3 mRNA expression was correlated with the degree of glioma malignancy. These results provide important clues to inform further studies of this novel gene and might be useful in diagnosing gliomas. The location of this protein may help investigators to discern high- and low-grade gliomas.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
LZ and YL conceived and designed the study, TR, ZW, GJ, WW acquired clinical data, LZ and LQ performed the operation and collected the sample, ZW collected and transferred the samples, JW made pathological diagnosis, XX, WL, and WW carried out the molecular genetic studies, JZ, LZ, and GJ participated in the sequence alignment, TR, ZW and WW followed the patients. WW and XX carried out statistical analysis, WW drafted the manuscript. All authors critically revised the article. All authors read and approved the final manuscript.

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