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PATTERNS OF EXPRESSION OF TSC-22 PROTEIN IN ASTROCYTIC GLIOMAS

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Aim: **To evaluate expression patterns of protein product of putative tumor suppressor gene *TSC-22* in human astrocytic tumors by immunohistochemical approach.** ***Methods:*** **Plasmid pET-23d-TSC22 was constructed for the expression of human TSC-22 protein in bacterial system, and polyclonal rabbit antibodies against recombinant TSC-22 were produced. Immunohistochemical analysis of TSC-22 and GFAP expression with the use of anti-human-TSC-22- and anti-human-GFAP-antibodies was performed on histological slides of astrocytic tumors.** ***Results:*** **Immunohistochemical analysis has shown that the number of cells expressing TSC-22 was significantly lower in glioblastoma tissues than that in diffuse astrocytoma. Double immunohistochemical staining of astrocytic tumors using anti-human-TSC-2- and anti-human-GFAP-antibodies showed that both TSC-22 and GFAP expression is co-localized in astrocytes.** ***Conclusion:*** **TSC-22 protein is expressed in astrocytes, but not in macrophage/microglial cells. In more aggressive forms of astrocytic tumors decreased expression of TSC-22 mRNA correlates with its lowered expression on protein level.**
Key words: TSC-22 **gene, TSC-22 antibodies, double immunohistochemical labeling, astrocytic gliomas.**

Astrocytic gliomas are the most common primary brain tumors and are divided histopathologically into three grades: diffuse astrocytoma (World Health Organization grade II), anaplastic astrocytoma (WHO grade III) and glioblastoma (WHO grade IV) [1]. Among them, glioblastoma is the most aggressive form, which may arise spontaneously (so called primary glioblastoma) or to progress from lower-grade gliomas (so called secondary glioblastoma). Inactivation of genes *TP53, p16INK4A, RB1, PTEN,* amplification/overexpression of *MDM2* and *EGFR* are the main genetic anomalies, which have been identified in astrocytic tumors [1, 2]. Some other nonrandom anomalies are also related to the peculiarities of gliomas, such as the cases of loss of heterozigosity on chromosomes 1p, 9p, 10p, 10q, 11p, 13q, 17p, 19q, and 22q. The potential tumor supressor genes have not been identified in these regions despite of the evidence on their existence obtained during last decade [3–10]. Therefore, malignant gliomas underwent an intense scientific scrutiny, and a search of new genes associated with initiation and progression of glial tumors is the aim of elucidating the molecular basis of glial tumourigenesis.

Earlier, differential hybridization of gridded cDNA libraries of human fetal and postnatal brain revealed several genes, differentially expressed in astrocytic gliomas and normal brain. *TSC-22* (TGF-β1 stimulated clone 22) was found among genes with the most decreased mRNA level in astrocytic tumors and was supposed to be a putative tumor suppressor gene [11]. Decreased content and even complete absence of TSC-22 mRNA were shown also in benign and malignant human salivary gland tumors, when compared to that in normal salivary gland [12]. These results suggest that down-regulation of *TSC-22* may play the important role in brain and salivary gland tumorigenesis. However, the conclusion about the changes of gene expression drawn only from the results based on RNA prevalence has to be taken with care. Since there is frequently considerable disparity in transcription and translation or functional activity, in the present work we carried out an analysis of *TSC-22* gene expression in astrocytic gliomas on the protein level.

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Abbreviations used: TSC-22 – TGF-β1 stimulated clone 22; cDNA – complementary DNA; ELISA – enzyme-linked immunosorbent assay; PBS – phosphate buffered saline; BSA – bovine serum albumin; GFAP – glial fibrillary acidic protein; SDS – sodium dodecyl sulfate.

MATERIALS AND METHODS

Glioma tissue samples were collected from A.P. Romodanov Institute of Neurosurgery (Kyiv, Ukraine) and Bordeaux hospital under the approval of the Institute Review Boards. Altogether, 5 glioblastomas and 10 diffuse astrocytomas were studied. One sample of meningioma tissue served as a negative control.

TSC-22 immunohistochemistry was done on formalin-fixed, paraffin-embedded sections with polyclonal anti-TSC-22 antibody obtained after immunization of rabbits by recombinant TSC-22.

PCR-product, encoding TSC-22 protein, was synthesized with specific primers on plasmid DNA from ICRFp507J1041 clone [11] containing full-length TSC-22 cDNA (Fig. 1). After digestion by NcoI and XhoI restrictases, PCR-product was cloned in pET-23d plasmid (Novagen, Germany) in-frame with His-tag sequence according to standard methods [13]. Forward primer AATTCCATGGAATCCCAATGGTGT (see forv in Fig. 1) contained two nucleotide substitutions as compared to nucleotide sequence of TSC-22 mRNA, which generated restriction site CCATGG (NcoI). Reverse

primer CCGCTCGAGTGCGGTTGGTCCTGAGC (see rev in Fig. 1) contained additional nucleotides at 5′-end, which generated restriction site CTCGAG (XhoI).

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            M  K  S  Q  W  C  R  P  V  A  M  D  L  G  V  Y  Q
    AATTGCATGAAATCCCAATGGTGTAGACCAGTGGCGATGGATCTAGGAGTTTACC
          ----------->
              forv
  L  R  H  F  S  I  S  F  L  S  S  L  L  G  T  E  N  A  S  V
AACTGAGACATTTTTCAATTTCTTTCTTGTCATCCTTGCTGGGGACTGAAAACGCTTCTG
  R  L  D  N  S  S  S  G  A  S  V  V  A  I  D  N  K  I  E  Q
TGAGACTTGATAATAGCTCCTCTGGTGCAAGTGTGGTAGCTATTGACAACAAAATCGAGC
  A  M  D  L  V  K  S  H  L  M  Y  A  V  R  E  E  V  E  V  L
AAGCTATGGATCTAGTGAAAAGCCATTTGATGTATGCGGTCAGAGAAGAAGTGGAGGTCC
  K  E  Q  I  K  E  L  I  E  K  N  S  Q  L  E  Q  E  N  N  L
TCAAAGAGCAAATCAAAGAACTAATAGAGAAAAATTCCCAGCTGGAGCAGGAGAACAATC
  L  K  T  L  A  S  P  E  Q  L  A  Q  F  Q  A  Q  L  Q  T  G
TGCTGAAGACACTGGCCAGTCCTGAGCAGCTTGCCCAGTTTCAGGCCCAGCTGCAGACTG
  S  P  P  A  T  T  Q  P  Q  G  T  T  Q  P  P  A  Q  P  A  S
GCTCCCCCCCTGCCACCACCCAGCCACAGGGCACCACACAGCCCCCCGCCCAGCCAGCAT
  Q  G  S  G  P  T  A  *
CGCAGGGCTCAGGACCAACCGCATAG
          <-----------
              rev
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Fig. 1. Nucleotide and deduced amino acid sequence of human TSC-22. Amino acid sequence designated by one letter code above the first position of codons. Positions of the primers used for PCR-product synthesis are indicated by arrows

E. coli cells BL21(DE3) were transformed with recombinant plasmid pET-23d-TSC22. This *E. coli* strain is deficient in both *lon* and *ompT* proteases and has the advantage for protein expression. Bacterial culture was grown at 37 °C to optical density of 0.5–0.7 (λ = 600 nm) and synthesis of recombinant protein was induced during 3 h by 1 mM IPTG. Obtaining of cell lysates and affinity purification of TSC-22, containing His_6 at the C-terminus, were carried out under native conditions on Ni-NTA agarose (Ni^{2+}-charged nitriloacetic acid-modified agarose) according to manufacturer's protocol (Qiagen, USA). Analysis of protein products was made by the electrophoresis in 12% SDS-polyacrilamide gel (SDS-PAAG) and Coomassie blue staining.

Polyclonal TSC-22-specific antibodies were produced as described previously [14] by immunization of male rabbits weighting 2–3 kg. 300 μg of recombinant TSC-22 in PBS with 50% Freund's adjuvant were injected into the rabbit — 10 subcutaneous injections along the spinal column, 30 μg each. Two immunizations were repeated in two week intervals by the same antigene quantities in the presence of incomplete Freund's adjuvant, and the boosting was repeated every two weeks for 8 weeks. Serum obtained from the rabbit was tested for immunoreactivity against TSC-22 protein by ELISA.

Double immunohistochemical labeling of histological slides by anti-human TSC-22 and anti-human glial fibrillary acidic protein (GFAP) antibodies was performed as described previously [15]. Paraffin was removed by successive incubation in toluene (2 x 5 min), 95% alcohol (5 + 3 min), and distilled water (5 min). To block non-specific labeling, slides were pretreated by 3% BSA in 1 x TBS (20 mM Tris-HCl, pH 7.5, 0.25 M NaCl) containing 0.2% gelatin. As the first primary antibody, rabbit polyclonal antiserum directed to human TSC-22 (dilution 1 : 2000) was incubated with slides overnight at 4 °C, and unbound antibodies were removed by 0.1% Tween 20 solution in 1 x TBS. Then, slides were incubated for 2 h at room temperature with secondary antibodies — fluoresceine-labeled anti-rabbit antibodies (Alexa Fluor® 488 goat anti-rabbit IgG (anti-rabbit FITC 488, Invitrogen A-11008)). Consecutive treatments of slides were made by second primary anti-human GFAP mouse antibodies (Sigma G3893) and rhodamine-labeled secondary anti-mouse antibodies (Alexa Fluor® 594 goat anti-mouse IgG (Invitrogen A-11005)). Slides were analyzed by fluorescent microscope (Carl Ceiss, Germany) with green filter for fluoresceine and red filter for rhodamine. Superposition of fluorescence pictures was made for the comparison of the cellular localization of TSC-22 and GFAP.

RESULTS AND DISCUSSION

Identification of genes that undergo alterations in expression during malignant transformation was a goal for many investigations. In previous publications, we described the changes of gene expression in astrocytic tumors [16–18]. However, the critical point of all such experiments is that the results based only on the changes of mRNA level are not enough for characterization of differential gene expression in normal and malignant tissues. In a lot of cases, significant differences between the abundance ratio of mRNA transcripts and the corresponding protein products were observed. Therefore, the present study was designed to assess the expression patterns of TSC-22 on the protein level in glial cells or auxiliary (microglial) cells and in astrocytic tumors of different grade of malignancy. The newly obtained data represent a necessary extension of the previously described changes of *TSC-22* gene expression on RNA level [11]. Also, the expression of glial fibrillary acidic protein — standard immunohistochemical marker of astrocytic gliomas has been studied simultaneously.

Recombinant TSC-22 protein was isolated from *E. coli* cells, transformed by the constructed plasmid, that contained TSC-22 cDNA. Recombinant TSC-22 protein after purification on Ni-NTA-agarose column was of expected size, about 20 kDa (Fig. 2). It has been used as antigen for generation of polyclonal anti-TSC-22 antibodies. Immunohistochemical analysis with the use of polyclonal anti-TSC-22 antibodies has been performed as well as double staining of histological slides with the use of anti-human TSC-22

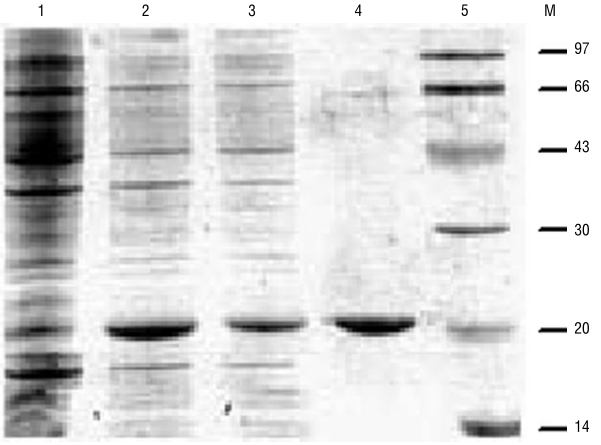

Fig. 2. Analysis of *E.coli* cell lysates in SDS-PAAG. 1 — total lysate of *E.coli* cells without IPTG induction, 2 — total lysate of *E.coli* cells with IPTG induction, 3 — soluble fraction of the lysate, 4 — purified TSC-22 eluted from Ni-NTA agarose, 5 — protein molecular weight marker (kDa)

polyclonal and anti-human GFAP monoclonal antibodies. Superposition of two flurescent patterns has demonstrated their similarity (Fig. 3, *a*). Hence, it may be concluded that TSC-22 protein is produced or accumulated in the same cells as GFAP protein.

Analysis of astrocytomas of different grades of malignancy has revealed that the quantity of TSC-22-positive cells was essentially lower in glioblastoma than in diffuse astrocytoma (see Fig. 3, *b*). These results indicated the existence of correlation between decreased expression of TSC-22 on mRNA level that we have detected earlier [11] and its decreased expression on protein level in more aggressive forms of astrocytomas. Immunohistochemical analysis has demonstrated the absence of TSC-22 expression in meningioma sample that served as negative control (see Fig. 3, *b*) confirms the data on very low expression level of TSC-22 mRNA in meningiomas [11].

TSC-22 belongs to the family of early response genes and was initially isolated from mouse fiblob-last cell line as a gene stimulated by transforming growth factor β1 (TGF-β1 stimulated clone 22) [19]. This gene encodes protein, which contains “leicine zipper” and is a transciptional repressor [20]. It was established earlier that TSC-22 expression level was essentially decreased in the salivary gland tumors, and that TSC-22 down-regulation increased a growth of salivary cancer cell line [12, 21]. Also it has been

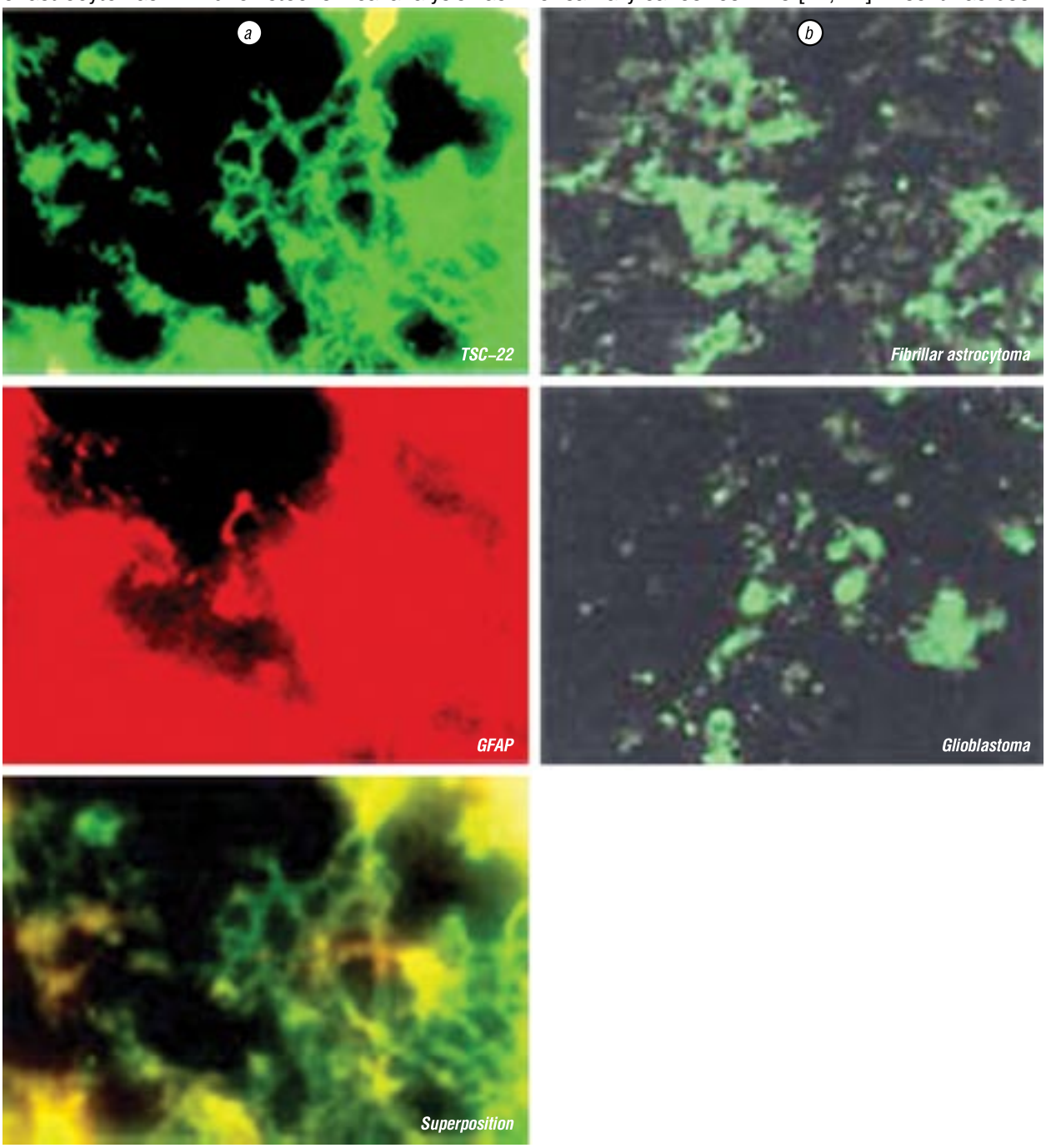

Fig. 3. Immunohistochemical detection of TSC-22 protein production in astrocytic tumors. *a*: double immunohistochemical labeling of diffuse astrocytoma section by antibodies against TSC-22 and GFAP, *b:* immunohistochemical labeling of diffuse astrocytoma and glioblastoma by antibodies against TSC-22

shown that subcellular localization of TSC-22 protein depends on induction of apoptosis [22].

Thus, the presented results along with the data obtained earlier that have shown the marked decrease of TSC-22 expression on mRNA level and protein level in human brain tumors along with the data of other authors on gene inactivation in the salivary gland tumors and negative role of TSC-22 protein in the cell proliferation process, indicate the potential suppressor role of this gene in tumor initiation and/or development.

Our previous analysis of genomic DNA from human brain tumors and from blood of healthy donors did not reveal any deletions in the locus of *TSC-22* gene [23]. It may be assumed therefore that the repression of the activity of this gene can exist on the level of regulation of expression. One of the reasons of tumor suppressor genes inactivation is the methylation of their promoters, as it was shown recently for the epithelial membrane protein 3EMP3 — another candidate on the glial tumor suppressor gene function [24]. Several sites for HpaII (CCGG) and high density of CpG-dinucleotides in *TSC-22* promoter region are the signs of so-called CpG-islands, frequently hypermethylated in tumors [25]. Analysis of *TSC-22* promoter region for possible methylation may help to elucidate the mechanisms of *TSC-22* gene inactivation in glial tumors.

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ОСОБЕННОСТИ ЭКСПРЕССИИ БЕЛКА TSC-22 КЛЕТКАМИ АСТРОЦИТАРНОЙ ГЛИОМЫ

Цель: **исследовать особенности экспрессии белкового продукта возможного опухоль-супрессорного гена *TSC-22* в астроцитарных опухолях человека.** ***Методы:*** **белок TSC-22 был экспрессирован в бактериальной системе с плазмидой pET-23d-TSC22 и применен в качестве антигена для получения поликлональных кроличьих антител. С их использованием проведен иммуногистохимический анализ экспрессии белков TSC-22 и GFAP в тканях астроцитарных опухолей человека (n = 15).** ***Результаты:*** **количество клеток, экспрессирующих TSC-22, значительно снижено в тканях глиобластомы по сравнению с таковым диффузной астроцитомы. При иммуногистохимическом анализе была выявлена коэкспрессия белков TSC-22 и GFAP в астроцитах.** ***Заключение:*** **белок TSC-22 экспрессируется в астроцитах, но не в макрофагах или клетках микроглии. Снижение уровня мРНК *TSC-22* коррелирует со сниженной экспрессией белка TSC-22 в более злокачественных формах астроцитом.**

Ключевые слова: **ген *TSC-22*, антитела против TSC-22, двойное иммуногистохимческое окрашивание, астроцитарные глиомы.**