Original Article

Protein and mRNA Levels of YKL-40 in High-Grade Glioma

(YK-40 / malignant glioma / biomarker)

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Abstract. Malignant gliomas are the most common type of primary malignant brain tumours, characterized by extreme proliferation and aggressive invasion. There is evidence for over-expression of the *YKL40* gene in high-grade gliomas. The high serum levels of the glycoprotein are associated with poor prognosis of various inflammatory and tumour processes. We investigated the *YKL40* mRNA level and protein expression in the tumour site and in the serum of high-grade glioma patients. The YKL-40 expression in 36 patients with glial tumours (astrocytoma grade III, glioblastoma) and 33 age-matched healthy persons was measured by gene analysis, immunohistochemistry and ELISA. YKL-40 serum levels in high-grade glioma patients compared to healthy subjects were significantly increased ($P \leq 0.05$). A wide range of variability in *YKL40* mRNA expression was found. YKL-40 staining *in situ* was more abundant in glioblastoma tissue than in anaplastic astrocytoma, with the lowest level in normal brain tissue. Our gene analysis revealed that in general, *YKL40* mRNA in glioma patients was over-expressed versus normal brain. A significant correlation between *YKL40* transcript and protein levels was observed ($P \leq 0.05$). It could be speculated that the YKL-40 protein might contribute to glioblastomas' specific biological characteristics that distinguish them from grade III gliomas. A complex investigation of *YKL40* expression was performed at the molecular and cellular levels in human high-grade gliomas. Serum YKL-40 concentrations increased with tumour grade and correlated positively with transcript rate, being the highest in glioblastoma. We provide evidence for a relationship between *YKL40* expression and the malignancy of glial tumours.

Introduction

Gliomas account for about 80 % of primary malignant brain tumours. Glioblastoma multiforme (GBM) is the most common type of glioma, which is highly aggressive, associated with poor survival and radioresistance (Schwartzbaum et al., 2006). The prognosis for patients with glioma is very poor – 30 % of patients under the age of 45 years survive for two years (Central Brain Tumour Registry of the United States). Appropriate treatment has not yet been identified. Informative prognostic and predictive markers for gliomas are pressingly needed. Serum markers that correlate with the tumour status and prognosis could significantly improve the prompt diagnosis and treatment.

YKL40 is one of the most over-expressed genes in high-grade malignant gliomas (Tanwar et al., 2002). The corresponding protein is a phylogenetically conserved chitin-binding glycoprotein, belonging to the family of chitinase-like proteins (Renkema et al., 1998). Due to a mutation of glutamic acid to leucine YKL-40 lacks chitinase activity (Fusetti et al., 2003).

Its name is based on the three NH2 terminal amino acids – tyrosine, lysine, and leucine. It is also known either as Chitinase 3-like-1 (CHI3L1) or breast regressing protein of 39 kD, or human cartilage glycoprotein-39 (HC gp39) (Johansen, 2006). The specific cellular receptors have not yet been identified, but it was suggested that the protein interacts with one or several signalling components on the plasma membrane (Johansen, 2006). YKL-40 was first identified from the whey pro-

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Abbreviations: CT – computed tomography, GBM – glioblastoma multiforme, MRI – magnetic resonance imaging, NTC – no template controls.

tein of bovine mammary secretions collected during the non-lactating period and was subsequently shown to be produced by human synovial cells, the MG-63 osteosarcoma cell line, chondrocytes, smooth muscle cells, macrophages, and neutrophils (Schultz and Johansen, 2010). The biological function of YKL-40 in cancer is unknown. Several studies reveal that it participates in extracellular tissue remodelling, migration, angiogenesis and inflammation (Francescone et al., 2011).

There is evidence that serum YKL-40 levels are high in patients with breast, ovary and colon cancer. The increased concentrations are associated with the disease progression, histopathological type and survival time (Johansen, 2006; Yamac et al., 2008; Stawerski et al., 2011). Other authors show that YKL-40 expression has no prognostic value in breast cancer (Roslind and Johansen, 2009). Some data indicate that serum glycoprotein levels are a reliable marker of the disease activity and prognosis in gliomas (Hormigo et al., 2006; Bernardi et al., 2012). Faibish et al. (2011) demonstrated that a monoclonal antibody against YKL-40 inhibited *in vitro* and *in vivo* tumour growth and angiogenesis of brain tumours. It could be speculated that YKL-40 might be a potential therapeutic molecular target for gliomas.

There is a lack of detailed and complex investigations of YKL-40 expression at the molecular and cellular levels in human gliomas. We have performed a study of the *YKL40* gene transcriptional activity in parallel with serum and tissue expression of the protein in high-grade gliomas. The purpose of the present study is to search for a correlation between the *YKL40* mRNA level and protein expression in the tumour site and in the serum of glioma patients.

Material and Methods

Patients

Thirty-six patients newly diagnosed preoperatively with high-grade glioma were included in the study. The median age for anaplastic astrocytoma patients was 60.4 ± 6.42 years and for GBM patients 57.93 ± 19.02 years. Symptoms of the disease preceded hospitalization by an average of 3.8 weeks. The most common clinical symptoms were the occurrence of a varying degree of central hemiparesis (91 %) and increased intracranial pressure (89 %). Preoperative evaluation of patients was assessed by the Karnofsky scale. Seventy-five % of the patients had a score of 60 points. Computed tomography (CT) or magnetic resonance imaging (MRI) was applied to identify the brain tumours. The study was approved by the University Ethics Committee (protocol №3/25.11.2009) in accordance with the Helsinki Declaration. Informed consent was signed by all examined individuals.

All patients had undergone surgical removal of the tumours at the Department of Neurosurgery of "St George" University Hospital in Plovdiv. No prior chemotherapy or radiotherapy was applied. A microsurgical resection of the tumour lesion was performed. In 91 % of the cases, total or subtotal lesionectomy was accomplished. Histological diagnosis determined 14 cases with astrocytoma grade IV (GBMs) and 22 cases with astrocytoma grade III (anaplastic astrocytomas) according to WHO grading.

Postoperatively, patients were treated with radiotherapy and chemotherapy. The duration of patients' follow-up was up to three years.

Control group

A control group of 33 healthy persons, aged 51.21 ± 16.46, was examined for YKL-40 serum levels. Clinical and routine biochemical tests were performed to assess their health status.

Sample collection and storage

1. Serum

Venous blood samples were collected from all patients in the morning before surgery (4.5 mmol/l blood, Monovette, Sarstedt, Nümberg, Germany) and centrifuged at 2500 rpm (Heraeus Labofuge 400R, Thermo Scientific Inc., Pittsburgh, PA) for 10 min. The serum was divided into aliquots and stored at -70 °C before analysis.

2. Tissue

Brain tissue was collected from all patients during surgical removal of the tumour. Control normal brain tissue was obtained *post mortem* from five individuals without indication for any brain pathology at the Department of Forensic Medicine. Both, tumour and normal brain tissues were placed in RNAlater (Quiagen, Austin, TX) at the time of surgical resection and stored at -20 °C until use. Frozen sections from the surgically removed tumours were examined prior to RNA extraction. Areas with no morphologic signs of necrosis, inflammation and neo-angiogenesis were selected. The percentage of tumour cells in each sample was 60–80 %. The histopathological examination was verified by two independent observers.

In parallel, brain tissue was fixed for 6–12 h in neutral formalin and then embedded in paraffin. Paraffin sections were used for routine histopathological examination and for immunohistochemical analyses.

Methods

1. RNA extraction

Total RNA was isolated from high-grade gliomas (15 astrocytomas grade III and 9 GBMs) and from five normal brain tissues. Samples of normal brain mRNAs were pooled, generating a constant control to be used in each experiment. Total RNA was extracted with GeneJET™ RNA Purification Kit (Fermentas, BIOSYSTEMS Ltd., Sofia, Bulgaria) according to the manufacturer's instructions. RNA concentration and purity was determined spectrophotometrically (A260/280) using

Spectrophotometer ND1000 (NanoDrop Technologies, Inc., Wilmington, DE).

2. ELISA

Serum YKL-40 concentrations were determined by ELISA assay, using a commercial kit (Quidel Corporation, San Diego, CA) according to the manufacturer's protocol. The validation of the method was performed in compliance with the international standard of quality and competence of medical laboratories (BDS/EN/ISO 15189). All samples were analysed in duplicates. YKL-40 levels were measured as absorbance at 450 nm in ELISA Sunrise Reader (Diamed Ltd., Sofia, Bulgaria).

3. Immunohistochemistry

Immunohistochemistry was performed with formalin-fixed, paraffin-embedded tissue samples after antigen retrieval. The avidin-biotin system was applied (Vectastain Elite ABC Kit, Vector Laboratories, Burlingname, CA). The sections were incubated at 37 °C for 1 h with primary goat anti-human chitinase 3-like antibody (YKL-40) (R&D Systems, Minneapolis, MN) and anti-goat IgG secondary antibody. The final detection was performed by freshly prepared DAB as a chromogen. Nuclei were counterstained with haemotoxylin.

4. Morphometric analysis

A morphometric analysis of the intensity of tissue expression was performed through the Quick-photo Micro 2.3. system. The area (μm^2), perimeter (μm), and expression level (%) of the glycoprotein were calculated. Only tumour tissues without necrotic areas were examined. Cells with strong granular cytoplasmic YKL-40 staining were scored. Ten consecutive fields were studied for each sample by two independent observers.

5. PCR analysis

cDNA synthesis

Aliquots of 2 μg total RNA were further purified by treatment with 3U of DNase I, RNase-free (Fermentas) at 37 °C for 30 min and one tenth aliquot of the foregoing reactions was used for cDNA synthesis. Reverse transcription was carried out using a RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas) at 44 °C for 1 h in the presence of oligo(dT)$_{18}$ primer.

Real-time PCR amplification procedure

cDNAs were analysed for the content of specific mRNA sequences by real-time quantitative PCR (qPCR) using Maxima® SYBR Green qPCR Master Mix (2X) (Fermentas). All qPCR were performed in a Rotor-Gene™ 6000 instrument (Corbett Life Science, Quiagen) and analysed with Rotor-Gene 6000 Series Software version 1.7.18. Assay components were optimized and consisted of 1× Maxima® SYBR Green qPCR Master Mix, 0.2 μM primers and 1.6 μl cDNA in a total reaction volume of 20 μl. The conditions consisted of an initial denaturation at 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 30 s followed by a melt analysis in which the temperature was increased by increments of 1 °C waiting for 5 s before each acquisition.

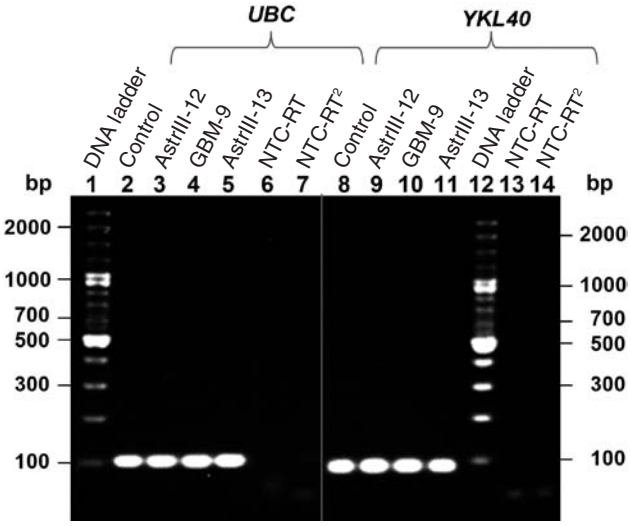

Fig. 1. Electrophoretic analysis of endpoint amplicons from RT^2-PCR.
Analysis of three samples is shown – AstrIII-12 and 13, and GBM-9. DNA fragments were separated in 2% agarose gel. Lanes 1 and 12, GeneRuler™ 100 bp Plus DNA ladder (Fermentas); lanes 2 and 8, control – a pool of normal brain samples; lanes 3, 5, 9 and 11, astrocytoma grade III samples; lanes 4 and 10, GBM-9; lanes 6 and 13, no template controls for reverse transcriptase reactions; lanes 7 and 14 – no template controls for real-time PCRs. A PCR product of 101 bp was amplified with the *UBC* primer pair (lanes 2–5), and an amplicon of 89 bp was obtained using primers for *YKL40* (lanes 8–11). No fragment was amplified in NTC reactions (lanes 6, 7, 13 and 14).

Water was used for the no template controls (NTC). Samples not treated with reverse transcriptase served as an RT-minus control. The CT values were used to calculate the fold change of expression units in the samples through the DeltaDelta Ct (ΔΔCt) relative quantification method. The expression of the UBC reference gene was applied to normalize mRNA levels. The primer sequences (forward, followed by reverse) in the PCR studies were: GACCACAGGCCATCACAGTCC and TGTACCCCACAGCATAGTCAGTGTT for *YKL40* (GenBank Identifier M80927); and TCCTGATCAGCAGAGGTTGATCTT and GGACCAAGTGCAGAGTGGACTCTT for *UBC* (NCBI Reference Sequence: NM_021009.5).

The specificity of RT^2-PCR reactions was ensured by melting curve (dF/dT) analysis and by agarose gel electrophoresis of the amplified products. A representative photo of an electropherogram of endpoint amplicons is given in Fig. 1. Clearly, only the expected fragment of 101 bp and 89 bp for *UBC* and *YKL40*, respectively, was amplified using the respective primer pair.

6. Statistical analysis

Normality of the continuous variables was examined by the Kolmogorov-Smirnov test and comparisons were

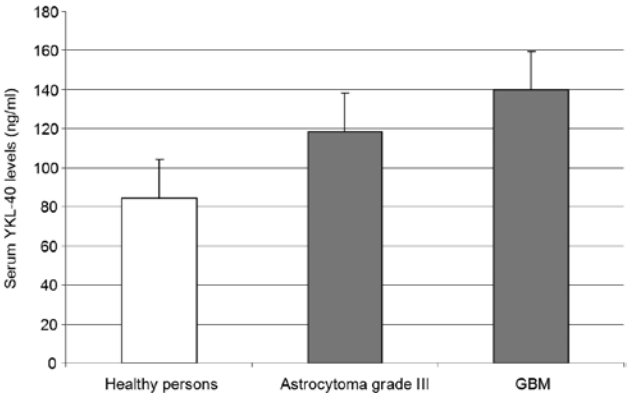

Fig. 2. Serum YKL-40 levels in astrocytoma grade III and GBM patients compared to control samples.

completed using Independent-Samples *t*-test. Pearson correlation coefficient was calculated to evaluate the correlation between the studied variables. Statistical analysis was carried out with the SPSS v 17.0 statistical software. The level of statistical significance of the null hypothesis was $P < 0.05$. All P values were two-tailed.

Results

Serum YKL-40 levels

Serum concentrations of YKL-40 were determined in 36 Bulgarian patients with astroglial tumours and compared to 33 age-matched healthy individuals.

The mean serum value of YKL-40 in healthy subjects was 84.52 ± 13.65 ng/ml, while the mean serum concentration of YKL-40 in anaplastic astrocytomas grade III was 127.6 ± 16.31 *vs* 139.68 ± 22.39 ng/ml in GBM patients. The levels of YKL 40 in the serum of glioma patients were significantly higher compared to those of healthy controls ($P \leq 0.05$) (Fig. 2).

Immunohistochemical expression of YKL-40

Immunohistochemistry determined considerable differences in the intensity and localization of the YKL-40 protein in the studied groups. The intensity of YKL-40 staining and its expression level (%) are presented in Fig. 3. The glycoprotein was found in single glial cells with feeble reactivity in the normal brain. Numerous positive malignant cells were labelled in grade III astrocytomas. The most intensive immunohistochemical reaction was detected in GBM, where large zones of tumour cells were stained for YKL-40.

YKL-40 mRNA level in gliomas

To investigate the transcriptional rate of *YKL40* in glioma samples we assessed the expression changes that occur at the mRNA level in high-grade gliomas compared to normal brain tissue. To this end, two-step real-time RT-PCR were carried out.

Our results revealed that all GBMs manifested elevated *YKL40* mRNA with 12-fold to 2300-fold increase relative to the normal brain (Fig. 4b). *YKL40* mRNA was detected in grade III astrocytoma samples with a range of -1.92 to 1000-fold change. Obviously, the obtained fold change of the *YKL40* gene expression in the studied samples spanned a wide range of values that contributed to the large SD for *YKL40* mRNA levels. Comparing different glioma cases, *YKL40* showed an average expression level in GBMs 1.66-fold greater than the average expression detected in grade III astrocytomas. As a whole, the differential gene expression of *YKL40* was higher in high-grade glioma samples in com-

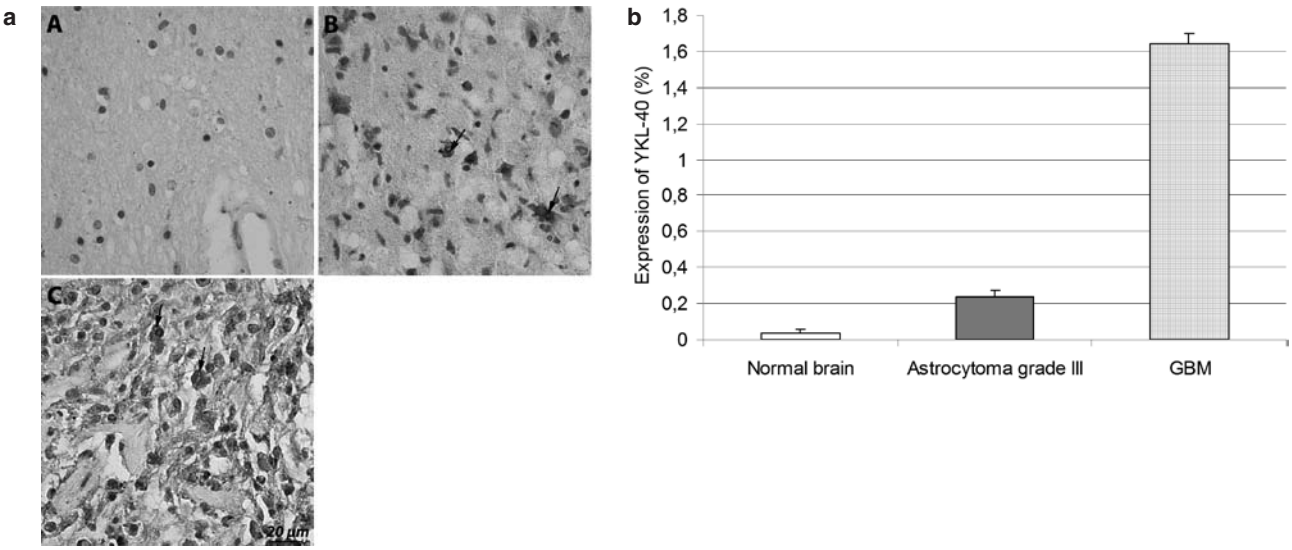

Fig. 3. Immunohistochemical staining for YKL-40 in normal brain and glioma tumours. Biotin-streptavidin peroxidase reaction.

a. In the normal brain, YKL-40 is expressed in single glial cells with feeble reactivity (**A**); numerous positive malignant cells are labelled in astrocytoma grade III (**B**); large zones of tumour cells are intensively stained for YKL-40 in GBM (**C**).

b. Morphometric analysis of tissue expression

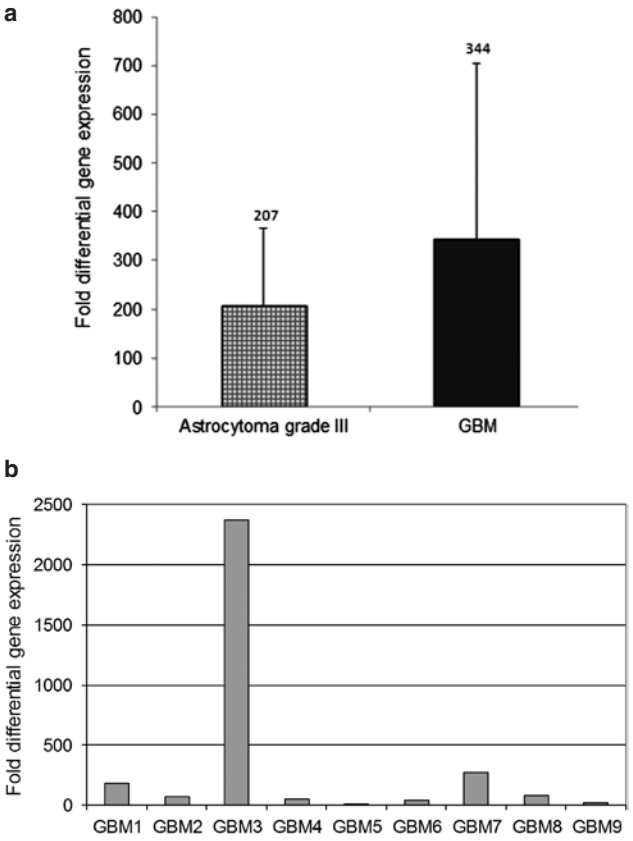

Fig. 4. RT^2-PCR analysis of high-grade glioma versus normal brain samples

a. *YKL40* tumour mRNA levels in high-grade gliomas as fold increase compared to normal brain.

b. Fold increase of *YKL40* mRNA level in nine particular GBM cases versus normal brain.

parison with normal brain tissue (Fig. 4a). A significant correlation between YKL-40 transcript and protein levels was observed ($P \leq 0.05$).

The *YKL40* mRNA level was higher in GBM patients compared to the normal brain (Fig. 4b). In three of the particular cases the gene expression was significantly increased. These patients had a shorter survival time compared to other GBM patients from the same group.

Discussion

Despite the recent advances in surgery, radio- and chemotherapy, the median survival of glioma patients remains approximately 14 months after diagnosis (Huber et al., 2013). Given that the clinical significance of YKL-40 expression and secretion in high-grade glioma is still unclear, we aimed to investigate the YKL-40 transcript and protein levels in these tumours.

Our study shows that serum YKL-40 levels are significantly higher in patients with high-grade gliomas in comparison with age-matched healthy subjects. These results are in agreement with other research groups (Tanwar et al., 2002; Hormigo et al., 2006). Bernardi et al. (2012) also observed that YKL-40 concentrations were increased in GBM patients. They found a wide range of variability in the glycoprotein levels. In our patients, we determined the same pattern of diversity.

In inflammatory diseases, the glycoprotein is generated by activated macrophages and neutrophils (Schultz and Johansen, 2010). However, we still lack knowledge regarding the exact cell source of YKL-40 in tumour lesions. Some authors reported that YKL-40 was produced by peritumoral macrophages in small-cell lung cancer and also in GBM (Shostak et al., 2003; Junker et al., 2005). In contrast, Horbinski et al. (2010) showed that YKL-40 was directly produced by neoplastic glial cells. Noteworthy, the increased serum concentration is a non-specific marker of gliomas because it could also be elevated in patients with several inflammatory diseases. Our previous investigation of inflammatory joint diseases showed a significant elevation of serum and synovial YKL-40 levels (Kazakova et al., 2013). In the present study, we performed immunohistochemical and qPCR analysis of YKL-40 in order to provide a particular evaluation of its local expression in gliomas.

Our results indicate that the *YKL40* mRNA level and protein expression in the tumour site and in the serum of glioma patients parallels with the malignancy grade of the tumours, suggesting a potential aggressive role of the protein in neoplastic development. Our data are in accordance with other investigations concerning the role of YKL-40 in breast and gastric cancers (Pan et al., 2013; Shao, 2013). Some researchers reported that immunohistochemical detection of YKL-40 could distinguish between anaplastic oligodendrogliomas and glioblastomas (Nutt et al., 2005). According to their study, YKL-40 staining provided a better histologic diagnosis. Our investigation shows that immunohistochemical manifestation of the glycoprotein could support the diagnostic procedure.

Recent studies reported that high expression of the glycoprotein promoted proliferation, angiogenesis and malignancy via phosphorylated-Akt signalling pathway (Antonelli et al., 2012). This pathway is involved in high-grade malignancy (Francescone et al., 2011). In addition, YKL-40 up-regulates VEGF and induces endothelial cell migration and tube formation (Shao et al., 2009).

We performed gene analysis to assess the transcriptional activity of *YKL40* in GBMs relative to anaplastic grade III astrocytoma and normal brain. The fact that *YKL40* is over-expressed in high-grade gliomas suggested that it could be an indicator of malignancy. We found a wide range of variability in *YKL40* mRNA expression. We could suppose that the diversity of the *YKL40* expression level among patients is due to the heterogeneity of these tumours and might correlate with the aggressive behaviour of the disease.

High variation in the *YKL40* expression levels among astrocytoma grade III and GBM samples from different patients was also detected by other authors (Tanwar et al., 2002). *YKL40* mRNA has been reported to be elevated in GBM samples examined in several independent laboratories by different methods (Lal et al., 1999; Mar-

kert et al., 2001; Tanwar et al., 2002; Francescone et al., 2011) and was proposed as a potential clinical marker for these tumours. Some researchers observed that increased serum levels of YKL-40 correlated with poor prognosis and survival in GBM patients (Pelloski et al., 2005). It could be speculated that the YKL-40 protein might contribute to GBM's specific biological characteristics which distinguish them from grade III gliomas. The utility of YKL-40 as a biomarker in these tumours is promising and could possibly serve as a potential therapeutic target.

Conclusions

In conclusion, a complex investigation of YKL-40 expression at the molecular and cellular levels in human high-grade gliomas has been performed. The real-time PCR and immunohistochemical data show that the mRNA and protein levels are significantly up-regulated in high-grade gliomas compared to the normal brain tissue. The serum YKL-40 concentration increases with tumour grade and correlates positively with the transcript rate, being the highest in GBM.

Our study provides evidence for a relationship between YKL-40 expression and the malignancy of glial tumours.

Acknowledgements

All experiments in the study comply with the current laws of Bulgaria. We declare no conflict of interest.

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