УДК 577.21:577.214.622 + 616–006.484.04

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EXPRESSION OF GENES BELONGING TO THE IGF-SYSTEM IN GLIAL TUMORS

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Increased expression of the insulin-like growth factor (IGF) family members, IGF1, IGF2, their receptors and binding proteins, or combinations thereof has been documented in various malignancies including gliomas. The results of multiple investigations suggest that the IGFs can play a paracrine and/or autocrine role in promoting tumor growth in situ during tumor progression but that these roles may vary depending on the tissue of origin. Enhanced IGF1 expression was not found in glioblastomas and it was supposed that IGF1 participation in the development of glial tumors may be substituted by protein products of highly expressed other genes, also participating in PI3K and MAPK pathways. Increased expression of IGF-binding protein genes in brain tumors makes the picture even more complicated. As other binding proteins, IGFBPs regulate the activity of their ligands by prolonging their half-life. The discrepancies arising from conflicting evidence on the results obtained by different laboratories in human gliomas are discussed. Our data highlight the importance of viewing the IGF-related proteins as a complex multifactorial system and show that changes in the expression levels of any one component of the system, in a given malignancy, should be interpreted with caution. As IGF targeting for anticancer therapy is rapidly becoming clinical reality, an understanding of this complexity is very timely.

Introduction. In recent years, evidences have been appearing that the members of IGF system may be involved in cancer development. The antisense strategies, directed to the components of IGF-signaling, are the subject of many clinical trials. All three IGF receptors (IGF1R, INSR and IGF2R) are very well known targets for anti-cancer therapy. Increased expression of IGF1 receptor same as its ligands may stimulate PI3K and MAPK signaling cascades leading to cell proliferation [1—3].

However, the role of IGFs, IGF receptors and IGF-binding proteins (IGFBPs) in tumor development is poorly understood to this time. Increased levels of IGF1, IGF2 and their receptors have been found in different tumor types (reviewed in [4]), although the data about *IGF1* and *IGF2* gene expression in astrocytic gliomas are quite ambiguous. There are several publications reporting enhanced expression of these genes in anaplastic astrocytomas and glioblastomas on the RNA or protein levels [5—8]. For example, Sandberg et al. [5] analysed the expression of *IGF* genes by slot blot and Northern blot hybridization and found several fold increased *IGF1* and *IGF2* mRNA levels in one anaplastic astrocytoma and three glioblastoma specimens analyzed as compared to different regions of human adult normal brain. In other work, these authors demonstrated by immunohistochemistry the production of IGF1-like peptide in tumor cells in two of three anaplastic astrocytomas and in three of four glioblastomas examined [6]. *In situ* hybridization and immunocytochemistry also have localized a production of both IGF1 and IGF2 mRNA and protein in small number of astrocytoma samples analyzed that was accompanied by the co-expression of respective type-1 and type-2 IGF receptors [7]. Immunocytochemical analysis of 39 astrocytic tumors of WHO grades II—IV revealed tumor cells expressing IGF1 and IGF1R in all tumor grades (however, authors do not show how many of samples being under investigation were positive) [8].

On the other hand, the expression of *IGF1* and *IGF2* genes in astrocytic tumors was not found in other investigations. Thus, *IGF2* mRNA was not detected by Northern analysis in 5 astrocytomas and 2 glioblastomas [9], and the expression of *IGF1* was not found by Northern analysis in two investigated gliomas (ependymoma and glioblastoma) [10]. Earlier, we also could not find the significant change of *IGF1* gene expression in glioblastoma by

Serial Analysis of Gene Expression (SAGE) [11]. Such contradictory results could be explained by not enough big quantity of samples being under investigations. Nevertheless, *IGF1* and *IGF2* genes were not presented also in the lists of the genes with significant expression changes in the articles on integrative genome-wide analysis of 81 [12] and 460 glioblastoma samples [13].

Expression of insulin-like growth factor binding protein (IGFBP) genes in brain tumors makes the picture even more complicated. The IGFBP family consists of six high-affinity members (IGFBP1−IGFBP6) and four low-affinity proteins (IGFBP7−IGFBP10) which contain on the NH_2-termini conserved «IGFBP motif» (GCGC-CXXC), share significant structural homology with IGFBP1−IGFBP6, and able specifically bind IGFs, although with relatively low affinity [14]. As other binding proteins, IGFBPs regulate the activity of their ligands by prolonging their half-life. The biological actions of IGFs may be regulated by IGFBPs either negatively or positively, depending on the tissue type and the physiological or pathological status, some of the IGFBPs also act by a mechanism independent of IGFs. Elevation of the activity of IGF1 and IGF2 in the absence of the increasing of their genes expression may be a result of the enhancement of stability and lifetime due to the interaction with IGFBPs [15]. In addition to functioning in extracellular fluids as simple carrier proteins regulating circulating IGF turnover, transport, and distribution, the locally produced IGFBPs act as autocrine/paracrine regulators of IGF action [16].

Here, we analyze the expression of IGF system members including all ten IGFBP genes in glioblastoma by different methods to clarify their expression patterns in this tumor.

Materials and methods. SAGE Genie database (http://cgap.nci.nih.gov/SAGE) was used for the comparison of gene expression in glioblastoma and human normal brain by Digital Gene Expression Displayer (DGED) tool. Two pools of SAGE-libraries (9 libraries of glioblastoma and 5 normal adult human brain libraries) were compared. Data, obtained by microarray analysis and available in Gene Expression Omnibus (GEO) site (http://www.ncbi.nlm. nih.gov/geo), were used also for the comparison of these genes expression in glial tumors and normal brain. DataSet files were searched by keywords «glioblastoma», «astrocytoma», and «normal brain». A DataSet represents a collection of biologically- and statistically-comparable samples processed using the same platform. In order to measure expression levels from different DataSet files accurately, normalization by several housekeeping genes was required. Scripts, written in Perl were used for the analysis of these data.

Glioblastoma surgical specimens were classified on the basis of examination of hematoxylin and eosin stained sections according to World Health Organization (WHO) criteria [17]. Surgical specimens of histologically normal brain tissue adjacent to tumors were used as a source of normal adult human brain RNA and protein. All patients were being treated at the hospital of A.P. Romodanov Institute of Neurosurgery (Kyiv, Ukraine). The study protocol was approved by the human ethics review committees of both institutions and a signed consent forms from patients were obtained. The tissue samples were stored at −70 °C until analysis. RNA was extracted from frozen samples as described in our previous articles [11, 18].

Equal amounts of total cellular RNA (5 μg each for 20 μl mixture) were transcribed into cDNA with an oligo (dT)/random hexamer primers and Revert Aid M-MuLV reverse transcriptase («Fermentas», Lithuania). Each semi-quantitative RT-PCR was performed in 25 μl reaction mixture containing cDNA synthesized on 50 ng of RNA (2 μl of 10-fold diluted cDNA), 2U Dream Taq Green DNA polymerase ((«Fermentas», Lithuania), manufacturer's buffer, 0.2 mM dNTPs, and 10 μM gene-specific primers (Table 1). Thermal cycling parameters were: initial denaturing step at 95 °C for 2 min, followed by 27−35 cycles of denaturation at 95 °C for 30 sec, annealing at 56 ⁰C for 30 sec, synthesis at 72 °C for 30 sec, and final extension incubation at 72 °C for 7 min. The number of cycles was chosen so that the PCR product amplification rate was in the linear phase. Amplified products were electrophoresed in a 1.5 % agarose gel. Densitometric analysis of PCR product bands was carried out by using the Scion Image 1.62c program. Relative expression levels of examined genes were estimated by normalization to the expression level of control gene, β-actin (*ACTB*). Data obtained with *ACTB* were used for calculation of P-value of gene expression changes

in glioblastoma by unpaired t-test assuming unequal variance.

Real-time PCR was performed with the iCycler iQ5 (BIO-RAD, USA). Reaction mixture (20 µl) contained 2 µl cDNA (quantity equivalent to synthesized on 50 ng of total RNA), 10 µl Maxima™ SYBR Green qPCR Master Mix («Fermentas») and 10 pmole of each *IGF1, IGF2* and *IGF1R* gene-specific primer (Table 1). The amplification procedure of target genes was as follows: initial denaturing step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at 56 °C for 15 sec and extension at 72 °C for 15 sec. Melting curve analysis was performed to confirm amplification of single bands. Reaction efficiency was calculated by R package named qpcR package [19]. Gene expression values (relative mRNA levels) were calculated based on the modified $\triangle\triangle$Cq method [20] as ratio of efficiency raised to power Ct of analyzed gene to efficiency increased to power Ct of reference gene (*ACTB*).

Results. Serial Analysis of Gene Expression (SAGE) revealed a very low level of *IGF1* gene expression in glioblastoma. Thus, tag TTTGAT-TAAT corresponding to three known long *IGF1* transcripts (7370 bases variant 1 mRNA, Ac.No NM_001111283; 7204 bases variant 2 mRNA, Ac.No NM_001111284, and 7321 bases variant 4 mRNA, Ac.No NM_000618) was found as only one tag per 101053 tags in one (SAGE_Brain_glioblastoma_B_R20) of nine adult glioblastoma

Table 1

Primers used in this work for the analysis of gene expression

Gene name	Primer sequences	Cycle number	PCR product size
IGF-I	For GTCCTCCTCGCATCTCTTC Rev ACATCTCCAGCCTCCTTAG	32	242
IGF-II	For ACACCCTCCAGTTCGTCT Rev ACTGCTTCCAGGTGTCATA T	35	249
IGF-IR	For ACAGAGAACCCCAAGACTGAGG Rev TGATGTTGTAGGTGTCTGCGGC	32	247
IGFBP1	For CGGAGATAACTGAGGAGGA Rev CACTGTCTGCTG TGATAAAATC	32	271
IGFBP2	For CTCAAGTCGGGTATGAAGG Rev GAGTAGAGGTGCTCCAGA	32	224
IGFBP3	For GCACAGATACCCAGAACT Rev CCATACTTATCCAC A CACCA	29	226
IGFBP4	For ACCTCTACATCATCCCCAT Rev TCAGACTCAGACTCCAC T	29	284
IGFBP5	For GACCGCAAAGGATTCTACAA Rev ACTGAAAGTCCCCGTCAA	29	130
IGFBP6	For GCAACTCCAGACTGAGGTC Rev CTCGGTTTTTTGTTGAGTGATG	32	353
IGFBP7	For CCATGACTACTTTTAACCATGCAG Rev GGTGTACTTGAGCTGTGAGGTC	29	366
IGFBP8	For GGCTTACCGACTGGAAGAC Rev GATAGGCTTGGAGATTTTGGG	25	259
IGFBP9	For CTGTGGTATGGGGTTCTC Rev TGGATGGTTTTGGTATTGTG	32	273
IGFBP10	For GCTCCCTGTTTTTGGAATG Rev CATTTCTTGCCCTTTTTCAG	29	231
ACTB	For AACTACCTTCACATCCATCA Rev GTACATACTCCTGCTTGCT	27	262

Table 2

Characteristics of DataSet files from Gene Expression Omnibus (GEO) repository used for the evaluation of gene expession changes in glioblastoma

File name	Microarray platform	Authors/Reference	Short description of the experiments
GDS1975	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	Freije et al., 2004 [21]	Large-scale gene expression analysis using the Affymetrix HG U133 oligonucleotide arrays on 85 diffuse infiltrating gliomas of all histologic types to assess whether a gene expression-based, histology-independent classifier is predictive of survival and to determine whether gene expression signatures provide insight into the biology of gliomas
GDS1815	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	Phillips et al., 2006 [22]	77 primary high-grade astrocytomas and 23 matched recurrences were profiled to identify changes in gene expression that relate to both survival and disease progression. Samples include WHO grade III and IV astrocytomas with a wide range of survival times. Novel prognostic subclasses of high-grade astrocytoma are identified and discovered to resemble stages in neurogenesis
GDS1096	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	Ge et al., 2005 [23]	Genome-wide expression profiling of 36 types of normal human tissues. Each RNA tissue sample pooled from several donors. 2503 tissue-specific genes were identified. Results provide baselines for interpretation of gene expression profiles of cancers.
GDS1962	GPL570 [HG-U133_ Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	Sun et al., 2006 [24]	mRNA expression data were collected from patients with brain tumor to improve diagnostic of gliomas on molecular level. 23 samples from epilepsy patients were used as non-tumor samples. 157 tumor samples included 26 astrocytomas, 50 oligodendrogliomas and 81 glioblastomas
GDS3069	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	Liu et al., 2007 [25]	Analysis of 12 primary brain tumor biopsies with some variation in their histological diagnoses. These results, together with those obtained from miRNA profiling by real-time PCR, provide insight into the relationship between endogenous fluctuations in miRNA and mRNA expression levels
GDS596	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	Su et al., 2004 [26]	Designed custom arrays that interrogate the expression of the vast majority of protein-encoding human and mouse genes were used to profile a panel of 79 human and 61 mouse tissues. The resulting data set provides the expression patterns for thousands of predicted genes, as well as known and poorly characterized genes, from mice and humans

SAGE-libraries from Cancer Genome Anatomy Project database. No one glioblastoma SAGE-library contains the tag CCCAAGACCC corresponding to the shortest *IGF1* transcript (949 bases variant 3 mRNA, Ac No NM_001111285).

Gene Expression Omnibus (GEO) Datasets (http://www.ncbi.nlm.nih.gov/gds) were used to increase the statistical significance of SAGE results. Obtained files represented the experimental data on gene expression according to microarray analysis. Altogether, there were found six DataSet files which contained data concerning gene expression in 225 glioblastoma and 71 normal brain samples (Table 2). To compare data from experiments with different microarray platforms, we used a normalization method proposed for real-time PCR by dividing expression level of every gene on geometric average of three housekeeping genes, *ACTB*, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and TATA-box binding protein (*TBP)* [27].

In GEO repository, *IGF1* gene set consists of three different probes corresponded to three nucleotide sequences in GeneBank (AU144912, M29644 and M37484) and represented different

regions of *IGF1* mRNA. Although the results were little bit different for each probe (Fig. 1), the average *IGF1* gene expression level was even 1,5-fold lower in glioblastoma than in anaplastic astrocytoma or human normal brain with P < 0.05 (the significance of differences was calculated using unpaired two-tailed t-test and assuming unequal variance (Table 3). Analysis of each DataSet file for each *IGF1* probe also did not reveal significant differences in *IGF1* expression between glioblastoma, anaplastic astrocytoma and normal brain as for example it is demonstrated for GDS1962 (GEO DataSet record 1962) file in Fig. 2. Real-time PCR showed also slightly decreased *IGF1* expression level (Fig. 3).

As it concerns *IGF2* gene, SAGE revealed about 30-fold increased average expression level in glioblastoma as compared to adult normal brain. However, this increasing was not statistically significant (P = 0,293) due to the very high expression levels in two of nine glioblastoma samples, much more than the sample population median level. In GEO repository, *IGF2* gene set also consists of three different probes corresponding to nucleotide sequences in GeneBank: M17863, NM_000612 and X07868. In spite of some variations between different probes, the results show mainly that in average the expression level of *IGF-II* gene in glioblastoma is increased slightly as compared to anaplastic astrocytoma or normal brain (Fig. 4) and 1,6-fold increasing of average *IGF-II* expression in glioblastoma as compared to human normal brain is statistically significant (Table 3). Analysis of individual DataSet files for each *IGF2* probe showed that glioblastomas can be divided on two subgroups: one group has low expression level of *IGF2* gene just like normal brain samples, but other group was characterized by significantly increased expression of *IGF2* gene (Fig. 5, *a*). This division could be seen more distinctly if individual samples of 81 glioblastomas and 23 normal brains, analyzed in GDS1962 file, to arrange according to *IGF2* expression level in descending order: approximately one quarter of glioblastoma samples has increased *IGF2* expression level while other three quarters of glioblastoma samples have low expression level (Fig. 5, *b*). These microarray data are in a good concordance with results obtained by SAGE and were supported by real-time (Fig. 3) and semi-quantitative RT-PCR (Fig. 6).
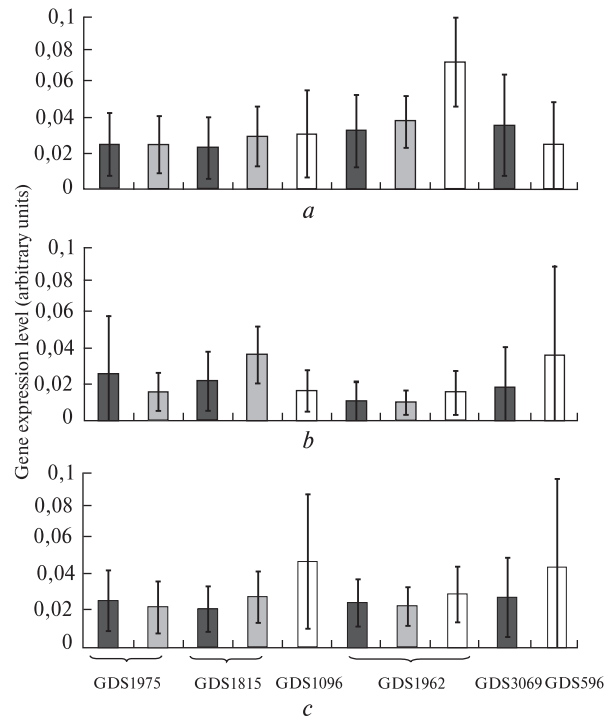

Fig. 1. Comparison of *IGF1* relative expression levels in glioblastoma, anaplastic astrocytoma and human normal brain according to microarray analysis data from six DataSet files of GEO repository (indicated below the bars in diagram). Data are presented for three probe sets of *IGF1* gene located on microarrays: AU14491 (*a*), M29644 (*b*) and M37484 (*c*). Samples of normal brain indicated by white bars, anaplastic astrocytomas — by grey bars, glioblastomas — by dark bars

IGF1 receptor is very well known target for anti-cancer therapy [28]. However, SAGE revealed only 1,4-fold not statistically significant increasing of *IGF1R* expression (P = 0,510) and microarray techniques showed even 1,2-fold (P = 0,046) decreasing of its expression in glioblastoma as compared to normal brain (Table 3). Real-time RT-PCR (Fig. 3) revealed 1,7-fold increasing of *IGF1R* expression, but the result was not statistically significant (P = 0,545). Thus, it can be concluded that *IGF1R* expression do not differ significantly in glial tumors and human normal brain. Expression of insulin receptor (IR), which is used alternatively by IGF2 [29], was decreased in 1,5-fold in glioblastoma according to SAGE and microarray results. At the same time, the expression of *IGF2R* gene was increased in glioblastoma as compared to normal brain according to SAGE
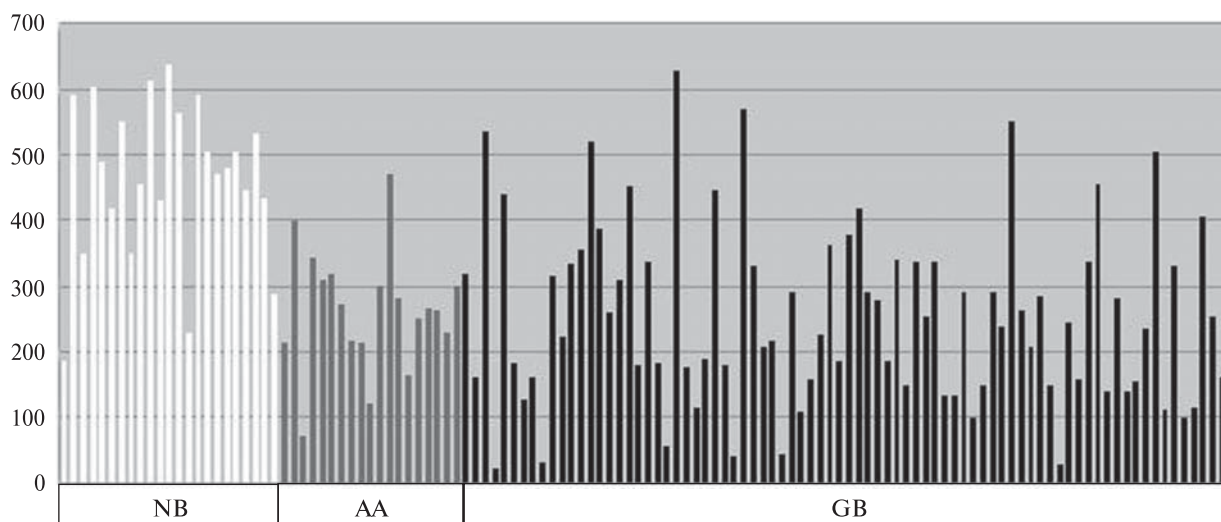
Fig. 2. *IGF1* expression in individual samples of glioblastoma, anaplastic astrocytoma and normal brain. Expression profile for *IGF1* gene (GeneBank: AU144912) obtained on the basis of the experimental data from DataSet record GDS1962 of GEO repository, submitted by Sun et al. [24]

(3,3-fold, P = 0,053) and microarray analysis data (1,9-fold, P < 0,001) (Table 3).

Comparison of *IGFBP* genes expression in glioblastoma and normal brain by SAGE revealed increased expression of all genes in glioblastoma except *IGFBP1* (Table 3). More than 3-fold up-regulation of *IGFBP2, IGFBP3, IGFBP4, IGFBP5,*

and *IGFBP7* genes in glioblastoma was statistically significant (P < 0,05) and confirmed by data of microarray analysis, which showed statistically significant increased expression levels also for *IGFBP8* and *IGFBP10* in addition to these five genes. Decreased expression of remained three genes (*IGFBP1, IGFBP6* and *IGFBP9*) in glioblas-

Table 3

Changes of the expression of IGF-system genes in glioblastoma according to three methods

Gene name	SAGE				Microarrays				RT-PCR			
	Average		GB/NB	P	Average		GB/NB	P	Average		GB/NB	P
	GB	NB			GB	NB			GB	NB		
IGF1	0.1	0.0	NaN	0.347	2.4	3.7	0.7	**<0.001**	1.07	1.14	0.94	**<0.001**
IGF2	66.4	11.2	5.9	0.363	11.4	7.0	1.6	**0.033**	0.65	0.19	3.36	0.052
IGF1R	3.1	2.2	1.4	0.510	6.3	7.6	0.8	**0.046**	0.88	0.83	1.06	**0.006**
IGF2R	9.7	3.0	3.3	0.053	10.6	5.6	1.9	**<0.001**	1.24	1.31	0.95	**<0.001**
IR	2.4	3.4	0.7	0.488	2.9	5.3	0.6	**<0.001**	1.15	1.17	0.98	**<0.001**
IGFBP1	1.4	2.4	0.6	0.506	1.6	2.2	0.7	0.058	1.17	0.62	1.88	**0.008**
IGFBP2	34.3	5.6	6.1	**0.023**	87.3	5.4	16.3	**<0.001**	2.00	0.77	2.58	**<0.001**
IGFBP3	26.8	7.2	3.7	**0.039**	47.9	8.4	5.7	**<0.001**	1.32	0.77	1.72	**0.001**
IGFBP4	19.2	0.8	24.0	**0.020**	13.7	8.2	1.7	**<0.001**	1.18	1.02	1.16	**<0.001**
IGFBP5	165.6	26.0	6.4	**0.032**	27.1	9.9	2.7	**<0.001**	1.73	0.64	2.71	0.072
IGFBP6	13.4	8.8	1.5	0.389	8.5	22.1	0.4	**0.004**	1.14	1.20	0.95	**<0.001**
IGFBP7	269.8	10.2	26.5	**0.014**	90.2	27.5	3.3	**<0.001**	0.95	0.64	1.48	**0.003**
IGFBP8	106.3	10.2	10.4	0.215	24.8	13.1	1.9	**<0.001**	0.89	0.52	1.72	**0.002**
IGFBP9	0.6	0.0	NaN	0.247	4.4	10.6	0.4	**<0.001**	1.45	1.60	0.90	**<0.001**
IGFBP10	37.6	6.6	5.7	0.103	27.1	13.5	2.0	**<0.001**	1.30	0.41	3.20	**0.001**

N o t e. GB — glioblastoma, NB — normal brain. P-value below the chosen threshold P < 0.05 indicated in bold.
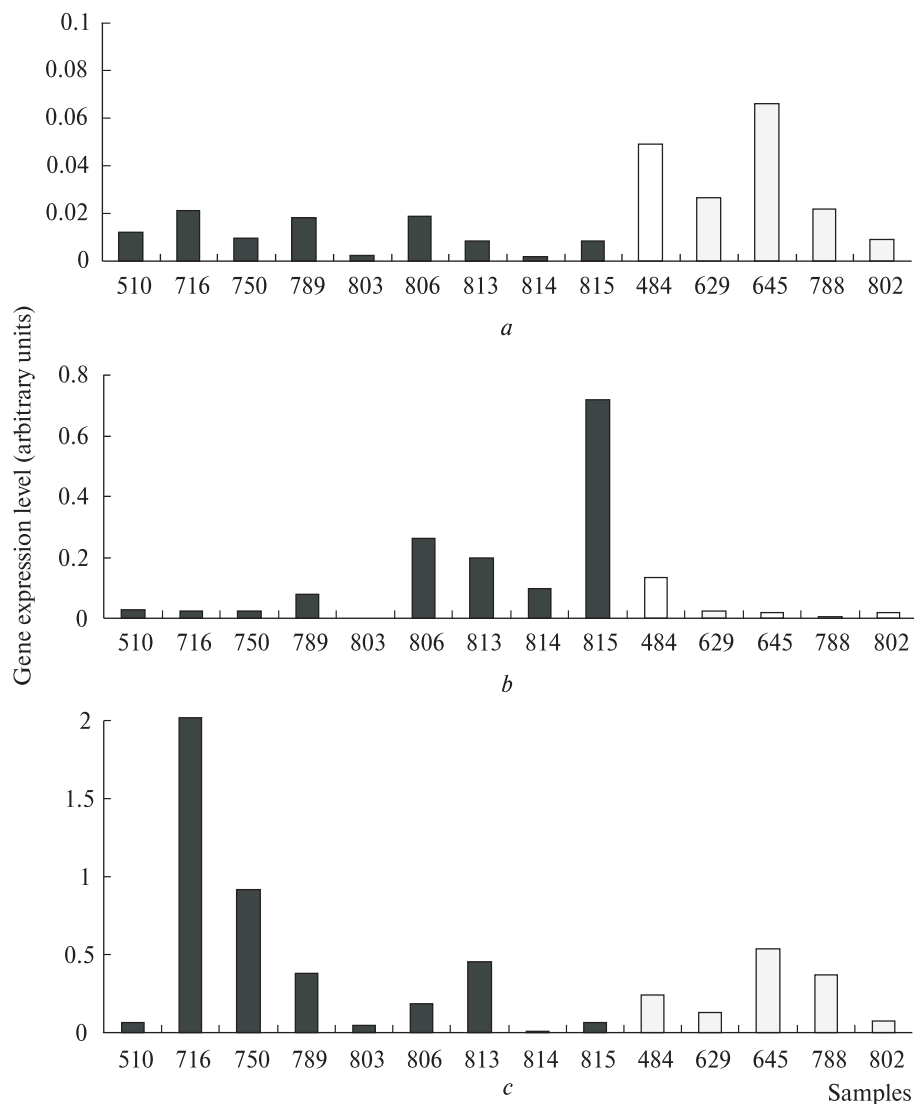
Fig. 3. Analysis of *IGF1* (*a*), *IGF2* (*b*) and *IGF1R* (*c*) genes expression in glioblastoma and human normal brain by real-tyme RT-PCR. Samples of normal brain indicated by white bars, glioblastomas — by dark bars

toma according to the results of microarray analysis in general were not in contradiction with SAGE results which showed some statistically nonsignificant decrease of the expression for *IGFBP1* or increase of the expression for *IGFBP6* and *IGFBP9* (Table 3).

Results of the *IGFBP* genes expression analysis by RT-PCR were in a quite good concordance with SAGE and microarray data. Semi-quantitative RT-PCR confirmed statistically significant increased expression of *IGFBP2, IGFBP3, IGFBP4, IGFBP7, IGFBP8,* and *IGFBP10* genes in glioblas-

toma (Fig. 6). Expression of *IGFBP5* was increased too, although P = 0,072. RT-PCR revealed also increased expression of *IGFBP1* gene in glioblastoma and this 1,88-fold increase was statistically significant (Table 3). Unlike RT-PCR, SAGE and microarray analysis results showed not statistically significant decrease of *IGFBP1* expression level in glioblastoma. Expression of *IGFBP6* and *IGFBP9* genes was not changed significantly in glioblastoma according to the results of RT-PCR and this in general does not contradict to SAGE and microarray analysis, as mentioned above (Table 3).
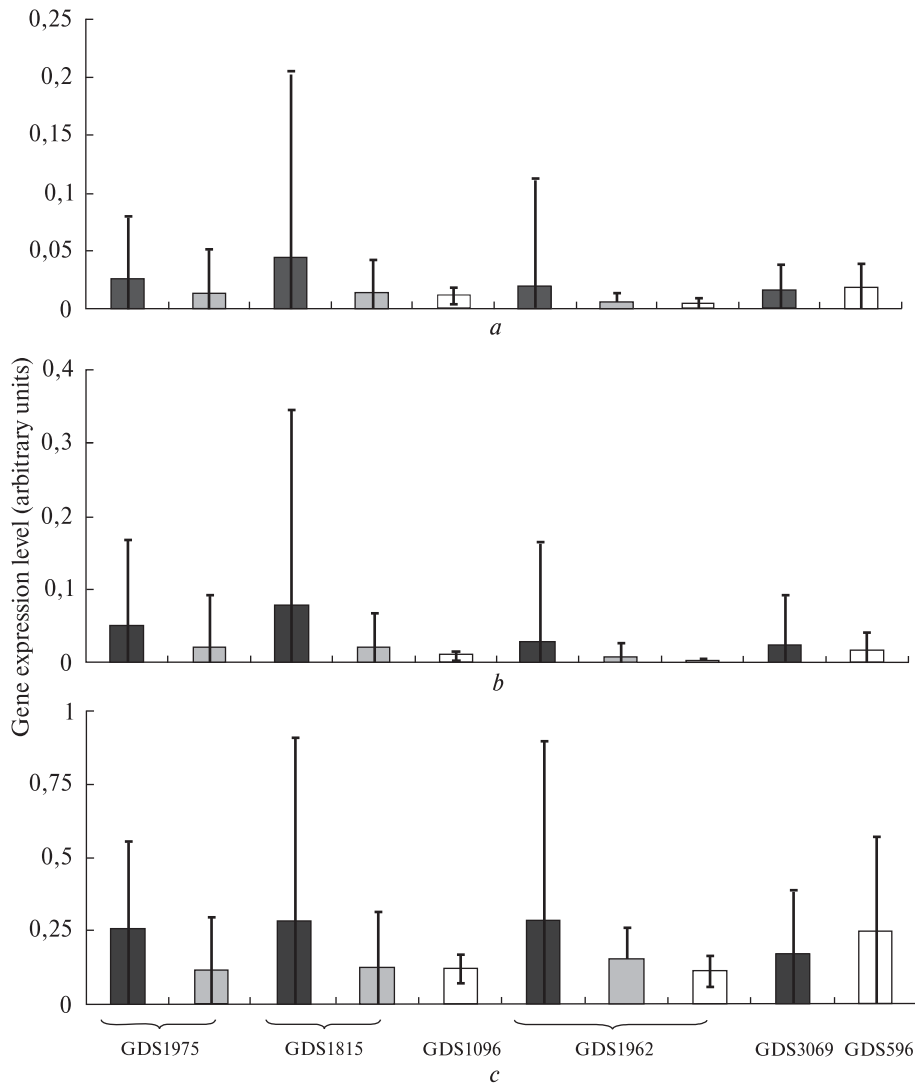
Fig. 4. Comparison of the relative expression levels of *IGF2* gene in glioblastoma, anaplastic astrocytoma and human normal brain according to microarray analysis data from six DataSet files of GEO repository (are indicated below the bars in diagram). Data are presented for three probe sets of *IGF2* gene located on microarrays: M17863 (*a*), NM_000612 (*b*) and X07868 (*c*). Samples of normal brain indicated by white bars, anaplastic astrocytomas — by grey bars, glioblastomas — by dark bars

So, the expression of seven *IGFBP* genes (*IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP7, IGFBP8,* and *IGFBP10*) was increased in glioblastomas according to three methods used for the analysis. The results for *IGFBP6* and *IGFBP9* genes differ slightly for three methods, but there were no contrast differences between these results. It is necessary to note that gene expression differences found by microarray analysis were more statistically significant: p-values were below the chosen threshold $P < 0.05$ for all genes analysed except

IGFBP1. This may be explained by larger sample numbers in both glioblastoma and normal brain groups analysed by this method as compared to SAGE or RT-PCR.

Discussion. The central role that the IGF system plays in initiating and promoting tumor progression makes it an attractive target for cancer therapy. Various strategies have been used to target components of this system in established animal and human tumor cell lines and in animal models of cancer; some of these strategies may be advanc-
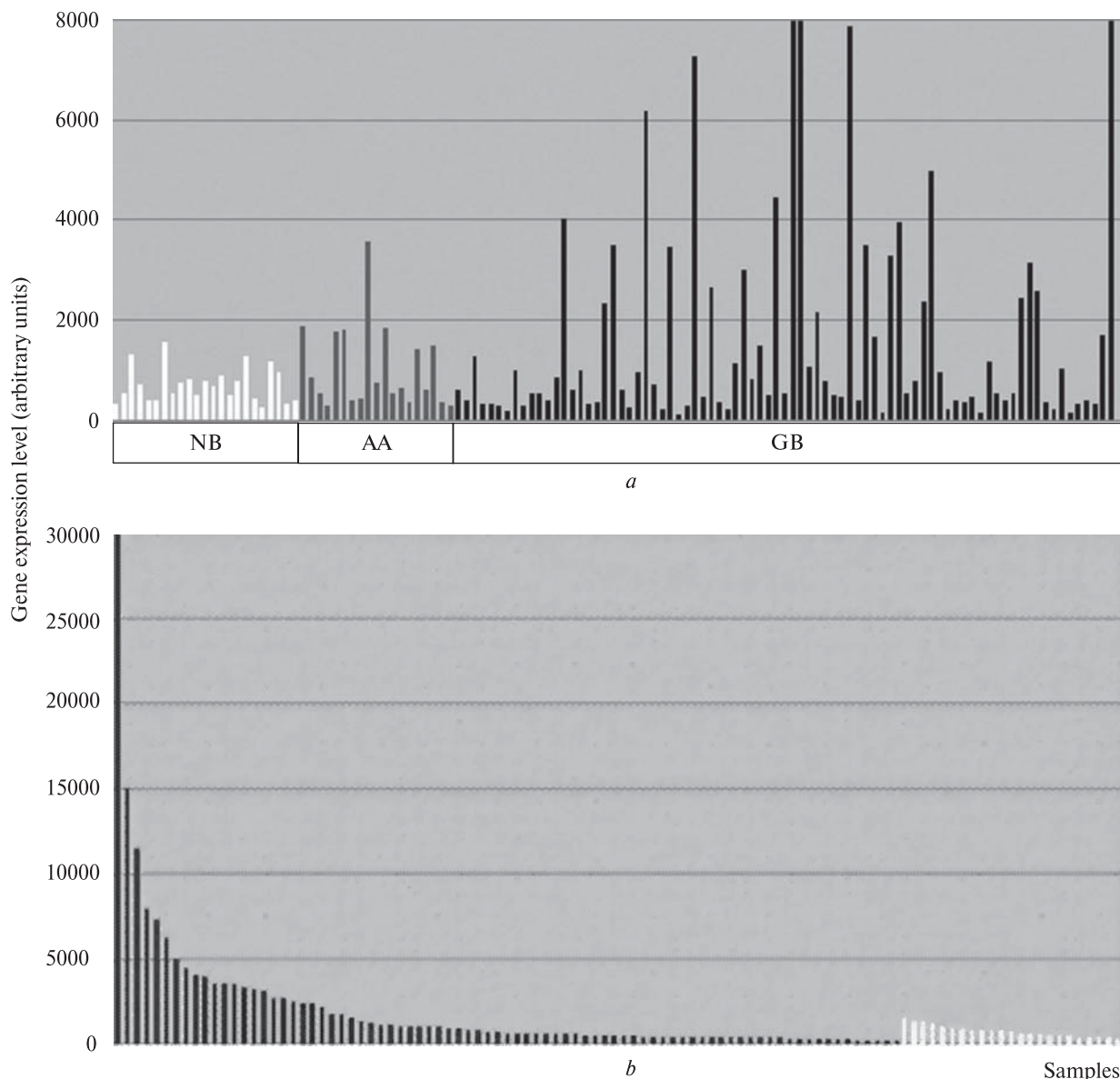
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Fig. 5. *IGF2* expression in individual samples of glioblastoma, anaplastic astrocytoma and normal brain: *a* − expression profile for *IGF2* gene (GeneBank: X07868) obtained on the basis of the experimental data from DataSet record GDS1962 of GEO repository, submitted by Sun et al. [24]; *b* − comparison of *IGF2* expression in glioblastoma and normal brain samples. Samples of normal brain indicated by white bars, glioblastomas − by dark bars

ing to clinical use. Among them IGF1 was targeted by different strategies including IGF1 peptide analogues [30], antisense oligonucleotides [31] and triple helix-expressing vectors [32].

Although increased expression of *IGF1*, *IGF2*, *IGF1R* or combinations thereof have been documented in various malignancies, these data show that while a correlation between *IGF1/IGF2*

expression levels and tumor progression could be consistently documented in some types of cancer (e.g. colorectal, hepatocellular and pancreatic carcinomas), no consistent correlation was seen in others (e.g. breast cancer) [4]. Taken as a whole, these studies suggest that the IGF1 role in a paracrine and/or autocrine promoting tumor growth may vary depending on the tissue of origin.
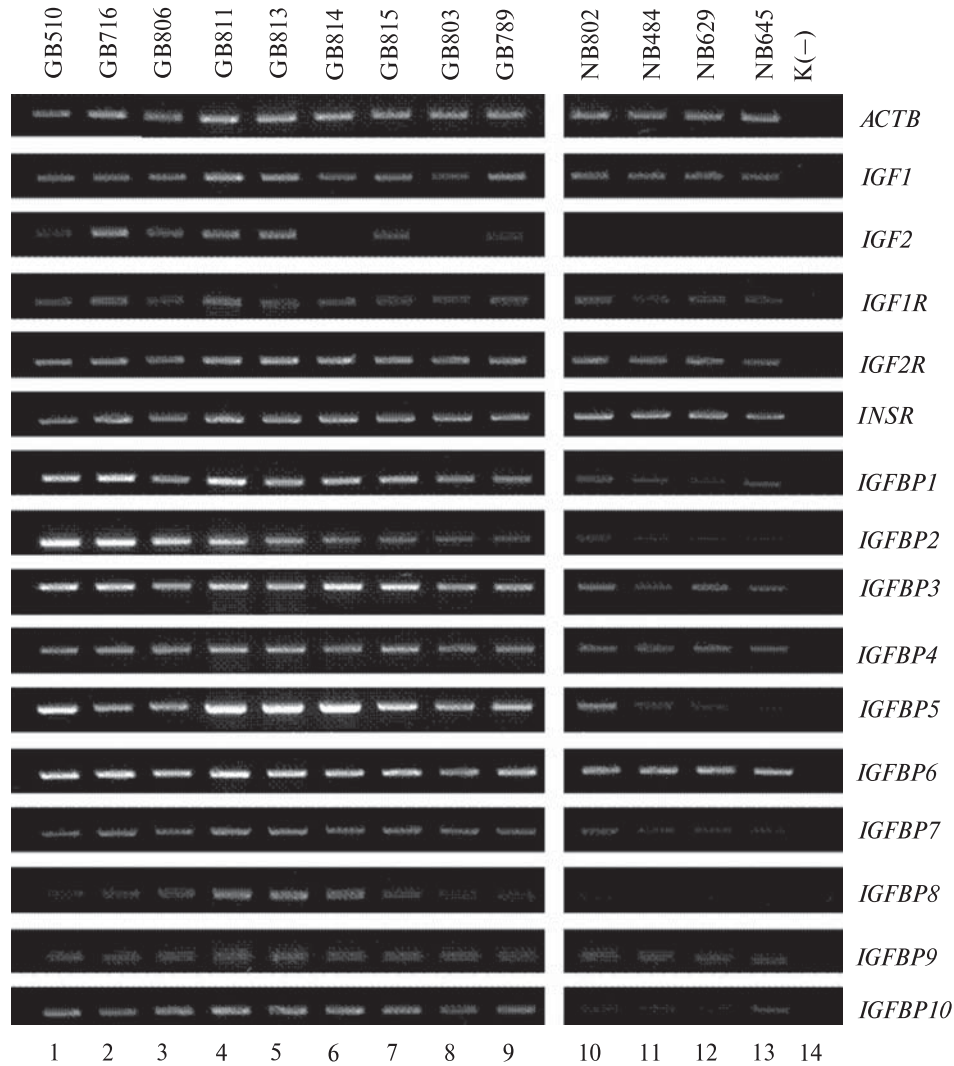
Fig. 6. Semi-quantitative PCR-analysis of IGF system genes expression in glioblastomas and normal brain. Control gene, *ACTB*, was used to determine relative expression level of analyzed genes in indivdual samples. Tissue and tumor types are indicated above each lane of the electrophoresis, numbers are the conditional numbers of RNA samples. GB − glioblastoma, NB − human normal brain, K(−) − control reaction without cDNA

Moreover, in some cases, conflicting results were obtained in different studies that analysed the same types of malignant tumors (e.g. gliomas). The references on *IGF1* gene expressions in glioblastoma are based on old papers, where authors used mostly slot blot hybridization or immunohistochemistry on comparably small numbers of clinical samples [5−10]. Immunohistochemical studies themselves are not very convin-cing: authors only mention the expression of *IGF1* in primary human astrocy-tomas [6] or write that *IGFI* mRNA can be seen in both astrocytoma and non malignant control human brain tissue [7]. The increased level of *IGF1* expression was mostly at the margin of glioblastomas and in perivascular zone and so as it was explained by authors [8, 9], could not be seen when the total RNA from the whole tumor was taken for *IGFI* mRNA measurement.

On the other hand, the expression of *IGF1* gene in astrocytic tumors was not found when we used SAGE or analysed data from the GEO repository. These data show that *IGF1* gene is expressed at a

low level in normal adult human brain and this level was not increased in glioblastoma. As it was mentioned above, other investigations also did not show the increased *IGF1* gene expression in this tumor [9, 10]. Taking into account quite big number of samples and different methods used in present investigation, the results of this study indicate that increasing of *IGF1* gene expression may be involved only in the formation of the limited part of astrocytic gliomas. Although the IGF1 was proposed as one of targets for glial tumor therapy and was supposed to become the alternative treatment for human glioblastoma [33], our results show why the anti-IGF1 treatment may not give positive results with gliomas, supposing that the development of these tumors is activated by some other way.

In contrast to *IGF1*, the expression of *IGF2* gene is up-regulated in glioblastoma although its expression level is relatively low as it was found previously in our work [34] as well as in other publications [35, 36]. Microarray analysis data show clearly the existence of the separate group of the glioblastomas overexpressing *IGF2* gene. This is in agreement with the results of Soroceanu et al. [37] who found that among 165 primary high-grade astrocytomas, 13 % of glioblastomas and 2 % of anaplastic astrocytomas expressed *IGF2* mRNA at the levels >50-fold the sample population median. Authors found that IGF2 can substitute for EGF to support the growth of glioblastoma-derived neurospheres and growth-promoting effects of IGF2 were mediated by the IGF1 receptor and phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3), a regulatory subunit of PI3K.

Survey of published data revealed only two old publications in which the author reported about the fourfold increase of IGF2 receptor, but not IGF1 receptor [38] or 2- to 5-fold higher cellular concentration of IGF2 receptor than the amount of IGF1 receptor [39]. Thus, IGF1 participation in cellular signaling pathways of glioblastoma may be substituted by IGF2 which may also stimulate both main signaling pathways, regulated by the extracellular signal-regulated kinase (ERK1/2) and protein kinase B-mediated (AKT).

When viewed together, all studies concerning the role of IGFs and IGF1R expression levels *per se* as indicators of tumor stage or predicators of disease outcome defy simple generalization and may be highly tumor-type specific. However as dis-

cussed above, the relevance of the IGF axis to cancer progression cannot be fully estimated by analysis of the expression levels of the IGF1R and its ligands only, because activation of the signaling pathway may occur through alternative mechanisms that bypass the requirement for receptor and/or ligand upregulation. For example, product of chitinase 3-like 1 (*CHI3L1,* other names *HC gp-39 or YKL-40*) gene with significantly increased level in glioblastoma [40] may also stimulate ERK1/2- and AKT-signaling pathways in a concentration range similar to the effective dose of IGF1. Just as IGF1, it activates two signal cascades, regulated by ERK1/2 and AKT and associated with mitogenesis control [41].

Results of the analysis of *IGFBP* genes expression in glioblastoma, obtained with three methods, demonstrate up-regulation of the majority *IGFBPs* in this tumor. Increased expression of *IGFBP2* [21, 42—48], *IGFBP3* [44, 47, 49, 50], *IGFBP4* [21, 49], *IGFBP5* [44, 48—50], *IGFBP6* [21, 44, 49], *IGFBP7* [51], and *IGFBP8* [44] genes in glioblastoma was reported previously in the studies using microarray analysis. It was shown that overexpression of *IGFBP5* gene correlates with the histological grade of human diffuse glioma: 83 % (58/70) of glioblastomas (WHO Grade IV) were immunopositive for IGFBP5, which was significantly higher than WHO Grade III gliomas (41 %, 41/101) or WHO Grade II gliomas (18 %, 13/72) ($p < 0.001$) [52]. Expression of *IGFBP1* to *IGFBP6* was analysed by PCR in glioblastoma cell lines T98G, A172, 86HG39 and U87MG and expression of *IGFBP2-IGFBP6* was found in all cell lines [36]. Higher content of *IGFBP1* mRNA in primary gliomas was demonstrated only in one work [53]. Authors found by RT-PCR that expression level of *IGFBP1* gene did not depend on grade of tumor malignancy.

Produced in tumor cells IGFBPs may stabilize insulin-like growth factor (s), IGF1 and/or IGF2, and drive their activation in glial tumors. On the other hand, some of the IGFBPs inhibit IGF actions or may act by a mechanism independent of IGFs, as reviewed by Mohan and Baylink [16]. It was documented that IGFBP1 increased migration of Chinese hamster ovary cells and trophoblast cells and affected apoptosis of breast cancer cells independently of IGF1 by activating α5β1 integrin—FAK—ILK—PI3-K—Akt signaling cascade [54].

Function of other inhibitory protein, IGFBP2, could be complex depending on the cell type and

cellular microenvironment. As reviewed by Fuku-shima and Kataoka [55], IGFBP2 has been considered as an inhibitory factor of IGF actions, particularly of IGF2, but binding of the IGFBP2/IGF complex to cellular surface proteoglycan may result in concentration of IGFs on the cell surface thus enhancing their actions. Authors supposed, that since binding of IGFs by IGFBP2 has growth-and/or migration-inhibitory effects, other mechanisms must be taken into account if attempt to find the correlation between overexpression of *IGFBP2* and malignant phenotypes of glioblastoma and proposed that IGFBP2 exerts influence on stimulation of cell proliferation and/or migration in an IGF-independent manner. Other study supported this suggestion and provided definitive evidence that IGFBP2 plays a key role in activation of the AKT pathway and collaborates with K-Ras or platelet derived growth factor B (PDGFB) in the development and progression of astrocytoma and oligodendroglioma [56].

IGFBP3 is known to block IGF action and inhibit cell growth. In addition, it possesses both growth-inhibitory and -potentiating effects on cells that are independent of IGF action and are mediated through specific IGFBP3 binding proteins/receptors located at the cell membrane, cytosol, or nuclear compartments and in the extracellular matrix. Transferrin and type I alpha collagen were characterized as these IGFBP3 binding proteins [57, 58]. *IGFBP3* was found among hypoxia-induced genes by the comparison of gene expression profiles of the U251 malignant glioma cell line under normoxic and hypoxic conditions, but the role of increased expression of *IGFBP3* gene in glioma tumorigenesis is unclear [59].

It was revealed that IGFBP4, a negative modulator of IGF1, displayed IGF1-independent anti-angiogenic effect on glioblastoma cells in response to their treatment by dibutyryl cyclic AMP (dB-cAMP) [60].

IGFBP5 could also stimulate cell migration through interaction with cell surface heparin sulfate proteoglycans and to determine cell fates by regulating apoptotic molecules (bax, bcl-2) and activating p38 MAP kinase and ERK 1/2 signal transduction pathways [61].

IGFBP6 is a relatively specific inhibitor of IGF2 actions. Overexpression of IGFBP6 inhibits tumor growth by inducing apoptosis and recently IGFBP6 was listed as a marker for cell senescence because it produces growth arrest with features of senescence but without the expression of cell differentiation markers [62].

IGFBP7 plays a negative role in the growth of cancer cells, including breast cancer, human prostate cancer, human cervical carcinoma (HeLa), murine embryonic carcinoma (P19), and osteosarcoma (Saos-2) cells [63]. Expression of IGFBP7 has been found to be up-regulated in human colorectal cancer and glioma cell lines and down-regulated in prostate and breast cancer cells. IGFBP7 is exclusively associated with laminin-stained glioblastoma vessels but was not observed in the vessels from nonmalignant brain [51]. IGFBP7 regulated glioma LN18 and LN443 cells proliferation and growth but not cell survival and promoted the migration of these cells through regulating the AKT (decreased phosphorylation) and ERK1/2 (enhanced phosphorylation) signal transductions [64].

IGFBP8(CTGF), *IGFBP9(NOV)* and *IGFBP10 (CYR61)* belong to the family of genes encoding CCN (cysteine-rich CYR61/CTGF/nephroblastoma-overexpressed gene) proteins which has been shown to play an important role in many processes, including proliferation, migration, adhesion, extracellular matrix regulation, angiogenesis, tumorigenesis, fibrosis, and implantation. CCN proteins share a modular structure and have in their N-termini four conserved domains with sequence homologies to insulin-like growth factor binding proteins (IGFBPs) [65]. Expression of these three genes was analysed by real-time PCR in gliomas of different malignancy grades and normal human brain by Xie et al. [66]. Authors found enhanced expression of *IGFBP8 (CCN2)* in 31 and *IGFBP10 (CCN1)* in 27 from 40 glioblastomas analysed, while only 7 glioblastomas had high levels of *IGFBP9 (CCN3)* mRNA. Significant correlation existed between *IGFBP8* and *IGFBP10* mRNA levels with tumor grade and survival of glioblastoma patients , but statistical analysis showed no difference between the clinical and pathological features and expression level of *IGFBP9* in gliomas. Results obtained in this work suggest that IGFBP8 and IGFBP10 may play some role in the progression of gliomas, but IGFBP9 is involved neither in their development nor progression. Furthermore, IGFBP9 had antiproliferative activity and suppressed the growth of glioma cells [67]. It was shown that IGFBP8 (CTGF)

was associated with oncogenic activities and drug resistance in glioblastoma. Overexpression of IGFBP8 caused the U343 glioblastoma cells to survive for longer than 40 days in serum-free medium and resist antitumor drugs including tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand, VELCADE (bortezomib, proteasome inhibitor) and temozolomide [68]. Xie et al. [69] demonstrated that IGFBP10 acts as an oncogene through the integrin-linked kinase (ILK) to stimulate β-catenin-TCF/LEF and AKT signaling pathways. Authors showed that forced expression of *IGFBP10* in U343 glioblastoma cells accelerated their growth in liquid culture, enhanced their anchorage-independent proliferation in soft agar, and significantly increased their ability to form tumors in nude mice.

As a summary, we have shown that *IGF1* gene expression is increased only in few cases of glioblastoma but predominantly it is not higher that in normal brain. IGF1 participation in cellular signaling pathways of glioblastoma may be substituted by increasing expression of *IGF2* or *IGF1R*, which may also stimulate both main signaling pathways, regulated by ERK1/2 and AKT. Several other genes with significantly increased expression may also stimulate these pathways in glioblastoma. Up-regulated IGFBPs may activate IGF, even if the latters do not increase their expression leading to the anti-apoptotic consequences in glial tumors. On the other hand, increased production of some IGFBPs leads to enhance their IGF-independent effects which may play an important role in the development of gliomas.

Obtained results highlight the importance of viewing the IGF-related proteins as a complex multifactorial system and show that changes in the expression levels of any one component of the system, in a given malignancy, should be interpreted with caution. Similar to the experience with other biology-based therapies, effective targeting of the IGF system may require a customized approach, where tumor profiling guides the selection of the appropriate drugs. As targeting of the IGF-family members for anti-cancer therapy is rapidly becoming clinical reality, an understanding of this complexity is very timely.

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ЭКСПРЕССИЯ ГЕНОВ, ОТНОСЯЩИХСЯ
К IGF-СИСТЕМЕ, В ГЛИАЛЬНЫХ ОПУХОЛЯХ

Повышенные уровни экспрессии отдельных членов семейства инсулиноподобных факторов роста (IGF) – IGF1 и IGF2, IGF-рецепторов, IGF-связывающих белков или их комбинации были обнаружены в различных новообразованиях, включая глиомы. Результаты множественных исследований свидетельствуют о том, что инсулиноподобные факторы роста могут стимулировать рост опухоли *in situ* аутокринным и/или паракринным способом, однако это действие может варьировать в зависимости от тканевого происхождения опухоли. Усиление экспрессии гена *IGF1* не было найдено в глиобластомах и предполагается, что участие IGF1 в развитии глиальных опухолей может быть замещено белковыми продуктами экспрессирующихся на высоком уровне генов, также участвующими в сигнальных путях MAPK и PI3K. Повышенный уровень экспрессии генов IGF-связывающих белков (IGFBP) в опухолях головного мозга делает картину еще более сложной. Как и другие связывающие белки, IGFBP регулируют активность своих лигандов, продлевая время их полужизни. В статье обсуждаются противоречивые результаты, описанные различными лабораториями для глиом. Полученные данные демонстрируют важность рассмотрения белков семейства инсулиноподобных факторов роста как сложную мультифункциональную систему и показывают, что изменения в уровне экспрессии любого компонента системы в упомянутой опухоли должны интерпретироваться с предосторожностью. В связи с тем, что выбор членов IGF-семейства в качестве мишени для противоопухолевой терапии быстро приобретает клиническую реальность, понимание сложности этой системы является весьма своевременным.

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ЕКСПРЕСІЯ ГЕНІВ,
ЩО НАЛЕЖАТЬ ДО IGF-СИСТЕМИ,
У ГЛІАЛЬНИХ ПУХЛИНАХ

Підвищений рівень експресії окремих членів родини інсуліноподібних факторів росту (IGF) – IGF1 та IGF2, IGF-рецепторів, IGF-зв'язуючих білків або їхньої комбінації був знайдений у різних новоутвореннях, включаючи гліоми. Результати множинних до-

сліджень свідчать про те, що інсуліноподібні фактори росту можуть стимулювати ріст пухлини *in situ* ауто-кринним та/або паракринним способом, однак ця дія може варіювати в залежності від тканинного походжен-ня пухлини. Посилення експресії гена *IGF1* не було знайдено в гліобластомах і передбачається, що участь IGF1 у розвитку гліальних пухлин може бути замінена білковими продуктами генів, що експресуються на високому рівні, які також приймають участь у сигналь-них шляхах MAPK та PI3K. Підвищений рівень екс-пресії генів IGF-зв'язуючих білків у пухлинах голов-ного мозку робить картину ще більш складною. Як і інші зв'язуючі білки, IGF-зв'язуючі білки регулюють активність своїх лігандів, продовжуючи час їхнього півжиття. У статті обговорюються суперечливі резуль-тати, описані різними лабораторіями для гліом. Отри-мані дані демонструють важливість розгляду білків ро-дини інсуліноподібних факторів росту як складну мультифункціональну систему і показують, що зміни рівня експресії будь-якого компонента системи у да-ній пухлині повинні інтерпретуватися із пересторо-гою. В зв'язку з тим, що вибір членів IGF-сімейства як мішені для протипухлинної терапії швидко набуває клінічної реальності, розуміння цієї системи є вельми своєчасним.

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