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Expression analysis of genes involved in brain tumor progression driven by retroviral insertional mutagenesis in mice

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Retroviral tagging previously identified putative cancer-causing genes in a mouse brain tumor model where a recombinant Moloney murine leukemia virus encoding the platelet-derived growth factor B-chain (MMLV/PDGFB) was intracerebrally injected in newborn mice. In the present study, expression analysis using cDNA arrays revealed several similarities of virus-induced mouse gliomas with human brain tumors. Brain tumors with short latency contained on average 8.0 retroviral insertions and resembled human glioblastoma multiforme (GBM) whereas long-latency gliomas were of lower grade, similar to human oligodendroglioma (OD) and had 2.3 insertions per tumor. Several known and novel genes of tumor progression or cell markers were differentially expressed between OD- and GBM-like tumors. Array and quantitative real-time PCR analysis demonstrated elevated expression similar to *Pdgfrα* of retrovirally tagged genes *Abhd2*, *Ddr1*, *Fos*, *Ng2*, *Ppfibp1*, *Rad51b* and *Sulf2* in both glioma types compared to neonatal and adult normal brain. The retrovirally tagged genes *Plekhb1*, *Prex1*, *Prkg2*, *Sox10* and *1200004M23Rik* were upregulated in the tumors but had a different expression profile than *Pdgfrα* whereas *Rap1gap*, *Gli1*, *Neurl* and *Camk2b* were downregulated in the tumors. The present study accentuates the proposed role of the retrovirally tagged genes in PDGF-driven gliomagenesis and indicates that insertional mutagenesis can promote glioma progression.
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Introduction

Glioblastoma multiforme (GBM) is the most common solid brain tumor among humans. It either develops *de novo* (primary GBM) or by progression from a lower grade glioma (secondary GBM). Early alterations in secondary glioblastomas include overexpression of platelet-derived growth factor (PDGF) ligands and receptors that cause an autocrine growth stimulation (Fujimoto *et al*., 1988; Nister *et al*., 1988; Hermanson *et al*., 1992; Westermark *et al*., 1995). In order to further elucidate the role of PDGF in gliomagenesis, we have generated a mouse glioma model in which recombinant Moloney murine leukemia virus encoding the platelet-derived growth factor B-chain (MMLV/PDGFB) together with replication competent MMLV helper virus is injected intracerebrally in newborn mice (Uhrbom *et al*., 1998). The model is based on the idea that tumors evolve through a combination of autocrine growth stimulation and retroviral insertional mutagenesis of cellular genes. The majority of the brain tumors resemble human glioblastomas, and also other types of brain tumors similar to primitive neuroectodermal brain tumors (PNET) and oligodendrogliomas are found. When wild-type mice were infected with PDGFB virus at P0 (postnatal day 0), the latency period was highly variable and ranged from 13 to 42 weeks after injection. In a recent study, we collected all gliomas generated, cloned and sequenced the integration sites to identify candidate genes that cooperate with PDGF in the development of glioma (Johansson *et al*., 2004). This genetic screen yielded 66 candidate brain tumor loci (Btl). Some of these harbor genes with an established role in oncogenesis whereas others have not previously been implicated in neoplastic transformation or PDGF signaling.

The main objective of the present investigation was to identify markers for PDGF-induced gliomagenesis and tumor progression in PDGF-induced glioma using expression profiling. Specifically, we used 15 K cDNA arrays to find differentially expressed genes between MMLV/PDGFB mouse gliomas and normal adult brain. We also searched for genes differentially expressed between early (high-grade) tumors and long-latency, possibly slowly growing (low-grade) tumors. In addition, we analysed the expression profile of most of the previously identified candidate glioma genes (Johansson *et al*., 2004). Several of the differentially expressed genes have previously been implicated in brain tumor progression. Evidence is presented that both high- and low- grade tumors originate from cells of the oligodendrocyte lineage, possibly oligodendrocyte progenitors. Several of the previously reported candidate cancer-causing genes were differentially expressed in the brain tumors, a finding that further strengthens their possible involvement in gliomagenesis.

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Results

Brain tumors with long latency were of lower grade and had fewer retroviral integrations

From a material of 69 brain tumors that developed in virus-injected wild-type mice, nine tumors presented after a lag period of 30 weeks or more. Eight of these resembled well differentiated but malignant (WHO grade II) oligodendroglioma (OD-like) (Figure 1). In contrast, excluding five early PNET-like tumors located in the cerebellum (including tumor 11, depicted in Figure 1c in Uhrbom *et al*., 1998), nine mice that presented with tumors before 18 weeks were afflicted by GBM-like malignancies (Figure 1). The remaining 46 tumors that occurred after intermediate latency periods were most often similar to GBM. The number of retroviral insertions previously identified by inverse PCR amplification of genomic DNA (Johansson *et al*., 2004) were on average 2.3 per tumor for OD-like and 8.0 for GBM-like tumors (significant differences with paired *t*-test, $P < 0.05$) (data not previously published). Moreover, the corresponding number of insertions in a locus designated as a Btl were 0.5 and 2.4, respectively.

Analysis of gene expression in mouse glioma vs normal brain using cDNA arrays

Microarray analysis of RNA expression was performed following the experimental outline depicted in Figure 2. An RNA pool from 10 cerebral tumors, five OD-like and five GBM-like, was prepared. In addition, separate RNA pools were prepared from three late OD-like tumors and three early GBM-like tumors. A pool from cerebral tissue of six non-neoplastic adult mouse brains infected with MMLV alone served as a control. In the experimental setup, referred to as the Pooled experiment (Figure 2), tumor pools were analysed against the control. This procedure is used to find the genes that are most likely to be differentially expressed in virus-induced brain tumors (regardless of grade) compared to normal brain tissue. The findings are presented in Supplementary Material 1. A consistent finding was a strong overexpression of *Pdgfrα*. Transcript levels of several other cell-cycle-related genes were also increased, for example *Cdk4*, *Ccnd1* and *Ki67*. Six of the 26 Btl genes that were spotted on the array were present among the top 85 differentially expressed genes. These genes (presented in bold text in Supplementary Material 1) were *Eef1a1* (elongation factor Tu) *Ppfibp1* (PTPRF interacting binding protein 1), *Fos* (FBJ osteosarcoma oncogene), *1200004M23Rik*, *Sulf2* (sulfatase 2) and *Sdc3* (syndecan 3).

Analysis of gene expression in early vs late glioma using cDNA arrays

In the next experimental setup, termed the Individual experiment (Figure 2), we analysed four early GBM-like and four late OD-like tumors by direct comparison in order to identify genes that may be related to tumor progression. As expected, fewer genes were differentially expressed in comparison with the previous experiment (Supplementary Material 1), since these two populations

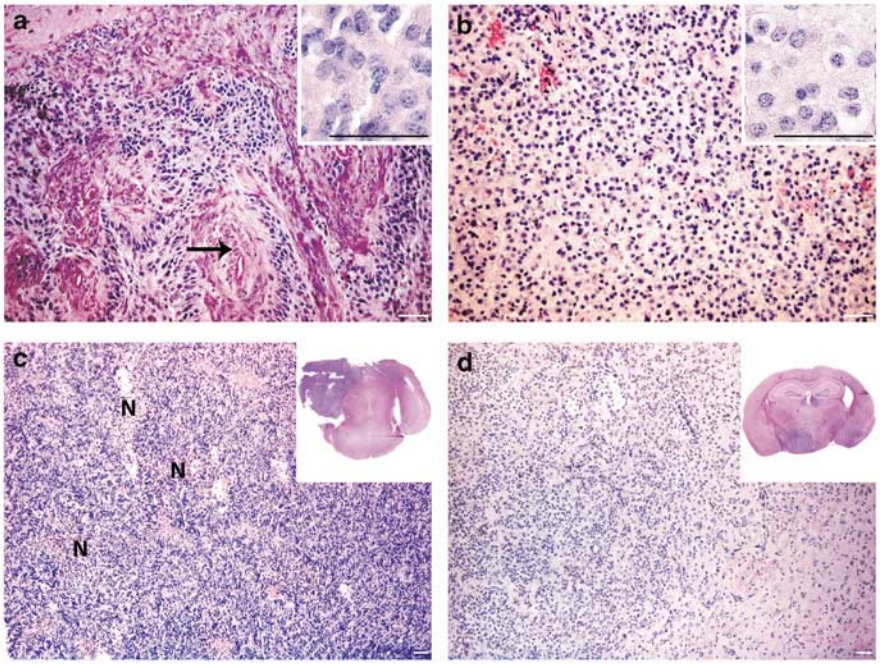

Figure 1 Photomicrographs of representative H&E-stained sections of MMLV/PDGF-induced mouse gliomas. Sections of two malignant GBM-like tumors (**a, c**) from mice killed between 14 and 18 weeks of age. Features like pseudopalisadation, microvascular proliferation (indicated by arrow) and necrosis (indicated by N) were commonly found. Sections of two less mitotic tumors resembling oligodendrogliomas (**b, d**) from mice killed after 30 weeks of age showing rounded homogenous tumor cells often surrounded by clear cytoplasm. Higher and lower magnifications of each tumor are presented within respective pictures. Sizebar in all pictures: 100 μm

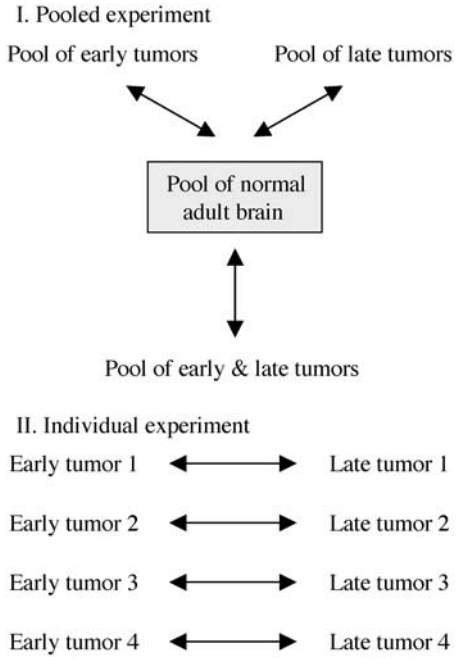

Figure 2 Experimental outline of the array experiments. (I) Pooled experiment. Pools of late and early, only early or only late tumors were, respectively, compared to normal adult brain. Equal amounts of total RNA were used for cDNA synthesis and hybridized (labeled with Cy3 or Cy5 with dye swaps) on the arrays. Differentially expressed genes in these tumors are presented in Supplementary Material 1. (II) Individual experiment. A direct comparison was made between one early and one late (randomly picked) tumor for each array. A total of four tumors in each group were compared (with dye swaps). Genes differentially expressed in these experiments are presented in Table 1

are more similar than tumor vs normal tissue. The expression profiles of the differentially expressed genes were further investigated using the results from the Pooled experiment described above in order to obtain more information about the relative expression between early and late tumors. For example, among the down-regulated genes, the expression of *Plp* (proteolipid protein) was highly reduced in the pool of early tumors and weakly reduced in the pool of late tumors compared to normal adult brain. *Lrp1* (low-density lipoprotein receptor-related protein 1), *Sparc* (secreted protein acidic and rich in cysteine) and *Ctsd* (cathepsin D) were increased in both tumor types compared to normal brain but more in the late tumor pool. Interestingly, while *Dnah5* (dynein heavy chain 5) was specifically induced, genes like *Cst3* (Cystatin C), *Gja1* (gap junction protein alpha 1/connexin 43), *Gstm1* (gluthatione S-transferase, M1), *Pink* (PTEN-induced putative kinase) and *Aldo3* (aldolase c/zebrin II) were downregulated in early tumors only. Moreover, a few genes were found to be upregulated in the late tumors and downregulated in the early tumors. The most distinct differences were found for *Cryab* (crystallin B, alpha), *Gnas* (guanine nucleotide binding protein, alpha stimulating), *Itm2b* (integral membrane protein 2, beta), *Lpd* (lipidosis-related protein lipidosin), *Sepp1* (selenoprotein P), *Mmd2,* (monocyte to macrophage differentiation associated 2) and *Fth* (ferritin heavy chain).

Quantitative real-time PCR validation

The group of retrovirally tagged genes that were found to be differentially expressed in the Pooled experiment was validated with quantitative real-time PCR (qrt-PCR). These experiments were performed on two separate RNA pools of the same five oligodendrogliomas and the five glioblastomas that were studied in the first array experiment. As a control, non-neoplastic adult brain was used. In addition, we included a pool of RNA from cerebral tissue of newborn wild-type mice. A total of 37 of the other tagged genes belonging to a Btl (Johansson *et al*., 2004) (including several that were not spotted onto the arrays) were also studied. The results from the qrt-PCR are presented as relative expression compared to adult brain and normalized to expression of the housekeeping gene *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) (Table 2). Seven of the Btl genes displayed the same expression pattern as *Pdgfrα*, that is, moderately induced in newborn brain and highly induced in both pools of mouse brain tumors relative to the expression in normal adult brain. Btl genes following this pattern were *Abhd2* (alfa/beta hydrolase domain containing 2), *Cspg4*/*Ng2* (chondroitin sulphate proteoglycan 4/neuron-glial 2), *Ddr1* (discoidin domain receptor family, member 1), *Fos*, *Ppfibp1*, *Rad51b* (DNA repair protein RAD51 homolog 2) and *Sulf2*. Five Btl genes, *Plekhb1* (pleckstrin homology domain containing, family B member 1), the mouse ortholog of *PREX1* (phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 protein), *Prkg2* (cGMP-dependent protein kinase II), *Sox10* (SRY-box containing gene 10) and *1200004M23Rik* had an equal or lower expressed in newborn brain compared to adult brain but were elevated in both tumor pools. Moreover, *Camk2b* (calcium/calmodulin dependent protein kinase II beta) and *Gli1* (GLI-Kruppel family member GLI) were strongly downregulated in the tumors compared to adult or newborn brain whereas *Rap1gap* (Rap1 GTPase-activating protein) showed reduced expression in the tumors only when compared to adult brain (Table 2). In order to validate the results from the Individual experiment, the expression of nine genes from Table 1 were analyzed with qrt-PCR using the same tumor pools and controls as described above (Table 2).

Immunohistochemical staining of cell-specific markers for the oligodendrocyte lineage

The high expression of *Sox10* mRNA (Table 2) and protein (Figure 3) in early as well as in late tumors suggests that the tumors are derived from cells of the oligodendrocyte lineage, since Sox10 is a marker for immature and differentiating oligodendrocytes in the CNS (Kuhlbrodt *et al*., 1998). Both types of tumors were also found to express *Ng2* mRNA (Table 2) and protein (Figure 3), which is another marker for oligodendrocyte precursors. These findings prompted us to analyse the expression of other oligodendrocyte lineage markers, viz. *Cgt*, *Olig2* and *Plp* (Figure 3).

Table 1 Genes differentially expressed in early vs late mouse brain tumors (Individual experiment), listed by category

Accession no. Genbank/Refseq	*Gene symbol*	*Gene description*	*Mean M (log_2) early/late fold change*	*Expression in early tumor vs normal adult brain*	*Expression in late tumor vs normal adult brain*
Cell cycle and growth regulators					
D12513	*top2a*	Topoisomerase (DNA) II alpha	1.51	+ + +	+ + +
X15666	*Rrm2*	Ribonucleotide reductase M2	1.33	+ + +	+
U02025	*Igfbp5*	Insulin-like growth factor binding protein 5	−2.04	ND	ND
U10120	*Nsf*	*N*-ethylmaleimide sensitive fusion protein	−1.77	− − −	−
U60593	*Ndrg1*	*N*-myc downstream regulated 1	−1.55	− − −	− −
AB033921	*Ndrg2*	*N*-myc downstream regulated 2	−1.43	− −	−
X76066	*Igfbp4*	Insulin-like growth factor binding protein 4	−1.30	NC	+
D76440	*Ndn*	Necdin	−1.22	− −	−
Transcription					
J00370	*Fos*	FBJ osteosarcoma oncogene	1.29	+ +	+
NM_144919	*Hdac11*	Histone deacetylase 11	−1.88	− − −	− −
AK009518	*Tef*	Thyrotroph embryonic factor	−1.51	− −	−
NM_145473	*AI481750*	RNA-binding protein pippin	−1.22	− −	−
Receptors and signal transduction					
AF212321	*Racgap1*	Rac GTPase-activating protein 1	1.20	+ +	+
M61896	*Gja1*	Gap junction protein alpha 1 (connexin 43)	−2.22	− −	NC
AF316872	*Pink*	PTEN-induced putative kinase	−2.00	− −	NC
AK007182	*Chn1*	Chimerin 1	−1.67	− − −	− −
NM_023168	*Grina*	Glutamate receptor, ionotropic, NMDA associated protein 1	−1.44	− −	−
NM_009790	*Calm1*	Calmodulin 1	−1.43	− − −	− −
AK003400	*Drd1ip*	Dopamine receptor D1 interacting protein, Calcyon	−1.31	− − −	− −
AF189817	*Plekhb2*	Pleckstrin homology domain containing family B (evectin) membrane 2	−1.29	− −	−
NM_007874	*Dp1*	Deleted in polyposis 1	−1.25	− −	−
X67469	*Lrp1*	Low density lipoprotein receptor related protein 1	−1.20	+	+ +
AK078311	*Camk2g*	Calcium/calmodulin dependent protein kinase II gamma	−1.20	− −	−
NM_175217	*Mmd2*[a]	Monocyte to macrophage differentiation associated 2	−1.05	−	+
AY519501	*Gnas*[a]	Guanine nucleotide binding protein, alpha stimulating (Gs alpha protein)	−1.02	−	+
Apoptosis					
U76253	*Itm2b*	Integral membrane protein 2B	−1.44	−	+
Stress or inflammatory response					
M73741	*Cryab*	Alpha B-crystallin	−2.04	−	+
L05670	*Clu*	Clusterin	−1.81	− −	NC
AK051454	*Pla2g7*	Phospholipase A2 group VII	−1.30	−	NC
Protein biosynthesis/degradation					
AY043479	*B3Gnt1*	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 1	2.59	+ +	+
AF229257	*Usp29*	Ubiquitin specific protease 29	1.96	+ +	+
ECM or ECM associated					
U77630	*Adm*	Adrenomedullin	1.25	+ +	+
X99807	*Sepp1*	Selenoprotein P, plasma, 1	−1.86	−	+
X53929	*Dcn*	Decorin	−1.22	NC	+
Cytoskeleton and cytoskeletal organization					
AF466704	*Dnah5*	Dynein heavy chain 5	2.64	+ +	NC
AF093542	*Tacc3*	Transforming acidic coiled-coil protein 3	1.26	+ + +	+
AB017026	*Osbpl1a*	Oxysterol binding protein-like 1A	−1.34	− − −	− −
Cell adhesion, motility or invasion					
U77330	*Sparcl1*	SPARC-like 1 (mast9, hevin)	−2.40	− −	NC
M59470	*Cst3*	Cystatin C	−1.96	− −	NC
X52886	*Ctsd*	Cathepsin D	−1.90	+ +	+ + +
J289016	*Clstn1*	Calsyntenin 1	−1.65	− − −	− −
X04017	*Sparc*	Secreted acidic cysteine rich glycoprotein (osteonectin)	−1.49	NC	+ +
Cell differentiation					
M15442	*Plp*	Proteolipid protein (myelin)	−2.70	− − −	−

Table 1 (*continued*)

Accession no. Genbank/Refseq	*Gene symbol*	*Gene description*	*Mean M (log_2) early/late fold change*	*Expression in early tumor vs normal adult brain*	*Expression in late tumor vs normal adult brain*
Glucose or lipid metabolism					
AK039267	*Aldo3/ ZebrinII*	Aldolase 3, C isoform	−2.02	− −	NC
NM_153781	*Pygb*	Brain glycogen phosphorylase	−1.78	− − −	−
AF249894	*Pfkm*	Phosphofructokinase	−1.72	− −	−
X51905	*Ldh2*	Lactate dehydrogenase 2, B chain	−1.45	− −	−
AB049821	*Bach*	Brain acyl-CoA hydrolase	−1.23	− − −	− −
AB050554	*Lpd*[a]	Lipidosis-related protein lipidosin (bubblegum)	−1.13	−	+
Cell homeostasis/ion transport					
X16646	*Atp1b1*	ATPase, Na+/K+ transporting, beta 1 polypeptide	−1.75	− − −	− −
M60170	*Fth*	Ferritin heavy chain	−1.69	−	+
AJ223584	*Atp2a2*	ATPase, Ca++ transporting	−1.25	− − −	− −
Other					
NM_011986	*Ncdn*	Neurochondrin	−2.27	− − −	− −
J04632	*Gstm1*	Glutathione S-transferase, mu 1	−2.08	− − −	NC
NM_024236	*Qdpr*	Quininoid dihydropteridine reductase	−1.47	− −	−
NM_134017	*Mat2b*[a]	Methionine adenosyltransferase II, beta	−1.03	− −	−
Unknown					
AK013267	*2810439F0-2Rik*	2810439F02Rik	1.61	++	+
AK078094	*D7Ertd715e*	DNA segment, Chr 7, ERATO Doi 715,	1.54	++	+
NM_178874	*1110063G1-1Rik*	1110063G11Rik	−1.58	− − −	− −
AF412297	*Ghitm*	Growth hormone inducible transmembrane protein	−1.56	− −	−

The top 60 genes ranked with the parametric empirical Bayes method were sorted after positive and negative fold change in protein function categories. M: mean log_2 fold change value. Reporters with M-values between −1.2 and 1.2 have been excluded. [a]Included since gene is present in Supplementary Material 1 or upregulated in late and downregulated in early tumors (despite exclusive expression levels). ND: not determined; NC: no or small change in average expression ($-0.5 < M < 0.5$); +: upregulated ($M > 0.5$); ++: highly upregulated ($M > 1.5$); +++: most highly upregulated ($M > 2.5$); −: downregulated ($M < -0.5$); − −: highly downregulated ($M < -1.5$); − − −: most highly downregulated ($M < -2.5$)

Olig2 was strongly upregulated in both early and late tumors whereas *Cgt* and *Plp* were upregulated in both tumor types but only in comparison to newborn brain.

Discussion

We have analysed the expression profile of mouse brain tumors induced by a PDGF-encoding retrovirus. We selected an array consisting of 15 247 unique cDNAs derived from embryonic libraries (Kargul *et al*., 2001) comprising many known and novel genes involved in growth and development. The experimental protocol allowed us to reveal genes that were differentially expressed in tumor tissue vs normal brain as well as those differentially expressed in short latency (high-grade) vs long latency (low-grade) tumors. Several of the differentially expressed genes in the Pooled experiment (tumor vs normal tissue) (Table 1) have previously been shown to be overexpressed in array studies of human brain tumor resections (Huang *et al*., 2000; Sallinen *et al*., 2000; Rickman *et al*., 2001; van den Boom *et al*., 2003). In addition to *Pdgfrα* and the cell cycle regulator genes (*Cdk4*, *Ccnd1*, and *Ki67*), *Top2a* (topoisomerase II alpha) and *Pcna* (proliferating cell nuclear antigen) were highly expressed; these have previously been found to correlate with malignancy in human astrocytomas (Lafuente *et al*., 2000; van den Boom *et al*., 2003). Genes known to be involved in glioma invasion, such as *Fn1* (fibronectin) and *Lgals1* (galectin-1), were also upregulated (Rajaraman *et al*., 1978; Ohnishi *et al*., 1998; Rorive *et al*., 2001; Yamaoka *et al*., 2000). Since normal adult brain was used as a control, the reduction in expression of neuronal markers was an expected finding. Similarly, *Prkcz* (protein kinase C, zeta), a negative regulator of Akt (Doornbos *et al*., 1999), was downregulated as well as *Tyro3* (protein tyrosine kinase 3) and *Fgfr2* (fibroblast growth factor receptor II), the two latter genes, are found to be downregulated or lost in human gliomas (Yamaguchi *et al*., 1994; Huang *et al*., 2000).

A number of genes that were differerentially expressed in early vs late tumors (Table 1) are known to regulate glioma progression. Sparc is known to both promote glioma invasion and delay tumor growth *in vivo* (Schultz *et al*., 2002). Human GBM cells transfected with cystatin C are found to lose their invasive growth and form small (if any) tumors when intracerebrally injected in athymic mice (Konduri *et al*., 2002). Ectopic expression of decorin has been shown to modulate brain tumor progression and increase survival of

Table 2 Relative expression of differentially expressed genes/Btl genes from qrt-PCR analysis

Gene	*Btl*[a]	*Neonatal brain*	*Late tumor*	*Early tumor*
Upregulated (Pdgfrα profile)				
Pdgfrα	[d]	**5.78** (4.83–6.92)	**42.22** (40.39–44.13)	**47.29** (41.28–54.17)
Fos	Btl-59[d,e]	**3.51** (2.71–4.55)	**20.02** (19.02–21.07)	**31.63** (27.42–36.48)
Cspg4/Ng2	Btl-49	**2.33** (2.13–2.55)	**27.22** (24.54–30.20)	**29.58** (28.00–31.24)
Ppfibp1	Btl-43[d]	**2.54** (2.09–3.09)	**12.73** (11.48–14.11)	**15.42** (13.12–18.12)
Sulf2	Btl-13[d]	**3.16** (2.68–3.72)	**11.42** (9.73–13.40)	**14.86** (12.93–17.07)
Rad51b	Btl-58	**4.36** (4.10–4.63)	**7.01** (6.34–7.75)	**11.08** (10.06–12.21)
Pdgfb/PDGFB[b]	—	**1.61** (1.37–1.89)	**6.22** (4.52–8.57)	**7.82** (6.30–9.70)
Abhd2	Btl-09	**1.78** (1.69–1.88)	**3.39** (2.89–3.98)	**5.78** (5.38–6.21)
Ddr1	Btl-25	**1.68** (1.64–1.72)	**2.71** (2.63–2.79)	**2.82** (2.71–2.93)
Upregulated (tumor specific)				
Sox10	Btl-08b	**0.69** (0.62–0.81)	**13.86** (12.77–15.04)	**14.62** (13.93–15.34)
1200004M23Rik	Btl-10[d]	**1.08** (0.85–1.31)	**8.28** (6.92–9.91)	**8.48** (6.37–11.29)
Plekhb1	Btl-07	**0.25** (0.22–0.28)	**6.65** (5.91–7.52)	**5.23** (4.89–5.59)
PREX1 mo	Btl-32	**1.17** (1.02–1.35)	**2.83** (2.46–3.26)	**2.80** (2.62–3.00)
Prkg2	Btl-02	**0.66** (0.61–0.71)	**3.00** (2.70–3.33)	**2.75** (2.64–2.87)
Downregulated (in both tumor types)				
Camk2b	Btl-53	**0.95** (0.89–1.02)	**0.48** (0.45–0.51)	**0.17** (0.16–0.18)
Neurl	Btl-20	**0.57** (0.52–0.63)	**0.72** (0.70–0.74)	**0.38** (0.35–0.41)
Gli	Btl-52c	**2.68** (2.46–2.92)	**0.41** (0.38–0.45)	**0.44** (0.43–0.45)
Rap1gap	Btl-12	**0.68** (0.65–0.71)	**0.90** (0.83–0.97)	**0.62** (0.57–0.68)
Other profiles				
Sdc3	Btl-26[d]	**16.72** (13.48–20.73)	**12.07** (11.03–13.21)	**9.43** (8.49–10.48)
Rhbg	Btl-33	**12.38** (11.68–13.12)	**4.41** (3.64–5.34)	**5.33** (4.96–5.73)
Trp53	Btl-01	**2.93** (2.60–3.30)	**2.98** (2.55–3.48)	**3.86** (3.64–4.09)
Eef1a1	Btl-50[d]	**3.99** (3.46–4.60)	**2.86** (2.59–3.16)	**3.57** (3.45–3.69)
Scarb1	Btl-40a	**3.07** (2.77–3.52)	**2.32** (1.96–2.75)	**3.07** (2.90–3.25)
Mars	Btl-52b	**1.89** (1.74–2.06)	**1.62** (1.57–1.67)	**2.47** (2.41–2.53)
Tax1bp2	Btl-47	**2.84** (2.28–3.54)	**2.42** (1.87–3.13)	**2.23** (1.85–2.69)
XM_130476[c]	Btl-31	**1.29** (1.26–1.32)	**1.48** (1.41–1.65)	**2.07** (1.95–2.20)
Rhcg	Btl-44	**5.63** (4.72–6.71)	**1.16** (1.06–1.27)	**1.87** (1.70–2.06)
Rab5c	Btl-55	**2.37** (2.13–2.64)	**1.95** (1.62–2.35)	**1.85** (1.64–2.09)
Map2k5	Btl-21	**1.22** (1.09–1.37)	**1.03** (0.98–1.10)	**1.69** (1.48–1.92)
Sppl2b	Btl-51	**3.02** (2.80–3.26)	**1.82** (1.70–1.95)	**1.64** (1.55–1.74)
Nfix	Btl-18	**4.56** (3.66–5.67)	**2.31** (2.22–2.41)	**1.54** (1.39–1.71)
Pea15	Btl-29	**1.56** (1.43–1.71)	**1.69** (1.43–2.00)	**1.48** (1.37–1.60)
2400010G15Rik	Btl-24	**1.31** (1.28–1.34)	**1.31** (1.22–1.41)	**1.48** (1.46–1.50)
Fmod	Btl-03a	**3.21** (2.56–4.03)	**1.26** (1.16–1.37)	**0.37** (0.32–0.43)
Fancc	Btl-60	**1.70** (1.63–1.77)	**1.38** (1.33–1.44)	**0.58** (0.54–0.63)
KIAA1337 mo	Btl-19	**1.24** (1.19–1.30)	**1.58** (1.38–1.81)	**0.91** (0.87–0.95)
JAZF1 mo	Btl-23	**6.88** (6.12–7.73)	**0.99** (0.96–1.02)	**0.92** (0.88–0.96)
Neurexin2	Btl-11	**2.38** (2.20–2.57)	**1.31** (1.18–1.46)	**1.16** (1.11–1.22)
P190RhoGAP	Btl-04	**2.73** (2.54–2.93)	**1.25** (1.16–1.34)	**1.14** (1.10–1.18)
Prkcabp1	Btl-08a	**1.82** (1.59–2.08)	**0.99** (0.88–1.12)	**0.99** (0.91–1.08)
Prkcbp1	Btl-05	**1.82** (1.64–2.02)	**0.92** (0.74–1.14)	**1.07** (0.93–1.23)
Slc30a5	Btl-62	**1.48** (1.25–1.75)	**1.25** (1.15–1.36)	**1.26** (1.18–1.34)
Sox5	Btl-27	**2.05** (1.85–2.27)	**1.24** (0.90–1.61)	**1.00** (0.95–1.05)
Sppl3	Btl-39	**2.34** (2.14–2.55)	**1.04** (0.96–1.12)	**1.01** (0.90–1.13)
Triad3	Btl-41	**1.67** (1.48–1.88)	**1.40** (1.24–1.58)	**1.32** (1.26–1.38)
Differentially expressed genes from array experiment				
Sparc	[e]	**1.02** (0.81–1.32)	**2.79** (2.65–2.94)	**1.42** (1.21–1.66)
Igfbp4	[e]	**1.15** (1.01–1.31)	**1.73** (1.66–2.05)	**0.84** (0.70–1.00)
Chn1	[d,e]	**0.18** (0.14-0.22)	**0.50** (0.43–0.58)	**0.18** (0.17–0.19)
Gja1	[e]	**1.27** (1.06–1.52)	**1.14** (1.04–1.25)	**0.41** (0.39–0.43)
Pink1	[e]	**0.49** (0.41–0.58)	**0.95** (0.86–1.05)	**0.49** (0.44–0.54)
Cst3	[e]	**0.32** (0.28–0.40)	**1.15** (1.01–1.30)	**0.54** (0.51–0.58)
Fth	[e]	**0.50** (0.44–0.56)	**1.54** (1.36–1.75)	**0.62** (0.57–0.67)
Sepp1	[e]	**1.27** (1.01–1.60)	**1.63** (1.52–1.75)	**0.57** (0.51–0.66)
Aldo3	[e]	**0.26** (0.23–0.29)	**1.27** (1.03–1.57)	**0.63** (0.59–0.67)

Expression is presented in absolute values realtive to adult brain (set to 1.0) and normalized to Gapdh expression. Within parentheses are the minor and major values when standard deviations from triplicate measurement are withdrawn or added; [a]Which brain tumor loci (Btl) number the gene belongs to; [b]Expression of human PDGFB in the brain tumors is relative to expression of mouse Pdgfb in the normal adult and neonatal brain; thus only relative levels between adult and neonatal or late and early are valid; [c]Reference sequence accession number of the gene, National Center for Biotechnology Information (NCBI). [d]Among top 85 genes in pecled experiment (Supplementary Material 1); [e]Among top 60 genes in individual experiment (Table 1); mo: mouse ortholog

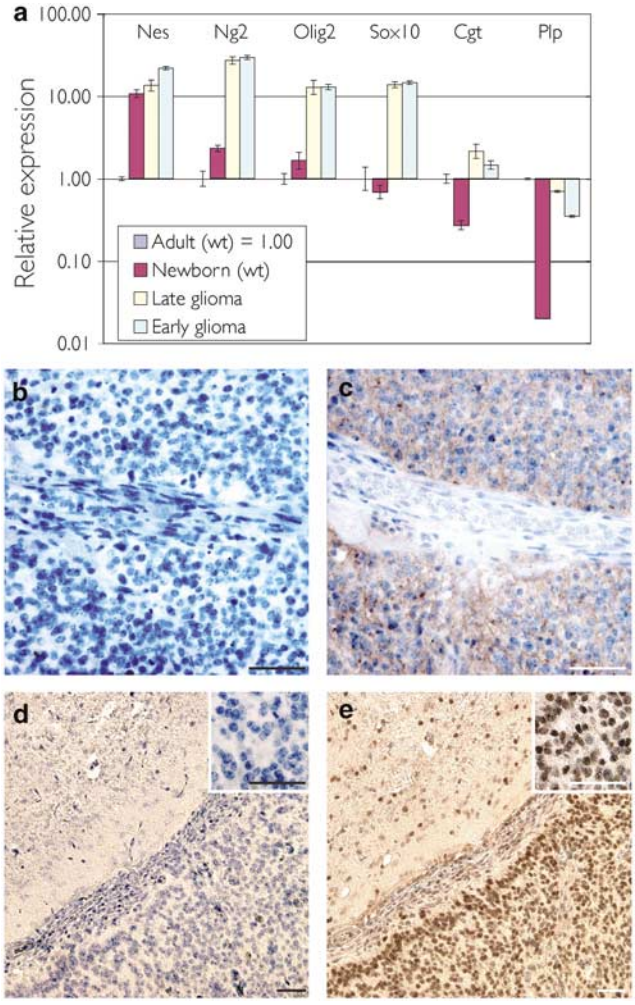

Figure 3 (**a**) Results from expression analysis using qrt-PCR. Absolute values of the relative expression (log scale) of the markers *Nestin*, *Ng2*, *Olig2*, *Sox10*, *Cgt*, and *Plp* in newborn brain (red) and in tumors from mice killed late (yellow) or early (light blue) compared to expression in adult brain (set to 1.0). Values are normalized to *Gapdh* expression for quantitative measurements. Error bars indicate standard deviation of triplicate measurements. (**c**) Section of a mouse brain tumor with Ng2-positive tumors cells surrounding a blood vessel (**e**) Positive immunostaining of Sox10 in nucleus of glioma cells (to the right) and normal oligodendrocytes (to the left). All sections were counterstained with hematoxylin. Negative controls of Ng2 (**b**) and Sox10 (**d**) were stained with hematoxylin and secondary antibody only. Sizebar in all pictures: 100 μm

glioma-bearing rats (Biglari *et al*., 2004). Similarly, *Ndrg2* (N-myc downstream regulated 2) has been reported to inhibit GBM cell proliferation (Deng *et al*., 2003). Here, we found that the expression of both *Ndrg1* and *Ndrg2* was decreased in both tumor types, but less so in the oligodendrogliomas. From a large-scale gene expression screen of human gliomas for histology-independent classification in four different prognostic groups, genes homologous to *Ndrg2* and *AI481790* (RNA-binding protein Pippin) from Table 1 had highest expression in the group with best survival. Similarly, *Top2a*, *Rrm2* (ribonucleotide reductase M2), *Tacc3* (transforming acidic coiled-coil protein 3) and *Adm* (adrenomedullin) were upregulated and correlated with poor prognosis (Freije *et al*., 2004). By inference, one may assume that other genes from Table 1 could act as potential regulators of progression of brain tumors.

Markers for early neural or oligodendroglial progenitor cells like nestin or Ng2 were expressed in early tumors suggesting that these tumor cells are derived from immature progenitors (Figure 3) (Uhrbom *et al*., 1998). The higher mRNA expression of nestin in early tumors (21.95) compared to late (13.55) further suggest that early tumors are derived from more immature cells than late tumors. However, the expression of the oligodendrocyte transcription factor gene *Olig2* was similarly increased in both early and late tumors and may indicate that even the GBM-like early tumors are derived from immature cells of the oligodendrocyte lineage, which are known to express PDGF α-receptors (Pringle and Richardson, 1993). This notion is strengthened by the finding that *Sox10* was highly expressed in both early and late tumors. Sox10, a transcription factor that is required for oligodendrocyte maturation (Stolt *et al*., 2002) and a marker for immature as well as mature oligodendrocytes (Kuhlbrodt *et al*., 1998), was highly expressed in glioma cells (Figure 3). *Cgt* (ceramide galactosyltransferase), reported to be more frequently expressed in human oligodendrogliomas than in astrocytomas (Popko *et al*., 2002), was present in both tumors but increased in late compared to early tumors. *Plp* (proteolipid protein), a marker for differentiated oligodendrocytes, showed an extremely low expression in newborn brain and was sparsely expressed in both tumors but more expressed in late ones (Table 1 and Figure 3). Altogether, these findings fit with the view that both early and late tumors are derived from cells of the oligodendrocyte lineage.

In all, 43 of the previously retrovirally tagged genes (Johansson *et al*., 2004) (excluding 12 Btl genes that were not differentially expressed on the array) were chosen for expression analysis by qrt, PCR (Table 2). Seven of these mimicked the expression profile of *Pdgfrα* and might be markers of a PDGF-responsive cell type that forms the tumors, or were upregulated secondary to PDGF stimulation. For example, *Fos* is a well-known PDGF-responsive gene (Cochran *et al*., 1984) and *Ng2* is upregulated in brain tumors (Chekenya *et al*., 1999) and coexpressed with PDGF alpha receptor in oligodendrocyte progenitors (Cochran *et al*., 1984; Nishiyama *et al*., 1996). Five of the tagged genes, *Abhd2*, *Prex1*, *Prkg2*, *Sox10* and *1200004M23Rik,* were found to be overexpressed in the tumors but not transcriptionally upregulated in newborn brain in contrast to *Pdgfrα* and a number of other genes. *Camk2b* was downregulated and its homolog, *Camk2g*, repressed in the Individual experiment (Table 1) was previously found attenuated during human astrocytoma progression but also in other types of cancer (Tombes *et al*., 1999; van den Boom *et al*., 2003). Expression of *Neurl*, a gene located on human chromosome 10q25, a region frequently deleted or found with loss of heterozygozity in astrocytomas and glioma cell lines (Nakamura *et al*., 1998), was also decreased.

Several of the previously tagged genes like *Sdc3*, *Rhbg*, *Eef1a1*, *Scarb1*, *Mars*, *Tax1bp2*, *XM_130476*, *Sppl2b*, *Pea15* and *Nfix* were elevated in both tumor types compared to adult brain but not always compared to their expression in newborn brain (Table 2). *Fancc* and *Fmod* were downregulated in the early tumors. Others showed no dramatic changes in expression in the tumors compared to normal adult brain and must, if involved in tumorigenesis, have been altered in minor tumor clones (i.e. not represented in the tumor pools) where they were retrovirally targeted.

Interestingly, there was a significantly higher number of retroviral insertions in the early, GBM-like tumors as compared to the late, OD-like tumors. In addition, only few common insertions were found in the latter group. A likely interpretation of this finding is that PDGF-mediated autocrine stimulation alone generally only gives rise to low grade oligodendrogliomas (Dai *et al*., 2001) and that the evolvement of higher grade tumors requires additional genetic events. In the present tumor model, these events are caused by insertional mutagenesis of host genes. Further studies of the possible involvement of the retrovirally tagged genes in glioma progression in mouse and man are therefore warranted. Several of the genes that were differentially expressed in the virus-induced mouse gliomas have previously been identified in human glioma expression profiles. This finding may be taken as additional support for the relevance of the present mouse model for studies of glioma biology.

Materials and methods

Tumor induction and histological analysis

The generation of MMLV/PDGFB-induced mouse brain tumors using wild-type C57BL/6 mice (purchased from Charles River Breeding Laboratories) and the cloning of provirally tagged sequences have previously been described and were performed in compliance with the local animal ethics committee (Uhrbom *et al*., 1998; Hesselager *et al*., 2003; Johansson *et al*., 2004). In this study, we included mice from several series of injections. Macroscopically confirmed tumor tissue was isolated directly after killing and frozen (−80°C). The adjacent tumor incorporating the brain was fixed at least 48 h in 4% formaldehyde in PBS (pH 7.4) and embedded in paraffin. Tumors of five mice killed early (14–18 weeks) and five mice killed late (30–42 weeks) (of 132 injected mice) were included. Pathological examination was conducted from hematoxylin and eosin-stained (H&E) 5-μm sections cut with a microtome (Microm, Heidelberg, Germany). Further information of brain tumor types or retroviral insertion sites is available in the Mouse Retroviral Tagged Cancer Gene Database (http://RTCGD.ncifcrf.gov).

Immunohistochemistry

Slides were deparaffinized in xylene and heated in a steamer for 45 min in 1 : 100 antigen unmasking solution (Vector Lab); then immersed in 1% H_2O_2 (Sigma) for 30 min and blocked in 1.5% normal goat serum in 0.05% PBS-T for 1 h at room temperature. Primary antibodies for Ng2 (Chemicon) and Sox10 (Chemicon) were diluted 1 : 100 and used for 1-h incubations at room temperature. Sections were further incubated for 1 h with secondary biotin antibodies (Dakocytomation). After incubation in AB solution mix for 30 min and staining with DAB according to the manufacturer's protocol (Vector Lab), sections were counterstained with hematoxylin and mounted in Immumount (Thermo Shandon). Sections were washed three times in 0.05% PBS-T between all incubation steps above and specificity was assured when compared against negative controls treated as described but incubated with blocking solution without primary antibodies.

RNA preparation and cDNA array experiments

Total RNA was isolated from frozen brain tumor tissues homogenized and extracted with an acid guanidinium thiocyanate–phenol–chloroform mixture (Chomczynski and Sacchi, 1987). Double extraction in chisam and precipitation with 2-butanol for 1 h in −20°C was followed by washing in 70% ethanol. RNA quality was confirmed by formaldehyde agarose gel electrophoresis UV spectroscopy and had 260/280 absorbance ratios higher than 1.95 in 10 mM TE buffer (pH 7.5). National Institute of Ageing (NIA) M15k.3 cDNA microarray slides were provided from the University Health Network Microarray Centre (Ontario Cancer Institute, Canada). cDNA from 3 μg of total RNA was synthesized and fluorescently labeled according to the indirect Micromax TSA labeling and detection kit protocol (Perkin Elmer, Life Sciences) for each sample. Probes of cDNA labeled with flourescein and biotin were purified with Microcon YM-30 columns (Millipore), mixed per pair and diluted in 45 μl hybridization buffer. Hybridization continued for 12–14 h in 65°C in a hybridization chamber (Corning). Cyanine 3 Tyramide and Cyanine 5 Tyramide were deposited onto the array for signal detection according to the TSA protocol. The microarray slides were scanned with a Gene Pix 4000B scanner (Axon Instruments Molecular Devices).

Array data storage and analysis

Image analysis was performed using the Genepix 5.0 software (Axon Instruments). Array data from spot intensities were stored and processed using a system for analysis of microarray data, BASE (Saal *et al*., 2002) modified at Linneaus Center of Bioinformatics, Uppsala University, Sweden. Statistical analysis was performed in the Linneaus Center of Bioinformatics Data Warehouse (http://www.lcb.uu.se/lcbdw.php) using R (http://www.R-project.org) packages from Bioconductor (Gentleman *et al*., 2004). In order to remove systematic variation present in the array data the datasets were normalized using print-tip lowess normalization (Yang *et al*., 2002). To find genes most likely to be differentially expressed, genes were ranked according to a parametric empirical Bayes approach (Lonnstedt and Speed, 2002). Average $\log_2$-values (M) from gene reporters spotted in duplicates on the arrays were collected but genes with large differences in ratios from duplicate spots and genes with non-reliable measurable values from more than one array were excluded from further analysis. Only published mouse-specific cDNA sequences of a reporter (National Institute of Aging/National Institutes of Health Mouse Genomics home page; http://lgsun.grc.nia.nih.gov) that were unique when searched with BLAST (National Center for Biotechnology Information) were included in the final list. Compared to the Individual experiment, the number of genes differentially expressed was larger in the Pooled experiment, where a cutoff *a posteriori* excluded reporters with *M*-values between −2 and 2. This coincided with an absolute log of odds score (*B*) above 4, presenting the 85 genes most likely to be

differentially expressed (in Supplementary Material 1). (A complete list of differentially expressed genes is available upon request.) In the Individual experiment, a lower threshold was used for cutoff since this system did not show as large differences as above, and average $\log_2$-ratios between -1.2 and 1.2 were excluded. Here, the lower cutoff ($B \geqslant 1$) presented 60 differentially expressed genes (Table 1).

Quantitative real-time PCR analysis

Preparation of cDNA from 0.5 μg DNase-treated (Amersham Biosciences) total RNA from tumor tissues was performed with ThermoScript RT-PCR System and oligo(dT_{20}) primer (Invitrogen). For each sample, qrt-PCR was performed using 4 μl of 1 : 4 diluted cDNA-mix, 2 × SYBR Green PCR Master Mix (Applied Biosystems, Perkin Elmer) with 0.2 μM of each tested oligonucleotide (ordered from Proligo, France). Sequences designed with Primer Express 1.5a software (Applied Biosystems, Perkin Elmer) are provided upon request. PCR reactions were run in triplicates on an ABI Prism 7700 sequence detection system instrument (Applied Biosystems, Perkin Elmer) with an initial 10 min at 95°C, followed by 45 cycles of two-step PCR at 95°C for 15 s and 60°C for 60 s. Continuous quantitative measurement of the PCR product was achieved by incorporation of SYBR Green dye into double-stranded DNA. The threshold cycle (C_T) values (indicating the fractional cycle number of amplified target calculated by the ABI Prism Sequence Detection Systems v1.7 software, Applied Biosystems, Perkin Elmer) of the samples were measured. To confirm specificity, PCR products were studied with agarose gel electrophoresis and to assure approximately equal amplification efficiencies, ΔC_T values were studied from serial dilutions of cDNA amplified with target and reference gene (Gapdh) primers. Relative changes in gene expression from quantitative PCR data were calculated from threshold cycle with the $2^{-\Delta\Delta C_T}$ method as reported (Livak and Schmittgen, 2001). Transcript levels were normalized against corresponding Gapdh levels and mean fold changes ± standard deviations were expressed relative to normal adult brain (set to 1).

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