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CHARACTERIZATION OF GENES WITH INCREASED EXPRESSION IN HUMAN GLIOBLASTOMAS

In the present study, we have used the gene expression data available in the SAGE database in an attempt to identify glioblastoma molecular markers. Of 129 genes with more than 5-fold difference found by comparison of nine glioblastoma with five normal brain SAGE libraries, 44 increased their expression in glioblastomas. Most corresponding proteins were involved in angiogenesis, host-tumor immune interplay, multidrug resistance, extracellular matrix (ECM) formation, IGF-signalling, or MAP-kinase pathway. Among them, 16 genes had a high expression both in glioblastomas and in glioblastoma cell lines suggesting their expression in transformed cells. Other 28 genes had an increased expression only in glioblastomas, not in glioblastoma cell lines suggesting an expression possibly originated from host cells. Many of these genes are among the top transcripts in activated macrophages, and involved in immune response and angiogenesis. This altered pattern of gene expression in both host and tumor cells, can be viewed as a molecular marker in the analysis of malignant progression of astrocytic tumors, and as possible clues for the mechanism of disease. Moreover, several genes overexpressed in glioblastomas produce extracellular proteins, thereby providing possible therapeutic targets. Further characterization of these genes will thus allow them to be exploited in molecular classification of glial tumors, diagnosis, prognosis, and anticancer therapy.

Introduction. Gliomas are the most common type of malignant brain tumors and various genetic alterations have been identified in human glioma tumorigenesis. Amplification and/or overexpression of the EGFR gene are a hallmark of primary glioblastomas (GBs) and they typically lack p53 mutations. In contrast, secondary GBs exhibit a high frequency of p53 mutations and rarely show EGFR amplification. These observations indicate at least two distinct genetic pathways involved in the development of GBs [1]. A number of other genetic alterations could be associated with one or other pathogenetic pathway.

A more comprehensive approach to find genes involved in the glioblastoma formation requires systematic study of gene expression. This allows identifying differences in gene regulation rather to merely consider structural alterations. Such changes in gene expression are important determinants of normal cellular physiology and, if disturbed, directly contribute to abnormal cellular physiology, including cancer.

As a result of extensive studies of the molecular pathogenesis of cancer, several novel regulatory pathways and networks have been identified. Delineation of these pathways has revealed some unique events, marked by morphological and histological changes of cells, and the expression of genes and proteins that accompany oncogenic transformation. Thus, the cell signature changes during cancer development. By reading these changes accurately, we can improve the early detection and diagnosis of individual cancers.

Significant technological advances that allowed profiling of transcription patterns have reshaped diagnostic biology. cDNA microarrays, oligonucleotide chips, sequencing of cDNA libraries, Digital Differential Display (DDD), and Serial Analysis of Gene Expression (SAGE) provide rapid identification of genes or proteins that are up- or down-regulated in a disease-specific manner. Gene expression profiles describe the transcriptional processes within a cell of a given type or state. The utility of these techniques is that they do not typically rely on a single gene, but rather the clustering of several genes that highlight disease versus non-disease profiles. The disparity in expression levels might not only be used to classify cancer and predict the outcome of treatment, but could also be used to identify multiple biomarkers for early cancer detection [2].

In an effort to identify the genes that might be used as molecular markers of glial tumors, we have

here analyzed gene expression in GB and normal adult human brain (NB) by Serial Analysis of Gene Expression. Our results, which are reported below, demonstrate that about 40 genes are expressed at markedly higher levels in glioblastomas compared with normal brain cells. Furthermore, several of the most differentially expressed genes produce extracellular proteins, thereby providing opportunities for therapeutic application.

Materials and methods. Nine SAGE libraries of human glioblastoma (GB), five SAGE libraries of GB cell lines, and five SAGE libraries of normal human brain (NB) were analyzed to compare gene expression in GB with that of NB by accessing SAGE NCBI web site http://www.ncbi.nlm.nih.gov/SAGE and using the search tool of Digital Gene Expression Displayer (DGED) provided by the SAGE Genie database [3]. Footnote to the Fig. 1 lists all libraries that were used in this study. Because of pooling of samples in some of the original SAGE libraries (GSM765 is a pool of five GB samples, GSM763 is a pool of two different NB samples), our comparison reflected levels of tumor-derived RNA from thirteen patients compared to RNA derived from six NB samples.

Samples of GBs were obtained from the A.P. Romodanov Institute of Neurosurgery (Kyiv). Tumors were classified on the basis of review of haematoxylin and eosin stained sections of surgical specimens according to World Health Organization (WHO) criteria [4]. Surgical specimens of histologically normal brain tissue adjacent to tumors were used as a source of normal adult human brain RNA.

RNA isolation from 12 GBs and Northern blot analysis were performed as described in our previous works [5, 6]. Densitometric analysis of hybridization signals was performed by the Scion Image 1.62c program.

The UniGene database of NCBI was searched to obtain the expressed sequence tags (ESTs) containing coding regions of corresponding mRNA. The selected cDNA clones were purchased from the Resource Center/Primary DataBase (RZPD) of the German Human Genome Project.

The following probes were used for hybridization: Annexin A1 (ANXA1) cDNA, clone IMAGp998 L168452; Beta-2-microglobulin (B2M) cDNA, clone IMAGp998I211214; CD74 antigen, invariant polypeptide of major histocompatibility complex, class II antigen-associated (CD74) cDNA, clone IMAGp998P143584; Complement component 1, q subcomponent, alpha polypeptide (C1QA) cDNA, clone IMAGp958J19169; HC gp-39, human cartilage glycoprotein-39 (CHI3L1) cDNA, clone IMAGE p998P09248; Sec61 gamma subunit (SEC61G) cDNA, clone IMAGp998G024700; Secreted protein, acidic, cysteine-rich (osteonectin) (SPARC) cDNA, clone IMAGp998E214660; Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 (SERPINA3) cDNA, clone IMAG p958K06246; and 500 bp long RT-PCR product of β-actin mRNA.

Results and discussion. Through information obtained by modern multifactor analyses of the differentially expressed genes among tumor groups, a combination of identified genes may help to define new or more relevant categories of tumor, and to establish stronger criteria for selecting cancer treatments for each individual patient [7]. To quantify the relative gene expression levels in GB and NB tissues by SAGE we used the public databases of Cancer Genome Anatomy Project (CGAP). In our previous work [8], the comparison of five GB SAGE libraries with two NB SAGE libraries, which were available that time, has revealed 117 genes with more than 5-fold difference at the $P \leqslant 0.05$ level.

Four new GB SAGE libraries have appeared recently in the SAGE Genie database. Comparing all nine GB tumor SAGE libraries, taken together, with five NB SAGE libraries, taken together (cut-off ratio: 5-fold change), the number of tags was 199, when the significance was set at $P \leqslant 0.05$. Mostly all SAGE tags matched GenBank entry and only two tags appeared to represent previously not described transcripts. About ninety percent of the matching transcripts corresponded to characterized mRNA sequence entries, whereas other tags matched uncharacterized expressed sequence tag (EST) entries. 129 tags corresponding to characterized mRNA without those encoding hypothetical proteins, ORFs, and mitochondrial genes, were selected for inclusion in a comprehensive database by following arbitrary criteria: the tag had to be overexpressed at a level of 10 tags per 200000 tags or greater; and the tag had to be overexpressed in at least 6 of the 9 (2/3) GB libraries. Criterion 1 was set to reduce the possibility of error, because tags found at a small number of copies could arise

from errors in sequencing a large copy number tag with a similar sequence. Criterion 2 was chosen to ensure the generality of findings, by excluding genes unique to particular tumor [9]. A ratio greater than 5 in a group of GBs as compared with NBs at a significance of $P \leqslant 0.05$ was defined as «overexpressed in tumors».

44 genes of 129 met the criteria for genes overexpressed in tumors, and these are included in Table 1. In this table, the last but one column gives for each gene (i.e., tag) the overall ratio for all of the GB samples, taken together as a group, to all of the NB samples, at a significance of $P \leqslant 0.05$. In the most cases, genes were overexpressed by more than 10-fold. Selecting a cutoff ratio of less than 5-fold change would lead to obtain more overexpressed genes, however, genes exhibiting the high differences in expression are likely more biologically relevant. Because members of this group were implicated in glioma development (for example, see [7, 10, 11]), the present article focuses on the study of overexpressed genes.

Increased expression of these 44 genes identified by SAGE could be supported either by GB tumor cells, or by host stromal cells, including infiltrating MAC/microglial cells present within tumor. To differentiate these two possibilities, we relied on the increased expression of particular gene in GB cell lines. When high gene expression was observed in GB tumors and also in GB cell lines, but not or very low in NB tissue as for the first 16 genes in the Fig. 1, such pattern suggested that the expression in the bulk tumor originated from transformed cells. Increased expression of 28 other genes observed only in GBs but not in GB cell lines, rather suggested that expression in the bulk tumors originated from host cells like MAC/microglial cells [7].

To assess the reliability of expression patterns, we arbitrarily selected eight differentially expressed transcripts and evaluated them by Northern blot analysis, when their expression level was sufficient, also allowing to detect alternative transcripts.

Expression patterns were usually reproducible between different samples. Out of eight genes with elevated expression in glioblastoma relative to normal brain as determined by SAGE, each was detected in the GB samples and was expressed at considerably lower levels in normal brain (Fig. 2). It is important to note, however, that there were differences in expression between individual tumors, with a few glioblastoma samples exhibiting either high or low amounts of individual transcripts. Such differences in gene expression undoubtedly contribute to the observed heterogeneity in the biological properties of tumors derived from the same organ [12, 13].

Gliomas are primarily derived from astrocytes, which take a variety of forms and subserve many different functions. However, microglial cells not only occur within and around brain tumors but also contribute significantly to the actual tumor mass. Studies by independent groups confirmed that approximately one third of all cells in glioma biopsies are labeled by MAC markers [14]. Although extraparenchymal brain MAC precursor cells also normally occur in the brain, many such phagocytes are presumably derived from intrinsic microglia. The number of microglia/MACs in gliomas exceeds that of other cells of the immune system. Close association of the monocyte/MACs with angiogenesis in human tumors is necessary for the continued growth of solid tumors, and acceleration of tumor growth accompanies neovascularization (for references, see [15]).

Of 28 genes observed only in GBs but not in GB cell lines (Fig.1), HLA-DRA, CD74, HLA-A, and B2M were among the most abundant 50 transcripts in monocytes [16], STAB1 gene products were localized to alternatively activated macrophages [17], CD99 plays a critical role in the diapedesis of monocytes [18], C1QA, a component of complement system is expressed in activated MACs [19]. HLA-DRA, LGALS3, HC gp-39, B2M, HLA-A, mannose receptor, C type 2, and CD74 were among the most abundant 50 transcripts in induced macrophages [16]. HC gp-39 (YKL-40), osteopontin (SPP1), and osteonectin (SPARC) genes express on the low level in monocytes (MO), but are significantly upregulated later in MAC differentiation being good markers of MO to MAC differentiation [16, 20].

Although comparing tumor with normal tissues shows differences in average gene expression, the protein products of genes are mostly polyfunctional, and which function (if any) of particular protein participates in glioma formation often remains unclear, however, the functional analysis and classification of genes overexpressed in glioblastomas lead to some interesting conclu-

Table 1

Genes with increasing of tag distribution between pools of 9 GB and 5 NB SAGE-libraries revealed by SAGE Digital Gene Expression Displayer (DGED)

N	Tag	Gene name	Gene symbol	Libraries		Tags		Tag Odds A:B	P
				A	B	A	B		
1	TGGGATTCCC	Chitinase 3-like 2	CHI3L2	9	0	256	0	NaN*	0.00
2	CCACAGGGGA	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1	8	0	169	0	NaN*	0.00
3	AGTGGTGGCT	Fibromodulin	FMOD	9	0	145	0	NaN*	0.01
4	TTAAATAGCA	Stress-associated endoplasmic reticulum protein 1	SERP1	6	0	140	0	NaN*	0.01
5	GTCAACAGTA	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABCC3	8	0	103	0	NaN*	0.04
6	TGCTCCTACC	Fc fragment of IgG binding protein	FCGBP	9	2	556	2	95.44	0.00
7	GTATGGGCCC	Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	9	2	826	4	70.92	0.00
8	ACCAAAAACC	Collagen, type I, alpha 1	COL1A1	8	2	1125	6	64.42	0.01
9	ACATTCTTTT	Glycoprotein (transmembrane) nmb	GPNMB	9	1	361	2	61.95	0.00
10	AGTACCTTAT	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	EGFR	8	1	163	1	55.93	0.01
11	GACCACCTTT	Microfibrillar-associated protein 2	MFAP2	6	1	153	1	52.50	0.01
12	ATCCTGAGTT	Major histocompatibility complex, class II, DQ beta 1	HLA-DQB1	9	1	125	1	42.89	0.04
13	GAAATAAAGC	Imunoglobulin heavy constant gamma 1 (G1m marker)	IGHG1	7	3	605	5	41.54	0.00
14	TTTGGTTTTC	collagen, type I, alpha 2	COL1A2	8	2	529	5	36.32	0.00
15	ATAATAAAGC	Retinoic acid receptor responder (tazarotene induced) 2	RARRES2	9	1	205	2	35.17	0.00
16	CATATCATTA	Insulin-like growth factor binding protein 7	IGFBP7	9	5	1189	13	31.42	0.00
17	TCACCAAAAA	Stabilin 1	STAB1	8	1	174	2	29.85	0.01
18	GCCCTTTCTC	Mannose receptor, C type 2	MRC2	9	1	159	2	27.28	0.02
19	GCCAACAACG	Nicotinamide N-methyltransferase	NNMT	9	2	153	2	26.25	0.03
20	CTTGGGTTTT	Insulin-like growth factor 2 (somatomedin A)	IGF2	9	3	289	4	24.80	0.00
21	GCTGCCCTTG	Tubulin alpha 6	TUBA6	9	3	259	4	22.22	0.00
22	ATCTTGTTAC	Fibronectin 1	FN1	9	3	192	3	21.96	0.01
23	AGAAAGATGT	Annexin A1	ANXA1	9	5	437	7	21.43	0.00
24	TAACTCTCCT	Scavenger receptor class A, member 3	SCARA3	9	3	241	4	20.68	0.00
25	AGAACCTTCC	Major histocompatibility complex, class I, A	HLA-A	9	5	395	8	16.95	0.00
26	GCAACAGCAA	Sec61 gamma subunit	SEC61G	9	5	576	12	16.48	0.00
27	ATCAAGAATC	Interferon, gamma-inducible protein 30	IFI30	9	2	185	4	15.87	0.04
28	GGATATGTGG	Early growth response 1	EGR1	9	3	275	6	15.73	0.01
29	AATAGAAATT	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1	8	4	438	11	13.67	0.00
30	GACTCTTCAG	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	SERPINA3	9	5	1239	32	13.30	0.00
31	GGGCATCTCT	Major histocompatibility complex, class II, DR alpha	HLA-DRA	9	5	814	22	12.71	0.00
32	AGCAGATCAG	S100 calcium binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))	S100A10	9	4	475	13	12.54	0.00
33	TTCACTGTGA	Lectin, galactoside-binding, soluble, 3 (galectin 3)	LGALS3	9	5	386	11	12.04	0.00
34	TTCTATTTCA	Moesin	MSN	9	5	254	8	10.90	0.05
35	TGCTGACTCC	Nestin	NES	9	4	284	9	10.83	0.03
36	GTTCACATTA	CD74 antigen (invariant polypeptide of major histo-compatibility complex, class II antigen-associated)	CD74	9	5	2959	99	10.29	0.00
37	GTTGTGGTTA	Beta-2-microglobulin	B2M	9	5	2830	95	10.25	0.00
38	CTCTAAGAAG	Complement component 1, q subcomponent, alpha polypeptide	C1QA	9	5	882	30	10.10	0.00
39	TTTGCACCTT	Connective tissue growth factor	CTGF	9	3	435	15	9.95	0.01
40	GGATGTGAAA	CD99 antigen	CD99	9	5	399	14	9.78	0.02

Continue table 1

N	Tag	Gene name	Gene symbol	Libraries		Tags		Tag Odds A:B	P
				A	B	A	B		
41	TAATTTTAAC	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1	9	5	336	12	9.61	0.04
42	TGGCCCCAGG	Apolipoprotein C-I	APOC1	9	3	755	27	9.60	0.00
43	ATGTGAAGAG	Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	9	5	2717	112	8.35	0.00
44	ACAAAGCATT	Insulin-like growth factor binding protein 5	IGFBP5	9	5	750	36	7.15	0.04

Note. Total tags in Pool (A): 791621, total tags in Pool (B):271584. F (expression factor): 5X. P (significance filter): $\leqslant 0.05$. NaN* stands for «not a number» and occurs when the denominator of the equation is 0, i.e., there are no sequences of a gene in pool B.

sions. In Table 2 the gene symbol was used to identify the gene in http://cgap.nci.nih.gov/SAGE/AnatomicViewer and find PubMed ID, which can be applied to retrieve a reference supporting the functional classification by searching Medline with the indicated number at http://www.ncbi.nlm.nih.gov/PubMed/medline.html [21]. A review of publications and databases on each gene was used to group genes with potentially similar function. The majority of genes with more than 5-fold increased activity in GB tumors, are related only to a few different groups: genes encoding proteins involved in angiogenesis, extracellular matrix (ECM), immune response, drug-resistance, IGF-axis, and MAP-kinase cascade.

Increased angiogenesis is a well-documented feature of glioblastomas. Several genes with a more than 5-fold increased expression reflect this phenomenon. Activation of EGFR (see more below in MAPK cascades) regulates vascular endothelial growth factor (VEGF) mRNA expression in GB cells by transactivating the proximal VEGF promoter. The other name of COL3A1, collagen of fetal and blood vessels, points to its participation in angiogenesis: the major cause of death in mutant mice was rupture of the major blood vessels, similar to patients with type IV Ehlers-Danlos syndrome. COL3A1 is essential for normal collagen I fibrillogenesis in the cardiovascular system and other organs. The fibrillar collagen found in most connective tissues has a triple-stranded rope like coiled structure and contains two alpha-1 polypeptide chains and one alpha-2 chain. COL1A1 encodes the major component of type I collagen. COL1A2 encodes the other component of type I collagen. FMOD participates in the assembly of the extracellular matrix as it interacts with type I and type II collagen fibrils and inhibits fibrillogenesis in vitro. MFAP2 is a major antigen of elastin-associated microfibrils. MRC2, mesenchymally expressed member of the macrophage mannose receptor family of endocytic receptors is a key player in cellular collagen interactions. FN1 is involved in cell adhesion and migration processes including host defense, blood coagulation, and wound healing. Hepatocytes and smooth muscle cells have collagen receptors; most other cells depend on fibronectin for binding to collagen. EGR1 is a transcription factor that is rapidly induced by various stimuli such as stress, inflammation or injury, mitogens, and differentiation factors and implicated in the regulation of cell growth, differentiation, and gene expression. Inhibition of EGR1 protein expression leads to block of angiogenesis, tumor growth and neovascularisation. SPP1 plays an important role in tumor growth through the enhancement of angiogenesis; it was predominantly observed in the microvasculature of glioblastomas associated with VEGF expression. SERPINA3 is a plasma protease inhibitor, angiogenesis is modulated by proteinases and antiproteinases released from tumor cells that carry out tissue remodelling. LGALS3 is differentially expressed in endothelial cells and may participate in tumor angiogenesis of human gliomas. CD99 plays a critical role in the diabetes's of monocots and triggers multifactorial events including T cell activation as well as cell-cell adhesion during hematopoietic cell differentiation. SPARC is also thought to be involved in angiogenesis and endothelial barrier function: it was suggested that SPARC plays a dual role in the VEGF functions, tumor angiogenesis and extravasation of tumors mediated by the increased permeability of endothelial barrier.

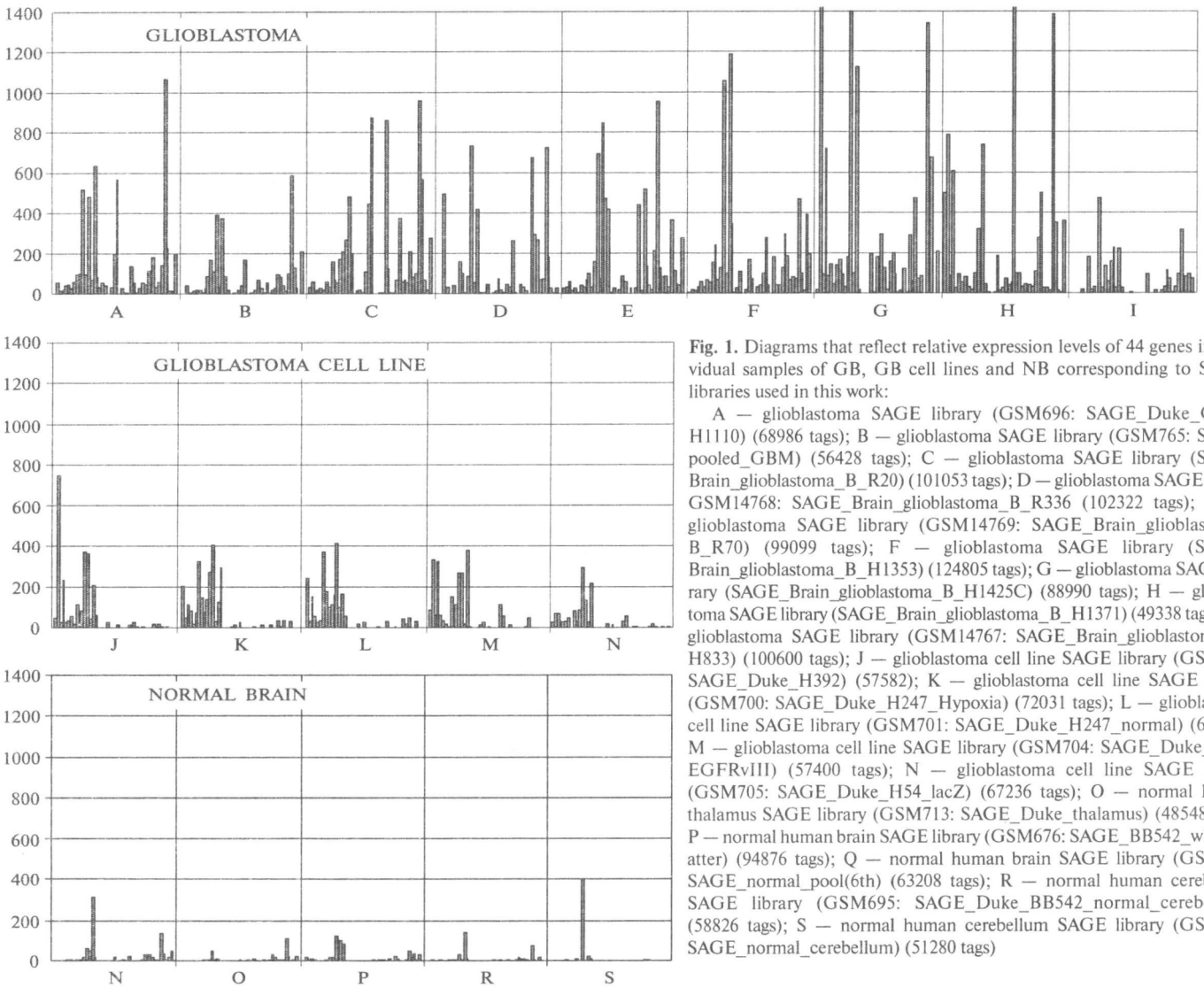

Fig. 1. Diagrams that reflect relative expression levels of 44 genes in individual samples of GB, GB cell lines and NB corresponding to SAGE-libraries used in this work:

A — glioblastoma SAGE library (GSM696: SAGE_Duke_GBM_H1110) (68986 tags); B — glioblastoma SAGE library (GSM765: SAGE_pooled_GBM) (56428 tags); C — glioblastoma SAGE library (SAGE_Brain_glioblastoma_B_R20) (101053 tags); D — glioblastoma SAGE library GSM14768: SAGE_Brain_glioblastoma_B_R336 (102322 tags); E — glioblastoma SAGE library (GSM14769: SAGE_Brain_glioblastoma_B_R70) (99099 tags); F — glioblastoma SAGE library (SAGE_Brain_glioblastoma_B_H1353) (124805 tags); G — glioblastoma SAGE library (SAGE_Brain_glioblastoma_B_H1425C) (88990 tags); H — glioblastoma SAGE library (SAGE_Brain_glioblastoma_B_H1371) (49338 tags); I — glioblastoma SAGE library (GSM14767: SAGE_Brain_glioblastoma_B_H833) (100600 tags); J — glioblastoma cell line SAGE library (GSM703: SAGE_Duke_H392) (57582); K — glioblastoma cell line SAGE library (GSM700: SAGE_Duke_H247_Hypoxia) (72031 tags); L — glioblastoma cell line SAGE library (GSM701: SAGE_Duke_H247_normal) (60663); M — glioblastoma cell line SAGE library (GSM704: SAGE_Duke_H54_EGFRvIII) (57400 tags); N — glioblastoma cell line SAGE library (GSM705: SAGE_Duke_H54_lacZ) (67236 tags); O — normal human thalamus SAGE library (GSM713: SAGE_Duke_thalamus) (48548 tags); P — normal human brain SAGE library (GSM676: SAGE_BB542_whitematter) (94876 tags); Q — normal human brain SAGE library (GSM763: SAGE_normal_pool(6th) (63208 tags); R — normal human cerebellum SAGE library (GSM695: SAGE_Duke_BB542_normal_cerebellum) (58826 tags); S — normal human cerebellum SAGE library (GSM761: SAGE_normal_cerebellum) (51280 tags)

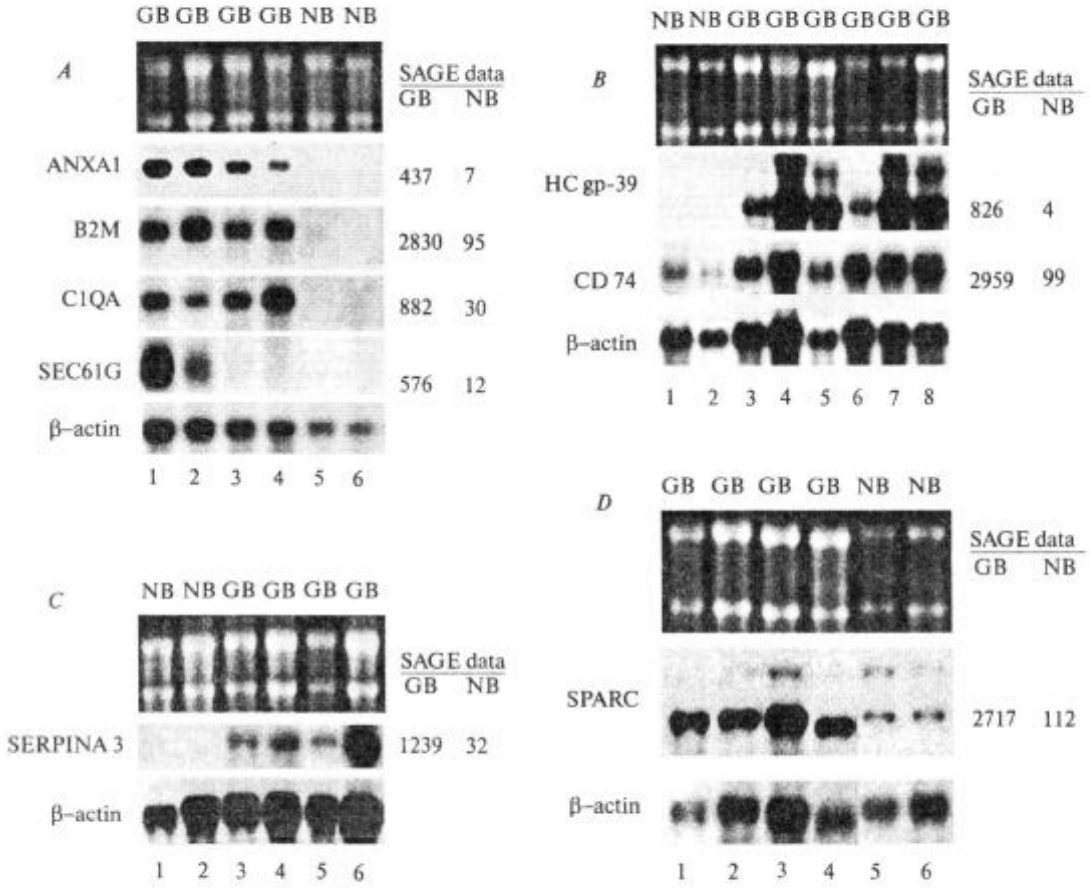

Fig. 2. Northern blot analysis of genes differentially expressed in glioblastoma. The top line of gels in each panel shows ethidium bromide stained gel. The number of SAGE tags observed in GB and NB SAGE-libraries is indicated to the right of each blot. *A* — hybridization of glioblastoma (GB) and normal brain (NB) RNAs with ANXA1, B2M, C1QA and SEC61G cDNA probes; *B* — hybridization of glioblastoma (GB) and normal brain (NB) RNAs with HC gp-39 and CD74 cDNA probes; *C* — hybridization of glioblastoma (GB) and normal brain (NB) RNAs with SERPINA3 cDNA probe; *D* — hybridization of glioblastoma (GB) and normal brain (NB) RNAs with SPARC cDNA probe

Changes in cell-cell interaction and in ECM genes expression are not surprising in glioblastoma. Functional changes in the immune system impact on tumor development [22] through a complex interplay between host and tumor: infiltrating host cells participate in the inflammatory and immune defense against tumor, whereas tumor cells produce immunosuppressive agents that promote host's tolerance. The group of immune-related genes encodes HLA-DRA MHC, one of the human leukocyte antigens (HLA) class II alpha chain paralogues, which consist of α- and β-chains, both anchored in the membrane; HLA-DQB1, beta subunit of heteromeric MHC class II proteins; IFI30, which has an important role in MHC class II-restricted antigen processing; CD74, HLA-DR invariant chain that is transiently associated with the MHC class II antigens in the endoplasmic reticulum and in endocytic vesicles; HLA-A, belonging to HLA class I heavy chain paralogues, a heterodimer consisting of a heavy chain and a light chain (B2M); SPP1, one of the key cytokines for type 1 immune responses mediated by macrophages; CD99 antigen that plays a critical role in the transport of MHC molecules. C1QA gene encodes the A-chain polypeptide, a major constituent of the human complement subcomponent C1q, participates in phagocytosis preceding antigen presentation. Annexin A1 is speculated to have immunosuppressive properties important for avoiding a host response to the tumor [23].

Table 2

Selected genes up-regulated in glioblastomas

Gene name	Gene symbol
Angiogenesis and fibrillogenesis	
Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	EGFR**
Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1*
Fibromodulin	FMOD**
Collagen, type I, alpha 1	COL1A1*
Microfibrillar-associated protein 2	MFAP2
Collagen, type I, alpha 2	COL1A2*
Mannose receptor, C type 2	MRC2
Fibronectin 1	FN1**
Annexin A1	ANXA1**
Early growth response 1	EGR1*
Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1**
Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	SERPINA3**
Lectin, galactoside-binding, soluble, 3 (galectin 3)	LGALS3**
CD99 antigen	CD99*
Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC**
Extracellular matrix (ECM) and ECM-related proteins	
Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	COL3A1*
Fibromodulin	FMOD**
Collagen, type I, alpha 1	COL1A1*
Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1**
Microfibrillar-associated protein 2	MFAP2
Collagen, type I, alpha 2	COL1A2*
Mannose receptor, C type 2	MRC2
Fibronectin 1	FN1**
Lectin, galactoside-binding, soluble, 3 (galectin 3)	LGALS3**
Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC**
IGF-axis	
Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1**
Insulin-like growth factor binding protein 7	IGFBP7**
Insulin-like growth factor 2 (somatomedin A)	IGF2**
Connective tissue growth factor	CTGF*
Insulin-like growth factor binding protein 5	IGFBP5
Gene name	Gene symbol
Immune response and complement pathways	
Fc fragment of IgG binding protein	FCGBP*
Major histocompatibility complex, class II, DQ beta 1	HLA-DQB1*
Immunoglobulin heavy constant gamma 1 (G1m marker)	IGHG1*
Annexin A1	ANXA1*
Major histocompatibility complex, class I, A	HLA-A**
Interferon, gamma-inducible protein 30	IFI30
Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	SPP1**
Major histocompatibility complex, class II, DR alpha	HLA-DRA*
CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	CD74**
Beta-2-microglobulin	B2M*
Complement component 1, q subcomponent, alpha polypeptide	C1QA
CD99 antigen	CD99*
MAPK cascades	
Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	EGFR**
Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1**
CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	CD74**
Protein tyrosine phosphatase, receptor-type, Z polypeptide 1	PTPRZ1
Drug resistance	
ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABCC3**
Fibronectin 1	FN1**

Continue table 2

Gene name	Gene symbol	Gene name	Gene symbol
Unclassified		Tubulin alpha 6	TUBA6
		Scavenger receptor class A, member 3	SCARA3
Chitinase 3-like 2	CHI3L2	Sec61 gamma subunit	SEC61G**
Glycoprotein (transmembrane) nmb	GPNMB*	S100 calcium binding protein A10 (annexin	
Retinoic acid receptor responder (tazarotene		II ligand, calpactin I, light polypeptide (p11))	S100A10*
induced) 2	RARRES2	Moesin	MSN
Stabilin 1	STAB1	Nestin	NES**
Nicotinamide N-methyltransferase	NNMT**	Apolipoprotein C-I	APOC1*

N o t e : Genes previously implicated in cancer and had patterns of expression that would be consistent with overexpression in cancer are marked by one or two asterisks; genes previously described in glioblastomas are marked by two asterisks.

MHC class I molecules play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen. Total loss of HLA class I antigens, aberrant reduction or loss of HLA-A, B, C/B2M molecules were found in different types of cancer. Decrease in the expression of HLA antigens is considered a characteristic of tumor progression. On the other hand, six out of fifteen glioma cell lines exhibited high levels of MHC class I and B2M molecules and a panel of 18 primary glioma cell explants exhibited high expression of class I HLA-A, B, C. It has been speculated that one of the approaches in gene therapy for glioblastoma may involve the transfer of costimulatory molecules, such as HLA, into glioma cells [24]. Our results on overexpression of MHC genes show that therapeutic recruitment of HLA to treat such diffusely infiltrative brain tumors, as astrocytic gliomas must be considered premature.

ABCC3/multidrug resistance-associated protein 3; (MRP3) is a member of the superfamily of ATP-binding cassette (ABC) transporters. Three ABC efflux transporters (ABCB1, ABCC3, and ABCB5) showed significant negative correlations with multiple drugs, suggesting a mechanism of drug resistance. It was postulated that the drug resistance is induced by the attachment of very late antigen-4 (VLA4) on leukemic cells to fibronectin on bone marrow stromal cells [25].

Insulin-like growth factor (IGF) system plays a crucial role in normal cell proliferation and malignant transformation. Insulin-like growth factors I and II are anabolic regulators in astrocytes and neurons, both normal and malignant brain growth, and are thought to play a pivotal role in the proliferation of brain tumors. Gliomas express IGF-II, which is a mitogen for many cell types and whose activity is regulated by genomic imprinting. Abnormal imprinting of IGF-II may contribute towards tumorigenesis and the modulation of aberrant imprinting status may result in new therapeutic approaches. IGF binding proteins (IGFBPs) are considered as primary modulators of metabolism, because of their ability to bind both IGF-I and IGF-II. All seven IGFBPs are expressed to a variable extent in brain tumors and glioblastoma cell lines. Both inhibitory and stimulatory actions have been described for the IGFBPs, each with distinct mechanisms. IGFBPs that are soluble in extracellular fluids decrease IGF activity: they prevent IGFs from activating the type 1 IGF receptor on the cell surface. IGFBPs can also intensify IGF activity. How this occurs is still unclear, but it seems to involve IGFBP association with the cell surface. Cell-associated IGFBP5 binds more IGF-II than IGF-I, because of a greater affinity of IGF-II for the IGFBP and because there are approximately 2-fold more binding sites for IGF-II [26]. IGFBP7 belongs to low-affinity IGFBPs. The activity of IGFBP7 was more than 20-fold increased in malignant mesothelioma cells [27]. CTGF belongs to a group of immediate-early genes, which are expressed after induction by growth factors, have significant sequence homology to the IGFBPs and together with IGFBP7, supposed to constitute a subfamily of IGFBP genes whose products bind IGFs with low affinity. Although the increasing of IGF-I gene expression was found neither in this study, nor by other investigators [28], the cellular responses to chitinase 3-like 1 (YKL40; HC-gp39) are similar to those elicited by IGF-1. Both proteins act synergistically

with respect to their growth-stimulating activity, both suppress the cytokine-induced secretion of MMPs [29]. It is not known which signaling pathways are affected in this system, but IGF-1 is a strong activator of PI3K and could exert its protective effects in a similar mode to HC-gp39 (see also MAPK cascades below).

Tyrosine kinase signaling is known or suspected to be altered in different sorts of tumors. At least four genes, whose activity increased by more than 5-fold in GBs, are related to the mitogen-activated protein kinase (MAPK) cascades, which include the extracellular signal-regulated kinase 1 and 2 (ERK1/2) cascade that preferentially regulates cell growth and differentiation, as well as the c-Jun N-terminal kinase (JNK) and p38 MAPK cascades. HC-gp39 activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. Stimulation of fibroblasts with IL-1 or TNF-a in the presence of HC-gp39 resulted in a marked reduction of both p38 mitogen-activated protein kinase and stress-activated protein kinase/Jun N-terminal kinase phosphorylation [29]. MAPK activation in response to nerve growth factor is controlled by Ras that interacts with and modulates the activity of effector proteins. The best characterized Ras effector is the serine/threonine kinase Raf, which leads to the activation of ERK pathway that plays a major role in cell proliferation and differentiation. Other effectors for Ras include the lipid kinase phosphatidylinositol-3-kinase (PI3K) involved in cell survival, proliferation, and metabolism. The suppressive effects of HC-gp39 were dependent on phosphoinositide 3-kinase activity, and treatment of cells with HC-gp39 resulted in AKT-mediated serine/threonine phosphorylation of apoptosis signal-regulating kinase 1 [29]. Enhanced expression of CD74 is associated with activation of protein kinase C delta. Macrophage migration inhibitory factor (MIF) binds to the extracellular domain of CD74, and CD74 is required for MIF-induced activation of the extracellular signal-regulated kinase-1/2 MAP kinase cascade [30]. Upregulation of IGF-II expression is a component of the effector mechanism of TrkA activation [31]. Several of the signaling cascades initiated by activation of TrkA receptor, including the Raf/MEK/ERK pathway and the PLC/PKC pathway (for references, see [32-35]); EGFR is a tyrosine kinase, involved in signaling pathways related to MAP kinase and PI3K. Activation of EGFR triggers mitogenic signaling. Inactivation of EGFR kinase significantly reduced PGE2-induced ERK2 activation, c-fos mRNA expression, and cell proliferation. EGFR binds to c-src and apparently has an important role in oncogenesis.

Although several transcripts were not expected to be differentially expressed in malignant tumors, the majority of gene products described here, have previously been shown to be dysregulated in neoplastic cells. 27 of the 44 potential glioblastoma tumor markers were previously implicated in cancer and had patterns of expression that would be consistent with overexpression in cancer cells (in table 2 they marked by one or two asterisks). The increased expression of 18 genes was previously described in glioblastomas (in table 2 they are marked by two asterisks).

Previously, we have characterized genes, with significantly (more than 5-fold) increased expression in bulk GB tumors and in GB cell lines [8]. However, both constituents of tumor, primary tumor tissue and «normal» host cells as macrophages, participate in the tumor growth and development. Taking into account as the tumor signature both the tumor-derived and host-derived overexpressed genes leads to consider a broader pattern of tumor markers.

In conclusion, both in vitro and in vivo experiments suggest that all genes found in this study as significantly overexpressed in GB tumors and GB cell lines, or only in GB bulk tumors, represent important molecular targets for characterization of astrosytoma development and should be included in the panel of tumor markers used for histopathological diagnosis and serological surveillance procedures. Moreover, several of the most differentially expressed genes such as beta-2-microglobulin, IGF-II, IGFB5, IGFBP7, galectin 3, chitinase 3-like 1 (YKL-40), chitinase 3-like2 (YKL-39), SERPINA3, SPARC/osteonectin, and SSP1/osteopontin produce extracellular proteins, thereby providing opportunities for clinical application. Further characterization of these genes will allow them to be exploited in molecular classification of glial tumors, diagnosis, prognosis, and anticancer therapy.

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РЕЗЮМЕ. Данные, имеющиеся в публичной базе данных по серийному анализу генной экспрессии (SAGE), были использованы для идентификации потенциальных молекулярных маркеров глиобластомы. 44 из 129 генов, которые проявили более чем пятикратную разницу уровня экспрессии при сравнении девяти SAGE-библиотек глиобластом и пяти SAGE-библиотек нормального головного мозга, повышали экспрессию в глиобластомах. Большинство соответствующих белков принимают участие в ангиогенезе, иммунном взаимодействии хозяин—опухоль, множественной устойчивости к лекарственным препаратам, формировании экстраклеточного матрикса, путях передачи сигнала в системе инсулиноподобных факторов роста (IGF) или MAP-киназы. Высокий уровень экспрессии 16 генов как в глиобластомах, так и в клеточных линиях глиобластом свидетельствует о том, что экспрессия этих генов происходит в трансформированных клетках. Экспрессия остальных 28 генов повышается только в глиобластомах, а не в клеточных линиях глиобластом, скорее всего в хозяйских клетках. Большинство этих генов входят в верхнюю часть списка транскриптов, активированных в макрофагах, а также вовлеченных в иммунный ответ и ангиогенез. Изменения экспрессии генов как в опухолевых, так и в хозяйских клетках могут быть использованы как молекулярные маркеры при анализе опухолевой прогрессии астроцитарных глиом, а также для выяснения механизма заболевания. Кроме того, значительная часть генов продуцируют экстраклеточные белки, таким образом давая возможности для клинического использования. Дальнейшая характеристика генов с повышенной экспрессией позволит использовать их для молекулярной классификации глиальных опухолей, диагностики, прогностической оценки и противоопухолевой терапии.

РЕЗЮМЕ. Дані, наявні в публічній базі даних по серійному аналізу генної експресії (SAGE), були використані для ідентифікації потенційних молекулярних маркерів гліобластоми. 44 з 129 генів, що виявили більш ніж п'ятикратну різницю рівня експресії при порівнянні дев'яти SAGE-бібліотек гліобластом та п'яти SAGE-бібліотек нормального головного мозку, підвищували експресію в гліобластомах. Більшість відповідних білків залучені до ангіогенезу, імунної взаємодії хазяїн—пухлина, множинної стійкості до лікарських препаратів, формування екстраклітинного матриксу, шляхів передачі сигналу в системі інсуліноподібних факторів росту (IGF) або MAP-кінази. Високий рівень експресії 16 генів як в гліобластомах, так і в клітинних лініях гліобластом свідчить про те, що експресія цих генів відбувається в трансформованих клітинах. Експресія інших генів підвищується лише в гліобластомах, а не в клітинних лініях гліобластом, скоріш за все в хазяйських клітинах. Більшість з цих генів входить до верхньої частини списку транскриптів, активованих в макрофагах, а також залучених до імунної відповіді та ангіогенезу. Зміни експресії генів як в пухлинних, так і в хазяйських клітинах можуть бути використані як молекулярні маркери в аналізі пухлинної прогресії астроцитарних гліом, а також для з'ясування механізму захворювання. Крім того, значна частина генів продукує екстраклітинні білки, таким чином даючи можливості для клінічного використання. Подальша характеристика генів з підвищеною експресією дозволить використовувати їх для молекулярної класифікації гліальних пухлин, діагностики, прогностичної оцінки та протипухлинної терапії.

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