RESEARCH ARTICLE

Microarray Analysis of the Hypoxia-induced Gene Expression Profile in Malignant C6 Glioma Cells

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Abstract

Hypoxia is commonly featured during glioma growth and plays an important role in the processes underlying tumor progression to increasing malignancy. Here we compared the gene expression profiles of rat C6 malignant glioma cells under normoxic and hypoxic conditions by cDNA microarray analysis. Compared to normoxic culture conditions, 180 genes were up-regulated and 67 genes were down-regulated under hypoxia mimicked by $CoCl_2$ treatment. These differentially expressed genes were involved in mutiple biological functions including development and differentiation, immune and stress response, metabolic process, and cellular physiological response. It was found that hypoxia significantly regulated genes involved in regulation of glycolysis and cell differentiation, as well as intracellular signalling pathways related to Notch and focal adhesion, which are closely associated with tumor malignant growth. These results should facilitate investigation of the role of hypoxia in the glioma development and exploration of therapeutic targets for inhibition of glioma growth.

Keywords: Glioma - hypoxia - microarray - differentially expressed genes

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Introduction

Malignant glioma, the most common primary tumor in the central nervous system (CNS) and the most aggressive brain tumor with very poor prognosis, is characterized by rapid growth, intense angiogenesis, vascular malformations and recurrent tendency. Of those pathological features, the variant necrotic regions, especially existed in glioblastoma multiforme (GBM), are regarded as the evidence of intratumoral hypoxia. With the accumulation of researching report, hypoxia is suspected to play an important role in glioma development and progress.

Many genes or proteins involving in tumorigenesis, angiogenesis and invasiveness are regulated by hypoxic condition. When GBM-derived neurosphere cultures were grown in 1% oxygen, the stem-like side population and the percentage of cells expressing CD133 respectively increased over fivefold and threefold, which were paralleled by an increase in clonogenicity (Bar et al., 2010). In glioma, VEGF, DLL4 and other angiogenesis-related molecules were reported to correlate with hypoxia (Xu et al., 2010; Li et al., 2011). Hypoxia promoted angiogenesis in GBM via upregulation of HIF-1α expression, which resulted in inducing recruitment of various tumor-associated angiogenic progenitor cells (Du et al., 2008). In vitro and in vivo researches also suggested that hypoxia increased the ability of GBM cell migration and invasion by activation of tumor cell c-met/SF/HGF and other pathways (Eckerich et al., 2007). Introducing HIF-1α- targeted small interfering RNA into the glioma cells significantly suppressed cell migration and invasion (Fujiwara et al., 2007).

Although more and more evidences indicate that hypoxia plays an important role in increased glioma cell invasion, chemoresistance and radiation resistance, the exact mechanism has not been ultimately clarified. cDNA microarray analysis provides an effective method to observe the differentially expressed genes under different conditions. By cDNA microarray analysis, Ha et al. (2006) identified 46 up-regulated genes and 23 down-regulated genes in melatonin-treated PBMCs, which provide a foundation for further studies on the function of melatonin. In this study, we used cDNA microarray to identify the differentially expressed genes (DE genes) to explore the potential therapeutic targets by comparing the gene expression profiles of rat C6 glioma cell line under normoxic and hypoxic conditions.

Materials and Methods

Rat C6 glioma cell culture

The rat C6 glioma cell line was obtained from Chinese Type Culture Collection (Chinese Academy of Sciences, Shanghai, no. TCR-1). C6 glioma cells were cultured in DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Gibco, USA) at 37 °C in a 95%

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air and 5% CO_2 atmosphere. C6 cells were grown to 80% confluence and exposed to either normoxic or hypoxic conditions for 24 h. Hypoxia was mimicked by addition of $CoCl_2$ (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 100 μM (Dai et al., 2008).

RNA extraction and reverse transcription

Total RNA was extracted by RNA easy Plus Mini Kits (Qiagen, CA, USA). The NanoDrop 1000 was used for accurately measuring RNA concentrations (OD260), protein contamination (ratio of OD260/OD280). The integrity and purity of RNA was assessed by electrophoresis on a denaturing agarose gel. Total RNA was transcribed to cDNA using a Superscript ds-cDNA synthesis kit (Invitrogen, USA) in the presence of 100 pmol oligo dT primers.

Microarray hybridization

ds-cDNA was cleaned and labeled by Cy3 in accordance with the NimbleGen gene expression analysis protocol (NimbleGen Systems, Inc., Madison, WI, USA). NimbleGen rat 12×135K cDNA microarray chip containing 26419 genes was used. Microarrays were hybridized at 42 °C during 16 to 20h with 4 μg of Cy3 labeled ds-cDNA in NimbleGen hybridization system. Following hybridization, washing was performed using the NimbleGen wash buffer kit. A total of six samples (three normoxic and three hypoxic samples) were analyzed by hybridizing one normoxic sample with one hypoxic sample for a total of three hybridization experiments.

Scanning and data analysis

After being washed in an ozone-free environment, the slides were scanned at 5 μm/pixel resolution using an Axon GenePix 4000B scanner. Scanned images (TIFF format) were then imported into NimbleScan software (version 2.5) for grid alignment and expression data analysis. After normalization of expression data, DE genes were identified through Volcano Plot filtering. A twofold increase in mRNA expression was arbitrarily used as a threshold value for differential expression. Hierarchical clustering was performed using the Agilent GeneSpring GX software (version 11.5).

GO analysis, the functional analysis associating DE genes, and pathway analysis were performed using the standard enrichment computation method. GO categories are derived from Gene Ontology (www.geneontology.org). Gene Ontology comprises three structured networks of defined terms that describe gene product attributes. The P-value, which denotes the significance of GO Term enrichment in the differentially expressed gene list, less than 0.05 is recommended. The less the P-value is, the more significant of the GO Term is.

Quantitative real-time RT-PCR assay

Quantitative real-time RT-PCR assay was performed for the interested up-regulated genes ANPEP and DLL3, and down-regulated genes ACTG1 and PDPK1. The cDNA transcribed from extracted RNA was subjected to quantitative Real-time PCR with a Bio-Bad iCycler system using the indicated primers synthesized by Qiagen (Valencia, CA). Reactions were performed in a final volume of 20μl with reagents provided by a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA). The mRNA levels of target genes were normalized to those of GAPDH mRNA. The series of measurements was performed three times.

Results

DE genes induced by hypoxia in rat C6 glioma

To assess the gene expression variation between the compared hypoxic and normoxic arrays, Scatter-Plot analysis (Figure 1a) and Volcano Plot filtering (Figure 1b) were performed. By further structural annotation, hybridization identified 180 up-regulated genes and 67 down-regulated genes by hypoxia. Among those DE genes, the up-regulated gene and down-regulated gene with highest absolute fold change were ANPEP and ANKRD28 with 3.92 and 15.34 folds change respectively.

Up-regulated DE genes by hypoxia

Among those 180 up-regulated genes in rat C6 glioma cell by hypoxia, 38 genes were described as hypothetical proteins, 73 genes were similar to some known genes, the rest 69 genes were clearly identified to have certain function.

To better annotate the functions associated with those DE genes, GO analysis is performed with GO categories which are derived from Gene Ontology (www.geneontology.org). 101, 112 and 110 up-regulated genes of those DE genes were identified to associate with biological process (BP), molecular function (MF) and cellular component (CC) respectively. Among those up-regulated DE genes, 47 genes are involved in multicellular organismal process, 65 genes are associated with cellular membrane component, and 13 genes have sequence-specific DNA binding transcription factor activity. Hypoxia significantly up-regulated three BP ontology: regulation of selenium ion, regulation of glycolysis and positive regulation of erythrocyte differentiation with enrichment score 2.689, 2.689 and 2.604 respectively,

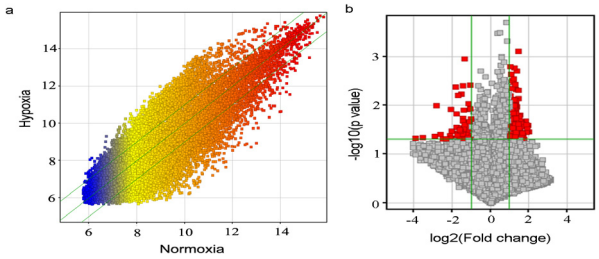

Figure 1. Expression Patterns of Up-regulated and Down-regulated Genes by NimbleGen 12×135K cDNA Microarray Chip. (a) Scatter-Plot. The values of horizontal and vertical axes are respectively the normalized signal values of normoxia and hypoxia sample (log2 scaled). The green lines are Fold Change Lines (The default fold change value given is 2.0). Genes above the top green line and below the bottom green line indicated more than twofold change of genes between two compared arrays. (b)Volcano plots. The vertical lines correspond to twofold up and down, respectively, and the horizontal line represents a P-value of 0.05. The red point in the plot represents the differentially expressed genes with statistical significance

Table 1. Up-regulated Genes by Hypoxia with Different Molecular Function in C6 Rat Glioma Cell

Gene ID	Gene symbol	Description	FC[a]	P
81641	ANPEP	Aminopeptidase N;cluster of differentiation antigen 13 (CD13)	3.92	0.028
29242	CAR4	carbonic anhydrase 4	3.52	0.02
292697	APOC2	apolipoprotein C-II	3.47	0.021
117261	SLC15A1	solute carrier family 15 (oligopeptide transporter), member 1 (Slc15a1), transcript variant 2	3.11	0.022
24197	ALPI	Alkaline phosphatase 1, intestinal, defined by SSR (Alpi)	3.04	0.021
171078	MLXIPL	MLX interacting protein-like (Mlxipl)	2.98	0.021
64055	GUCA2B	guanylate cyclase activator 2B (uroguanylin)	2.85	0.049
316052	EOMES	similar to Eomesodermin homolog	2.83	0.017
266807	DUOX1	dual oxidase 1	2.82	0.023
116569	LCT	lactase, transcript variant 1	2.71	0.039
64570	NAT8	N-acetyltransferase 8 (camello like)	2.63	0.049
83589	APBA1	amyloid beta (A4) precursor protein-binding, family A	2.63	0.036
315338	HOXC10	similar to Homeobox protein Hox-C10 (Hox-3.6)	2.62	0.028
290257	CTSG	similar to Cathepsin G precursor (Vimentin-specific protease) (VSP)	2.6	0.04
80840	GPR12	G-protein coupled receptor 12	2.6	0.01
24784	SLC9A3	solute carrier family 9, member 3	2.57	0.024
368158	RORC	similar to Nuclear receptor ROR-gamma (Nuclear receptor RZR- gamma) (Thymus orphan receptor) (TOR)	2.56	0.023
679701	BARX2	BarH-like homeobox 2	2.54	0.009
83567	MCHR1	G protein-coupled receptor 24	2.54	0.032
25146	CYP17A1	cytochrome P450, family 17, subfamily a, polypeptide 1	2.51	0.005
54276	NEUROD2	neurogenic differentiation 2	2.5	0.02
25470	KCNA5	potassium voltage-gated channel, shaker-related subfamily, member 5	2.43	0.009
498286	SLAMF1	similar to signaling lymphocytic activation molecule (predicted)	2.41	0.01
291100	IRF4	Interferon regulatory factor 4 (Lymphocyte-specific interferon regulatory factor) (Transcriptional activator PIP)	2.41	0.009
24796	SPN	Sialophorin (CD43) (W3/13 antigen)	2.39	0.008
503254	LYG1	Similar to Lysozyme g-like protein 1 (Predicted)	2.38	0.036
298079	ALDH1B1	aldehyde dehydrogenase 1 family, member B1	2.38	0.024
83509	SLC7A7	solute carrier family 7 member 7	2.36	0.036
25681	COL10A1	procollagen, type X, alpha 1	2.35	0.025
313507	TAL1	T-cell acute lymphocytic leukemia-1 protein homolog	2.31	0.049
116660	PTPRN	protein tyrosine phosphatase, receptor type, N	2.31	0.019
364241	KCNK5	Potassium channel, subfamily K, member 5	2.29	0.023
114125	DLL3	delta-like 3 (Drosophila)	2.29	0.031
364994	DPEP3	dipeptidase 3	2.29	0.017
85253	PLG	Plasminogen	2.23	0.039
94199	DPEP1	dipeptidase 1 (renal)	2.23	0.042
25097	PLD2	phospholipase D2	2.22	0.016
116778	PCDHA10	Cadherin-related neuronal receptor 10	2.22	0.045
25342	OXTR	oxcytocin receptor	2.22	0.018
350719	FEZF2	zinc finger protein 312	2.21	0.025
64621	AKP3	intestinal alkaline phosphatase-II (IAP-II)	2.21	0.045
25545	SEPW1	selenoprotein W, muscle 1	2.21	0.035
296512	FMN1	Formin-1 isoforms I/II/III	2.18	0.028
315388	TMPRSS6	Transmembrane protease, serine 6 (Matriptase-2) isoform 1	2.17	0.026
287638	DLX3	distal-less homeobox 3	2.17	0.004
362268	RBPJL	recombining binding protein suppressor of hairless-like (Drosophila) (predicted)	2.17	0.021
89833	FTCD	Formimidoyltransferase-cyclodeaminase	2.16	0.045
292850	KLK10	Kallikrein 10	2.16	0.043
24506	INS2	insulin 2	2.15	0.048
245917	SYCN	Syncollin	2.15	0.03
25040	GIP	gastric inhibitory polypeptide (Gip), Glucose-dependent insulinotropic peptide	2.13	0.023
83513	MMP24	matrix metalloproteinase 24 (membrane-inserted)	2.13	0.021
54259	INPP5D	Inositol polyphosphate-5-phosphatase D, 145 kDa	2.09	0.036
690037	FOLR4	Similar to folate receptor 4 (delta) isoform 1	2.08	0.018
497881	TLX3	T-cell leukemia homeobox protein 3 (Homeobox protein Hox-11L2) (Respiratory neuron homeobox protein)	2.08	0.002
83826	NR5A1	nuclear receptor subfamily 5, group A, member 1, transcript variant 1 (Nr5a1)	2.07	0.04
60449	GNB3	guanine nucleotide binding protein, beta 3	2.07	0.036
116680	PTPRU	protein tyrosine phosphatase, receptor type, U	2.06	0.011
116638	SLC17A7	solute carrier family 17 (brain-specific sodium-dependent inorganic phosphate cotransporter), member 7	2.06	0.013

[a]the ratio of normalized intensities between hypoxia and normoxia groups

Table 2. Down-regulated Genes by Hypoxia with Different Molecular Function in C6 Rat Glioma Cell

Gene ID	Gene symbol	Description	FC[a]	P
365320	RRM1-PS1	Ribonucleotide reductase M1	11.66	0.046
303824	IGF2BP2	similar to IGF-II mRNA-binding protein 2 (predicted)	6.26	0.039
24944	LAMP2	lysosomal membrane glycoprotein 2	5.71	0.048
361580	GTF2H1	General transcription factor II H, polypeptide 1 (Predicted), isoform CRA_b	5	0.049
360678	ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha	3.93	0.046
360602	LUC7L3	similar to cisplatin resistance-associated overexpressed protein (predicted)	3.49	0.015
308776	PDE8A	phosphodiesterase 8A	3.4	0.048
312703	PEX5	peroxisome biogenesis factor 5 (predicted), Peroxisomal targeting signal 1 receptor	3.3	0.036
689343	LOC689343	similar to Pyruvate kinase isozymes M1/M2 (Pyruvate kinase muscle isozyme)	3.22	0.006
361555	ANKRD27	similar to VPS9-ankyrin repeat-containing protein isoform 1	3.02	0.033
306417	SH3RF1	putative scaffolding protein POSH (Sh3md2)	3	0.043
25193	CCND3	cyclin D3	2.96	0.031
81663	GNA12	guanine nucleotide binding protein, alpha 12	2.94	0.049
25183	GDI1	guanosine diphosphate dissociation inhibitor 1	2.92	0.029
65134	STX5	syntaxin 5a	2.73	0.004
287876	ACTG1	actin, gamma, cytoplasmic 1	2.72	0.021
295647	GCA	similar to grancalcin	2.71	0.018
304914	RABGAP1L	similar to rab6 GTPase activating protein (GAP and centrosome-associated)	2.7	0.039
114859	EIF2AK4	eukaryotic translation initiation factor 2 alpha kinase 4 (predicted)	2.69	0.031
678774	LOC678774	similar to TBC1 domain family member 22A	2.66	0.032
117596	ATP6V1B2	ATPase, H transporting, lysosomal V1 subunit B2	2.65	0.03
300438	LDLR	low density lipoprotein receptor	2.63	0.032
30369	LIG3	ligase III, DNA, ATP-dependent	2.62	0.042
289350	RAB3GAP2	RAB3 GTPase activating protein subunit 2	2.59	0.025
293484	EIF3C	similar to eukaryotic translation initiation factor 3, subunit 8	2.58	0.049
361407	COG4	similar to component of oligomeric golgi complex 4	2.56	0.048
302661	OFD1	similar to oral-facial-digital syndrome 1 gene homolog	2.38	0.039
117060	UBR5	progestin induced protein (Dd5): similar to Ubiquitin-protein ligase EDD1 (Hyperplastic discs protein homolog)	2.33	0.035
365841	SLC25A44	similar to CG5805-PA	2.32	0.026
308703	LRRK1	leucine-rich repeat kinase 1 (predicted)	2.28	0.046
689226	LOC689226	similar to ubiquitin-conjugating enzyme E2R 2, transcript variant 1	2.23	0.047
140940	ARF3	ADP-ribosylation factor 3	2.17	0.049
679869	TCF7L2	similar to transcription factor 7-like 2 (T-cell specific, HMG-box) isoform 2	2.15	0.033
296461	OSBPL2	oxysterol binding protein-like 2	2.07	0.03
294429	RANBP2	similar to RAN binding protein 2	2.04	0.019
81745	PDPK1	3-phosphoinositide dependent protein kinase-1	2.03	0.037

[a]the ratio of normalized intensities between hypoxia and normoxia groups

which include DE genes INS2 and MLXIPL, GIP and SEPW1, TAL1 and INPP5D respectively. Anchored to membrane is the most significant hypoxia-induced CC ontology with enrichment score 3.709, which include ALPI, CAR4, DPEP3, AKP3 and DPEP1. Exopeptidase activity is the most significant hypoxia up-regulated MF ontology with enrichment score 2.883, which include ANPEP, DPEP3, FOLR4 and DPEP1. The information of up-regulated DE genes in rat C6 glioma cell by hypoxia was presented in Table 1. To further confirm the up-regulated genes, mRNA levels of two interested genes ANPEP and DLL3 were examined by quantitative real-time RT-PCR and similar results as microarray were achieved (Figure 2).

Down-regulated DE genes by hypoxia

Among those 67 down-regulated genes in rat C6 glioma cell by hypoxia, 46 genes were similar to some known genes, and the rest 21 genes were clearly identified to have certain function. 43, 41 and 51 down-regulated genes were identified to associate with BP, CC and MF ontology respectively. Among those down-regulated DE genes, 15 genes are involved in establishment and maintenance of cellular component location, 35 genes are associated with intracellular component, and 47 genes have binding function. Hypoxia significantly down-regulated genes involved in intracellular signalling pathway of BP ontology with enrichment score 3.030, including GNA12, ARF3, GDI1, LRRK1, ARHGDIA, EIF2AK4, SH3RF1, UBR5, TCF7L2, GCA and PDPK1. Intracellular membrane-bounded organelle is the most significant CC ontology affected by hypoxia with enrichment score 3.316 which include 29 genes such as STX5, LAMP2, LDLR, PEX5, and so on. Protein binding is the most significant MF ontology affected by hypoxia with enrichment score 4.395, which include 38 genes such as SH3RF1, ACTG1, CCND3, IGF2BP2 and so on. The information of down-regulated DE genes in rat C6 glioma cell by hypoxia was presented in Table 2. Quantitative real-time RT-PCR assay verified the down-regulated level of mRNA for the interested genes ACTG1 and PDPK1 (Figure2).

Pathway analysis

Base on the latest KEGG (Kyoto Encyclopedia of Genes and Genomes) database, we provided pathway analysis which allows to determine the biological pathway.

Table 3. Pathway Analysis for DE Genes Induced by Hypoxia in Rat C6 Glioma

Pathway.ID[a]	Definition[b]	P[c]	Count[d]	GENES[e]
				Up-regulated Degenes
rno04974	Protein digestion and absorption	0.002	4	KCNK5//SLC15A1//SLC7A7//SLC9A3
rno00790	Folate biosynthesis	0.002	2	AKP3//ALPI
rno04614	Renin-angiotensin system	0.008	2	ANPEP//CTSG
rno00340	Histidine metabolism	0.012	2	ALDH1B1//FTCD
rno04740	Olfactory transduction	0.016	13	OLR120//OLR1389//OLR1568//OLR1579//OLR1640//OLR172//OLR285//OLR299//OLR417//OLR67//OLR70//OLR727//OLR812
rno04330	Notch signaling pathway	0.043	2	DLL3//RBPJL
				Down-regulated DE genes rno05213
Endometrial cancer		0.012	2	PDPK1//TCF7L2
rno04145	Phagosome	0.022	3	ACTG1//ATP6V1B2//LAMP2
rno04510	Focal adhesion	0.023	3	ACTG1//CCND3//PDPK1
rno04520	Adherens junction	0.023	2	ACTG1//TCF7L2
rno05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.023	2	ACTG1//TCF7L2
rno05215	Prostate cancer	0.033	2	PDPK1//TCF7L2

[a] Pathway identifiers used in KEGG; [b] definition of the PathwayID; [c] the enrichment P-value of the PathwayID used Fisher's exact test. The P-value cut-off is 0.05; [d] the count of the DE genes' entities directly associated with the listed PathwayID; [e] the DE genes associated with the PathwayID

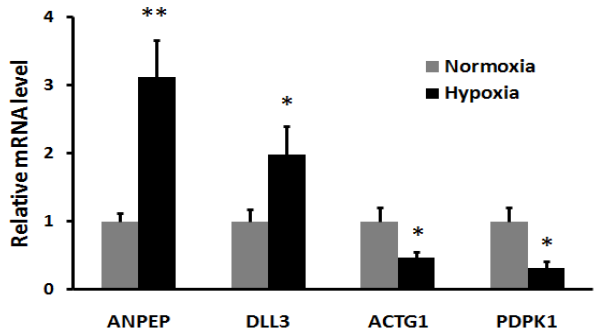

Figure 2. Quantitative Real-time RT-PCR Assay for ANPEP, DLL3, ACTG1 and PDPK1. The mRNA levels of ANPEP and DLL3 were up-regulated under hypoxia, while those of ACTG1 and PDPK1were down-regulated. * and ** indicate P<0.05 and P<0.01, respectively, in comparison with normoxia group

Among those up-regulated DE genes in rat C6 glioma cell, six biological pathways were identified, including protein digestion and absorption, folate biosynthesis, renin-angiotensin system, histidine metabolism, olfactory transduction and notch signaling pathway. Among those down-regulated DE genes in rat C6 glioma cell, six biological pathways, involved in endometrial cancer, phagosome, focal adhesion, adherens junction, arrhythmogenic right ventricular cardiomyopathy and prostate cancer, were identified. The DE genes associated with these pathways were presented in Table 3.

Discussion

Hypoxia, an existed pathophysiological feature in glioma, is one of the hot researching point because of its effect on tumorigenesis, angiogenesis, invasion, chemoresistance and radioresistance. However, the hypoxia-induced gene expression changes are not fully recognized up to now. In this study, we identified the DE genes between normoxia and hypoxia in rat C6 glioma cells by using cDNA microarray. To our knowledge this is the first time that cDNA microarray chip containing more than 25000 genes was used on the glioma cell line under hypoxic conditions. The cDNA microarray analysis revealed 180 hypoxia up-regulated genes such as ANPEP, CAR4, EOMES, NEUROD2, etc., and 67 hypoxia down-regulated genes such as IGF2BP2, LAMP2, ARHGDIA, and so on.

With realization of the important role of glioma stem cell during tumorigenesis, malignant progress and recurrence, factors that regulate glioma stem cell differentiation have been widely studied (Kolenda et al., 2011). In this study, we identified 28 hypoxia up-regulated genes and 4 hypoxia down-regulated genes that are associated with developmental process and cell differentiation. Of those genes, NEUROD2, DLL3, TLX3, HOXC10, GIP, OXTR, PCDHA10, RAB3GAP2 and PEX5 are involved in neurogenesis.

NEUROD2, neurogenic differentiation factor 2, is one of the basic helix-loop-helix (bHLH) transcriptional factors which play important roles in neuronal differentiation and survival. During the differentiation from a progenitor cell to a neuron, NEUROD2 and related bHLH transcription factors induce neuronal differentiation (Ravanpay et al.,2010). Dysregulated expression or loss of function of NEUROD family members contributes to transformation of specific primitive neuroectodermal tumor (Rostomily et al.,1997). Moreover, NEUROD2 was mainly found to be expressed in endovascular invasive cells which is similar to invasive cancer cells (Westerman et al., 2002). Here, we firstly demonstrated that NEUROD2 was induced by hypoxia in C6 glioma cells. Our finding implicated that up-regulation of NEUROD2 by hypoxia may be involved in glioma cell differentiation, proliferation or invasion.

DLL3, Delta-like 3, is a Delta family member of Notch ligands expressed broadly in developing nervous system (Henke et al.,2009). DLL3 can bind and activate Notch-1 or another Notch receptor. DLL3 expression was also detected in glioma tissues. Compared with their pediatric counterparts, the expression array data in adult grade II diffuse astrocytomas also revealed a significant difference in the expression of DLL3 and other genes

putatively involved in neural stem cell maintenance and CNS development, suggesting that their more aggressive behavior may be due to derivation from less differentiated cell type (Jones et al., 2011). PTEN and DLL3 expression were also suggested as a robust two-gene prognostic model in glioma, which implicated that Akt and Notch signaling are hallmarks of poor prognosis versus better prognosis gliomas, respectively (Phillips et al., 2006).

In this study, we observed that DLL3 gene was 2.29 folds up-regulated by hypoxia in C6 glioma cells in vitro and identified the significance of Notch pathway including DLL3 and RBPJL genes. The Notch signaling pathway is an evolutionarily conserved, intercellular signaling mechanism essential for proper embryonic development. Based on the importance of Notch pathways in GBM (Cooper et al., 2010; Li et al., 2011), the dependence of DLL3 expression and the activity of the DLL3-promoter in the dorsal neural tube on the bHLH transcription factors (Henke et al., 2009), and the direct transcriptional regulation of DLL3 gene by the RBPJL product predicted in some instances (González et al., 2010), we thereby postulate that DLL3 may be involved in the maintenance of glioma stem cell properties under hypoxia, and a number of genetic interactions of DLL3, NEUROD2 and RBPJL is possibly existed.

The formation of abnormal tumor vasculature and glioma cell invasion along white matter tracts are believed to be the major factors responsible for the resistance to treatment. Therefore, investigation of angiogenesis and invasion in glioblastoma is essential for the development of a curative therapy (Onishi et al., 2011). Although both angiogenesis-dependent and angiogenesis-independent invasion phenotypes coexist in malignant glioma, these factors simultaneously regulating angiogenesis and invasion are fascinated. In our microarray analysis results, significant changes of some genes promoting angiogenesis such as ANPEP, involving in tissue remodeling and tumor invasion such as COL10A1, PLG, MMP24 and LAMP2 were observed in rat C6 glioma cells under hypoxia. Among those genes, ANPEP is thought to contribute to both angiogenesis and tumor invasion.

ANPEP, also known as aminopeptidase N/CD13, is a broad specificity aminopeptidase which may be involved in the metabolism of regulatory peptides of diverse cell types (Potolicchio et al., 2005). It is highly expressed on myeloid cells and is a reliable marker of the myeloid lineage of normal and leukemic cells (Kern et al., 2006; Winnicka et al., 2010). ANPEP plays multiple roles in tumor invasion (Mawrin et al., 2010) and angiogenesis (Di Matteo et al., 2011). However, researches about the role of ANPEP for glioma angiogenesis and invasion are absent. To our knowledge, this is the first time that up-regulation of ANPEP gene was observed in glioma cells under hypoxia. We postulate that ANPEP may contribute to the regulation of tumorigenesis, angiogenesis and invasion of glioma under hypoxic condition.

Tumor cellular metabolic processes are different under hypoxia compared with that under normoxia. Cellular carbohydrate metabolic process is especially concerned because of the vigorous proliferation of tumor cell and the insufficient nutrition supply. Tumor cells rely more on glycolysis than on oxidative phosphorylation for glucose metabolism, and targeting metabolic pathways in tumor cells has become a topic of considerable interest (Vlashi et al., 2011). In this study, we also identified in C6 glioma cells 7 hypoxia up-regulated genes and 15 hypoxia down-regulated genes related with cellular metabolic processes. Among those genes, MLXIPL, INS2, LYG1,CCND3, TCF7L2 and LOC689343 are involved in cellular carbohydrate metabolic processes. MLXIPL, INS2 and LOC689343 regulate the glycolysis.

Even in the presence of oxygen, malignant cells often highly depend on glycolysis for energy generation, a phenomenon known as the Warburg effect. INS2 product increases cell permeability to monosaccharides and accelerates glycolysis. MLXIPL, a carbohydrate response element binding protein (ChREBP), is a glucose-responsive transcription factor that plays a critical role in the glucose-mediated induction of gene products involved in glycolysis and lipogenesis (Iizuka et al., 2008). Our results demonstrated that hypoxia induced upregulation of MLXIPL and INS2 with 2.98 and 2.15 folds respectively, which implicated that activated glycolysis may be required for efficient glioma cell proliferation under hypoxia.

LOC689343, similar to pyruvate kinase (PK) M1/M2, is a key kinase in the process of glycolysis. The isoforms PKM1 and PKM2 are alternatively spliced products of the PKM2 gene. PKM2 gene transcription is activated by HIF-1, interacts directly with HIF-1α subunit, then promotes transactivation of HIF-1 target genes by enhancing HIF-1 binding and p300 recruitment to hypoxia response elements (Luo et al., 2011). The tetramer/ dimer ratio of PKM2 determines whether glucose carbons are degraded to lactate with production of energy (tetrameric form) or are channeled into synthetic processes (dimeric form) (Kumar et al., 2010). In tumor cells, abnormal expression of PKM2 protein is observed. The metastatic pancreatic cancer cells contained about fourfold more PKM2 protein and about 3.5-fold more dimeric PKM2 than the non-metastatic cells (Kumar et al., 2010). By microarray analysis, we found that LOC689343 was down-regulated more than 3 fold in rat C6 glioma cells under hypoxia. This is not coincidence with other research. Shimizu et al. (2004) reported that PK was up-regulated by hypoxia in human astrocytoma cells. Reason of this difference is not clear. Because the PKM2 change was different in different cell types under the same condition, and the activation of PKM2 is determined by the tetramer/dimer ratio which is affected by tumor microenvironment conditions (Kumar et al., 2010), the PKM2 subtype should be further studied in C6 glioma cells under hypoxia in order to definite its functional change although its mRNA level decreased.

In summary, we identified the up-regulated and down-regulated genes that are more than twofold differentially expressed by comparing a set of normoxic and hypoxic samples through cDNA microarray analysis in rat C6 glioma cell line. GO analysis and Pathway analysis revealed that these genes are involved in cell differentiation and adhesion, metabolic process, immune and stress response, signal transduction, and so on, which implicated the complicated effect of hypoxia on glioma cell. Whether these differentially expressed genes could

be found in other cell lines under the same conditions, and the related function or role of these genes in glioma malignant progress should be clarified for future studies.

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