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Upregulation of *ASCL1* and inhibition of Notch signaling pathway characterize progressive astrocytoma

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Astrocytoma is the most common type of brain cancer constituting more than half of all brain tumors. With an aim to identify markers describing astrocytoma progression, we have carried out microarray analysis of astrocytoma samples of different grades using cDNA microarray containing 1152 cancer-specific genes. Data analysis identified several differentially regulated genes between normal brain tissue and astrocytoma as well as between grades II/III astrocytoma and glioblastoma multiforme (GBM; grade IV). We found several genes known to be involved in malignancy including Achaete-scute complex-like 1 (*Drosophila*) (*ASCL1*; Hash 1). As *ASCL* has been implicated in neuroendocrine, medullary thyroid and small-cell lung cancers, we chose to examine the role of ASCL1 in the astrocytoma development. Our data revealed that *ASCL1* is overexpressed in progressive astrocytoma as evidenced by increased levels of *ASCL1* transcripts in 85.71% (6/7) of grade II diffuse astrocytoma (DA), 90% (9/10) of grade III anaplastic astrocytoma (AA) and 87.5% (7/8) of secondary GBMs, while the majority of primary *de novo* GBMs expressed similar to or less than normal brain levels (66.67%; 8/12). *ASCL1* upregulation in progressive astrocytoma is accompanied by inhibition of Notch signaling as seen by uninduced levels of HES1, a transcriptional target of Notch1, increased levels of HES6, a dominant-negative inhibitor of HES1-mediated repression of *ASCL1*, and increased levels of Notch ligand Delta1, which is capable of inhibiting Notch signaling by forming intracellular Notch ligand autonomous complexes. Our results imply that inhibition of Notch signaling may be an important early event in the development of grade II DA and subsequent progression to grade III AA and secondary GBM. Furthermore, ASCL1 appears to be a putative marker to distinguish primary GBM from secondary GBM.

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Introduction

Astrocytomas are the most common primary brain tumors and occur at an incidence of almost 12 per 100 000 people (Landis *et al*., 1999). Diffuse astrocytoma (DA) may be classified (as per WHO classification) as low-grade DA (grade II), anaplastic astrocytoma (AA; grade III) and glioblastoma multiforme (grade IV; GBM), in the order of increasing malignancy (Mischel and Vinters, 2001). Currently, these classifications are based on the observed histopathological characteristics of the tumor, which are sometimes subjective and inconsistent. GBM constitutes more than 80% of malignant gliomas (DeAngelis, 2001) and patients with GBM have a median survival of less than 1 year. Current treatments, including surgery, radiation therapy, and chemotherapy, unfortunately have not changed the natural history of these incurable neoplasms; and the prognosis of patients with GBMs has not improved significantly in the past 30 years (Davis *et al*., 1998). To find new diagnostic and therapeutic strategies, a better understanding of the biological pathway(s) leading to glial tumorigenesis is warranted.

Astrocytoma development is known to involve accumulation of a series of genetic alterations (Nagane *et al*., 1997) similar to other cancers. Identification of many of the genes involved in the astrocytoma development, using standard molecular approaches, has helped to understand the process of astrocytoma genesis and progression (Louis and Gusella, 1995). Frequent

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amplification of epidermal growth factor receptor (EGFR) (Brock and Bower, 1997; Hill *et al*., 1999), platelet-derived growth factor receptor (Maxwell *et al*., 1990; Fleming *et al*., 1992; Hermanson *et al*., 1992, 1996; Westermark *et al*., 1995), amplification of chromosome 12q region, which carries the cdk4 gene (Nagane *et al*., 1997; Hill *et al*., 1999) and alterations in chromosomes 1p, 9p, 10, 17p, 19q and 22q, have frequently been found in these tumors. In addition, mutations in the tumor suppressor gene *p53* were found to be associated with chromosome 17p alterations in low-grade and progressive astrocytoma (Maher *et al*., 2001; Phatak *et al*., 2002). Inactivation of the cdk inhibitor $p16^{INK4a}$ residing in chromosome 9p is very common in sporadic astrocytoma, occurring in 50–70% of high-grade gliomas and 90% of GBM cell lines (James *et al*., 1991; Olopade *et al*., 1992). LOH in chromosome 10 is one of the most frequent alterations in GBM and is accompanied by the loss of *PTEN/MMAC* gene (Li *et al*., 1997; Hill *et al*., 1999).

GBMs are of two types: primary GBM (*de novo* type), which manifests in older patients (mean age: 55 years) as an aggressive, highly invasive tumor, usually without any evidence of prior clinical disease, after a short clinical history of approximately less than 3 months, and secondary GBM (progressive type) is usually seen in younger patients (mean age: 40 years) and develops more slowly by malignant progression from DA (WHO grade II) or AA (WHO grade III). Although some differences in the genetic alterations between these two GBMs have been identified, they are not sufficient enough to be used as differentiating markers considering the fact that the two types of GBMs have almost comparable clinical, genetic and biological characteristics (Kleihues *et al*., 2002). However, it is likely that these subtypes would respond differently to specific novel therapies as they are developed in the future (Kleihues and Ohgaki, 1999).

Despite all this information, our understanding of astrocytoma development is not sufficient enough to improve prognosis for GBM patients. A more global, systematic understanding of expression patterns of various genes and their downstream gene products in astrocytoma will hopefully provide new diagnostic and therapeutic targets. Towards this, a number of studies have reported the gene expression profile of astrocytoma (Liau *et al*., 2000; Sallinen *et al*., 2000; Ljubimova *et al*., 2001; Rickman *et al*., 2001; Watson *et al*., 2001; Fathallah-Shaykh *et al*., 2002; Tanwar *et al*., 2002; Godard *et al*., 2003; Nutt *et al*., 2003; Wang *et al*., 2003).

In this study, we used cDNA microarrays containing 1152 genes to study the expression profile of DA. By using hierarchical cluster analysis, we have identified several genes that are differentially expressed between normal brain tissue and astrocytoma as well as between grade II/III astrocytomas and GBMs. A novel finding was that the elevated expression of Achaete-scute complex-like 1 (*Drosophila*) (*ASCL1*) and simultaneous inhibition of Notch signaling pathway describes the development and further progression of grade II DA to grade III AA and then to secondary GBM. Finally, we propose that ASCL1 could be used as a marker to differentiate secondary GBMs from primary GBMs.

Results

Hierarchical clustering of the complete data set

We have used microarray slides containing 1152 cancer-related genes in duplicate (GeneMAP™ Human Cancer Array) obtained from Genomic Solutions Inc. A total of seven tumor samples comprising three grade II/III astrocytomas and four GBMs (grade IV) were analysed. The 459 genes that had values in at least two samples in each group were used for further analysis. The normalized and log 2-transformed data containing values for these genes were subjected to an unsupervised, two-way, average-linkage hierarchical cluster analysis with uncentered correlation coefficient as similarity metric, using the Gene Cluster software. The Java TreeView software was used to display and analyse the results of the Gene Cluster program (Figure 1). This was carried out to see whether the two groups – grade II/III astrocytoma and GBMs (IV) – show distinct patterns of gene expression. The sample dendrogram clearly separates into two main branches: one consisting of grade II/III samples and other consisting of GBMs, exemplifying the inherent differences between them. In addition, genes with similar expression profiles were grouped together. Four clusters **A**, **B**, **C** and **D** were noteworthy (Figure 1). The cluster **A** essentially consists of genes upregulated in all grades of astrocytomas and the cluster **B** essentially consists of genes downregulated in all grades of astrocytomas. The other two clusters (**C** and **D)** consist of genes, which are differentially expressed between grade II/III astrocytomas and GBMs (Figure 1). The cluster **C** essentially consists of genes that are upregulated in grade II/III astrocytomas compared to GBMs. The cluster **D** consists of genes that are downregulated in grade II/III astrocytomas compared to GBMs (Figure 1).

Real-time quantitative RT–PCR analysis of selected genes

In our study, Osteonectin, an acidic, cysteine-rich, secreted protein (SPARC), was upregulated in majority of all grades of astrocytoma (grade II/III – 89% (8/9); secondary GBMs (IV) – 75% (3/4); primary GBMs (IV) – 91.7% (11/12); data not shown). Vimentin, a cytoskeleton protein, was also found upregulated in majority samples of all grades of astrocytomas (grade II/III – 70% (7/10); secondary GBMs (IV) – 100% (4/4); primary GBMs (IV) – 91.7% (11/12); data not shown). In addition, we identified β2-microglobulin to be upregulated more than 1.5-fold compared to normal brain samples in majority of GBMs (grade II/III – 27.2% (3/11); secondary GBMs – 66.67% (4/6) and primary GBMs (IV) – 100% (12/12); data not shown). Further, we found Semaphorin 4D (CD100) to be downregulated in astrocytomas, particularly in the majority of primary GBMs (grade II/III – 44.4% (4/9); secondary GBMs (IV) – 25% (1/4); primary GBMs (IV) – 80% (12/15); data not shown).

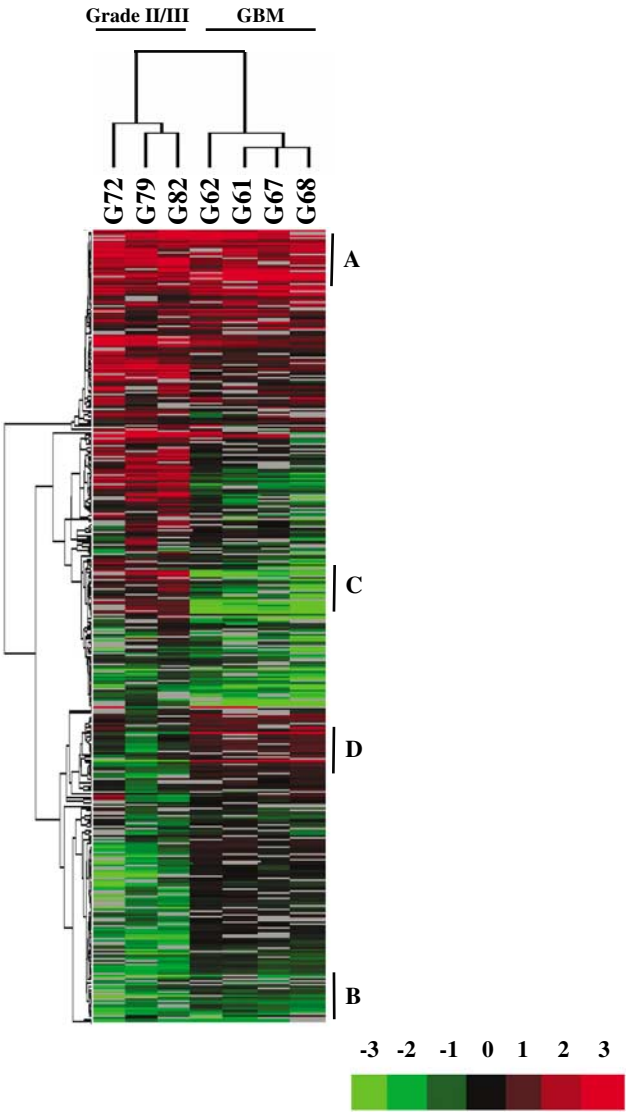

Figure 1 Two-way hierarchical clustering of microarray data. Normalized, log 2-transformed expression ratios of 459 genes from seven samples (three grade II/III astrocytomas and four GBMs) were analysed by two-way hierarchical clustering using Gene Cluster software and results were visualized by TreeView program. A dual color code was utilized with red and green indicating up- and downregulation, respectively, in the particular glioma sample compared to normal brain tissue. Four clusters named **A**, **B**, **C**, and **D** are marked. The scale at the bottom shows the relationship between color saturation and the log 2-transformed gene expression ratios

Differential regulation of ASCL1 distinguishes between primary and secondary GBM

We found the *ASCL1* to be an attractive candidate as it is differentially regulated in progressive astrocytoma (see below). *ASCL1* has been shown to be highly expressed in neuroendocrine (NE) cancers, medullary thyroid cancer and small-cell lung cancer (Ball *et al*., 1993). We found ASCL1 to be upregulated in majority of grade II DAs (85.71%; 6/7), grade III AA (90%; 9/10) and secondary GBMs (87.5%; 7/8) (Figure 2a). However, among primary GBMs, *ASCL1* upregulation was seen only in 33.33% (4/12) of the samples (Figure 2a). Increase in ASCL1 transcripts also correlated immunohistochemically with increased nuclear staining for ASCL1 in grade II DA (Figure 3d), grade III AA (Figure 3f) and secondary GBM (Figure 3h). Most of these samples also showed increased nuclear staining for p53, which is indicative of mutated p53 characterizing progressive astrocytomas and did not show staining for EGFR (Table 1). As expected, primary GBMs did not show detectable staining for ASCL1 (Figure 3j). The majority of these tumors overexpressed EGFR, while p53 immunoreactivity was noted in minimal number of cases (Table 1). Normal brain sections did not reveal immunoreactivity for ASCL1 (Figure 3), p53 and EGFR (data not shown). Table 1 describes the details about various astrocytoma samples used in this study, their staining pattern for various markers and their clinical parameters. These results suggest that the *ASCL1* expression could be used to define progressive astrocytoma and further to differentiate primary from secondary GBMs.

Inhibition of Notch signaling pathway in progressive astrocytoma

In the central nervous system (CNS), Notch signaling promotes differentiation of neural stem cells to astroglial cell lineage, while simultaneously inhibiting neurogenesis and oligodendroglial differentiation (Ge *et al*., 2002). Notch signaling causes transactivation of Hairy and Enhancer of Split (*HES*) genes, which in turn repress *ASCL1* expression through transcriptional mechanisms (Chen *et al*., 1997). Since our results show that there is upregulation of *ASCL1* in the majority of grade II DA, grade III AA and secondary GBMs, we hypothesized that Notch signaling may be inhibited during DA (grade II) development from astroglial cells, which would further provide suitable environment for progression to AA (grade III) and subsequently to secondary GBM. To test this hypothesis, we analysed the levels of various Notch pathway genes in the same set of samples. We found that the transcript levels of the Notch target gene *HES1*, a transcriptional repressor of *ASCL1*, remain similar to or less than that of normal brain tissue in 52.94% (9/17) of grade II/III astrocytoma (three out of seven grade II and six out of 10 grade III samples) and 85.7% (6/7) of secondary GBM (Figure 2b). Next, we looked at the levels of HES6 another member of the HES family of genes. HES6 has been shown to antagonize functionally HES1 and relieve positive bHLH factors like ASCL1 from inhibition by HES1 (Bae *et al*., 2000; Gibert and Simpson, 2003). We found that the level of HES6 transcripts is increased several fold in the majority of samples from grade II DA (71.43%; 5/7), grade III AA (66.67%; 6/9) and secondary GBM (71.43%; 5/7) (Figure 2c). Thus, the high level of HES6, which is expected to inhibit HES1, gives another explanation for induced expression of ASCL1 in majority of grade II/III astrocytomas and secondary GBMs in addition to inhibition of Notch signaling as seen by the uninduced levels of HES1.

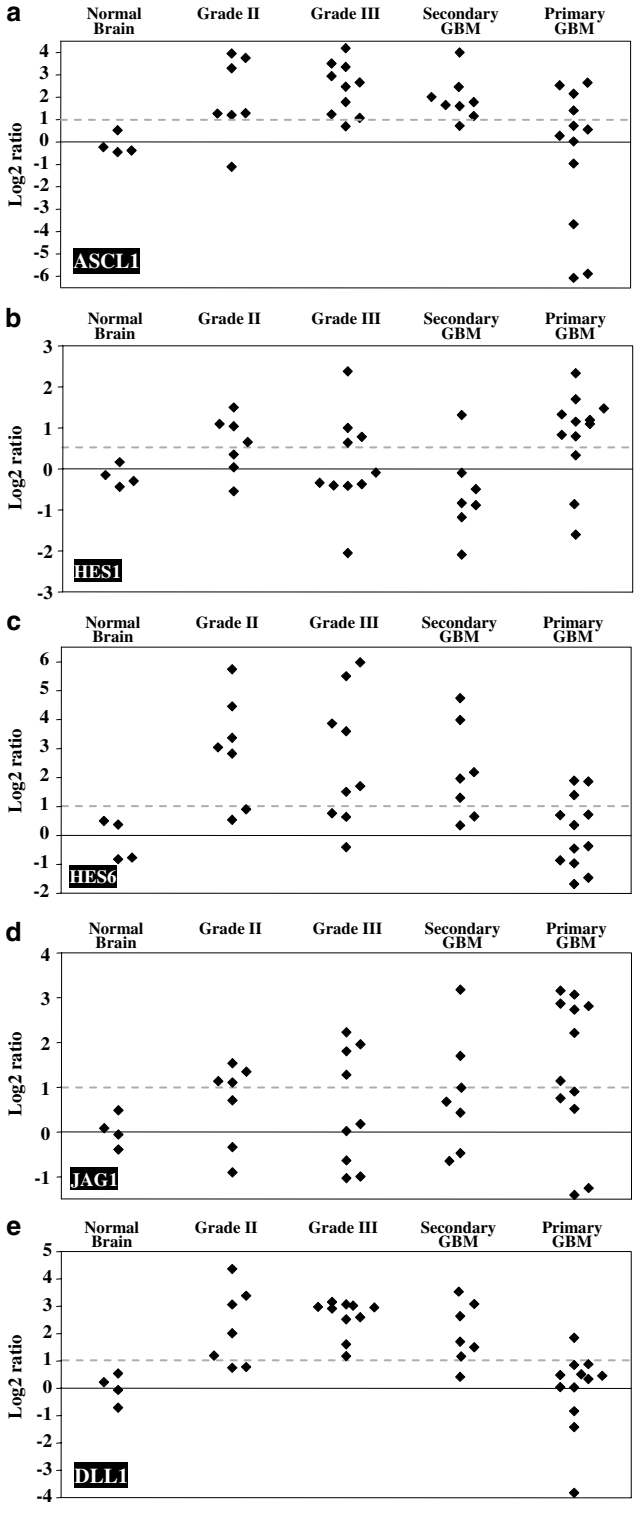

Figure 2 Scatter plots of differentially regulated Notch pathway genes during astrocytoma. Log 2-transformed gene expression ratios obtained from real-time quantitative PCR analysis are plotted for ASCL1 (**a**), HES1 (**b**), HES6 (**c**), JAG1 (**d**) and DLL1 (**e**). Each dot represents a data derived from one sample. A change in gene expression by twofold or more over its mean expression in normal brain sample was considered significant

Since Notch receptors get activated upon binding to their ligands, we then analysed levels of expression of Notch ligands Delta1 (Delta-like 1; DLL1) and Jagged 1 (JAG1) in astrocytoma samples. The levels of Jagged 1 transcript is found to be upregulated in 57.14% (4/7) of DA, 40% (4/10) of AA and 42.86% (3/7) of secondary GBMs (Figure 2d). Surprisingly, we found very high levels of Delta1 transcripts in the majority of samples analysed belonging to grade II DA (71.43%; 5/7), grade III AA (100%; 10/10) and secondary GBMs (85.71% 6/7) (Figure 2e). High levels of Delta1 seen in astrocytoma samples overexpressing ASCL1 can be explained by the fact that Delta1 is shown to be transcriptionally activated by ASCL1 (Heitzler *et al*., 1996). Increased levels of Notch ligand Delta1 is expected to activate the Notch signaling pathway. On the other hand, the presence of uninduced levels of Notch target gene *HES1* and very high levels of ASCL1 transcripts in these samples are suggestive of inhibition of Notch signaling. As the activity of notch ligands is known to be regulated by glycosylation of notch receptors by fringes (Haltiwanger and Stanley, 2002), we analysed the levels of different fringe molecules in different grades of astrocytoma. We did not find any significant change in the expression levels of Lunatic, Radical and Manic fringe in most samples, suggesting that fringe molecules may not have any role in inhibiting notch ligands during progressive astrocytoma development (data not shown). It is reported that Notch ligands Delta1 and Jagged 1 sequester Notch proteins in the endoreticulum or Golgi apparatus of neuronal precursors as intracellular heteromeric complexes and thus reduce the effective dose of Notch signaling (Sakamoto *et al*., 2002). Thus, the overexpression of Notch ligands is believed to inhibit Notch signaling by forming intracellular cell-autonomus ligand : receptor associations rather than activate the Notch pathway. Taken together, these results suggest that Notch signaling is inhibited early during the development of DA and the consequent elevation of ASCL1 may facilitate further progression to AA and later to secondary GBM.

In contrast to the above observation, we found no significant evidence for inhibition of Notch signaling in primary GBM development. The majority of primary GBM samples had similar levels of ASCL1 transcripts to that of normal samples (66.67%; 8/12) (Figure 2a). In good correlation, the expression of HES1 is increased in most primary GBM samples (75% had more than 1.5-fold transcripts than that of normal; 9/12) (Figure 2b). As expected, the levels of HES6 transcripts did not increase in primary GBMs. The expression levels are in the same range as normal samples in the majority of them (75%; 9/12) (Figure 2c). We found the Jagged 1 transcript levels going up in the majority of primary GBMs (58.3%; 7/12) (Figure 2d), the significance of which is not clear at present. The Delta1 transcript levels remain unchanged in the majority of primary GBMs (91.67%; 11/12) (Figure 2e**)**. These results suggest that Notch pathway remains activated in primary GBMs and indicates the possibility that Notch pathway has no role in the development of primary GBM.

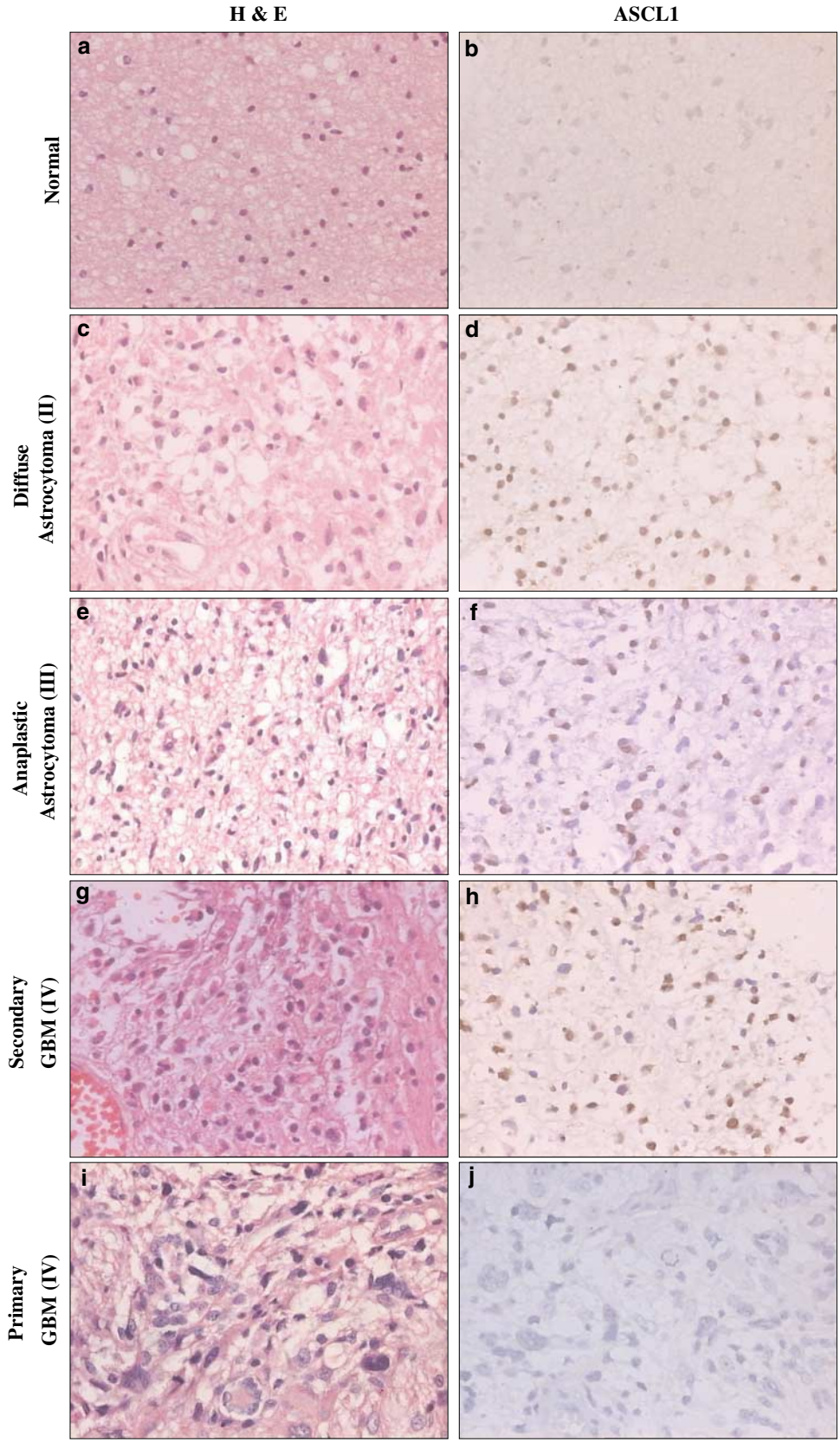

Figure 3 Immunohistochemical validation of ASCL1 overexpression in progressive astrocytoma. Sections from normal brain (**a** and **b**), grade II DA (**c** and **d**), grade III AA (**e** and **f**), secondary GBMs (**g** and **h**) and primary GBMs (**i** and **j**) were stained with H&E (**a**, **c**, **e**, **g** and **i**) and for ASCL1 (**b**, **d**, **f**, **h** and **j**). *Note* that grade II DA, grade III AA, secondary GBM but not Primary GBM samples are positive for ASCL1 staining

Table 1 ASCL1 expression characterizes progressive astrocytoma

S no.	*Sample ID*	*Age of patient*	*Duration of symptoms (in months)*	*MIB-LI (%)*	*IHC staining*			*Quantitative RT–PCR of* ASCL1	
					p53	EGFR	ASCL1	*Status*	*Fold change*
IV, Primary GBM									
1	95	55	3	10	+	+	–	↓	−1.95
2	122	61	2	16	–	+	–	↓	−12.86*
3	242	57	1	20	–	+	+	↔	1.98
4	64	67	1	18.5	+	+	–	↓	−67.68
5	65	70	6	18.5	+	+	+	↑	5.49
6	81	45	3	16.5	–	+	–	↔	1.47
7	61	50	3	18.5	–	+	–	↔	1.02
8	258	46	3	21	–	+	+	↑	6.27
9	75	25	0.5	32	+	+	–	↓	−59.72
10	156	53	3	16	–	+	+	↑	4.44
11	94	58	3	18	–	+	+	↔	1.21
12	110	43	4	11.5	+	+	–	↑	2.65
IV, Secondary GBM									
1	146	35	2	40	+	+	+	↑	15.99
2	195	43	3	28	+	–	+	↑	3.16*
3	197	18	4	13	+	–	–	↑	4.02
4	251	35	5	28	+	–	+	↑	2.05
5	254	48	5	14	+	–	+	↑	3.04
6	255	18	6	25	+	+	+	↔	1.64
7	160	40	5	15.5	+	–	+	↑	3.41
8	210	29	12	12	–	+	+	↑	5.78
III, AA									
1	79	32	5	9.8	+	–	+	↑	2.35
2	90	55	12	6	+	–	–	↑	7.70
3	93	49	3	5.5	+	–	+	↑	18.27
4	140	25	1	7	+	–	+	↑	3.44
5	172	21	10	8	+	–	+	↑	5.54
6	184	28	4	9.5	–	+	+	↔	1.62
7	246	30	3.5	6	+	–	+	↑	2.10
8	259	32	12	6	+	–	+	↑	6.34
9	277	30	3	10	+	–	+	↑	11.42
10	262	35	6	9	+		+	↑	10.21
II, DA									
1	91	33	5	3	+	–	+	↑	2.43
2	127	32	2	4	+	–	+	↓	−2.16*
3	248	30	24	1.5	+	–	+	↑	3.38
4	230	27	3	2	+	–	–	↑	13.47
5	263	43	5	3	+	–	+	↑	15.46
6	271	13	3	1.5	–	–	+	↑	9.78
7	234	28	11	3	–	–	+	↑	2.29

MIB-LI = MIB-1 labeling index; EGFR = epidermal growth factor receptor; ASCL1 = Achaete-scute complex-like 1. *Value was derived from semiquantitative RT–PCR. ↑Upregulated with respect to normal brain. ↓Downregulated with respect to normal brain. ↔No significant change with respect to normal brain

Discussion

In the present study, we used cDNA arrays to identify differentially expressed genes between normal brain tissue and astrocytoma as well as between different grades of astrocytoma. Many of the identified genes have known or suspected relevance to cancer, while some of them have no known functions. In accordance with previous findings, we found Osteonectin and Vimentin to be upregulated in the majority of all grades of astrocytoma (Sallinen *et al*., 2000; Ljubimova *et al*., 2001; Fathallah-Shaykh *et al*., 2002). Although differential regulation of β2-microglobulin has been reported in diffuse large-B-cell lymphoma (Jordanova *et al*., 2003) and multidrug resistance phenotype in tumor cells (Scheffer *et al*., 2002), we found β2-microglobulin to be upregulated in GBM. Another gene, Semaphorin 4D, which has a functional role in the immune system (Hall *et al*., 1996) as well as in the nervous system (Kumanogoh *et al*., 2002), is found to be downregulated in astroctyomas. However, the significance of its regulation in astrocytomas is not known. More importantly, we identified ASCL1 as a differentiating marker between secondary and primary GBM. Furthermore, ASCL1 upregulation and inhibition of Notch signaling were seen in grade II DA, grade III AA and secondary

GBMs, but not in primary GBMs, suggesting that these molecular changes may characterize progressive astrocytoma.

We provide evidence for the regulation of Notch signaling pathway during low-grade astrocytoma development from normal astroglial cells and further progression to AA and then to secondary GBM. During the development of CNS, the neural stem cells, which are common progenitor cells, proliferate and subsequently differentiate into three major cell types of the brain: neurons, astrocytes and oligodendrocytes (Qian *et al*., 2000). Several molecular mechanisms have been found to be involved in the differentiation of multipotent neural stem cells into different brain cell types. Neurogenic bHLH transcription factors like neurogenin 1/2 and Mash1, a murine homolog of *ASCL1*, have been shown to inhibit glial differentiation (Furukawa *et al*., 2000; Zhou *et al*., 2000; Nieto *et al*., 2001; Novitch *et al*., 2001; Satow *et al*., 2001; Sun *et al*., 2001). The cytokine leukemia inhibitory factor promotes astroglial differentiation through JAK-STAT signaling pathway (Johe *et al*., 1996; Bonni *et al*., 1997). Notch signaling has been shown to play a major role in the differentiation of several tissues including nervous tissue in many organisms (Ghysen *et al*., 1993; Artavanis-Tsakonas *et al*., 1995, 1999). While the Notch signaling inhibits the neuronal and oligodendroglial differentiation, it has recently been reported to drive instructively satellite glial cell differentiation in peripheral neural crest stem cells and to promote astrocyte differentiation in adult hippocampal NSCs (Morrison *et al*., 2000; Tanigaki *et al*., 2001). Binding of any of the Notch ligands, which include Delta1, Jagged1, and Jagged2, leads to a complex cleavage and activation of Notch proteins (Weinmaster, 1997; Artavanis-Tsakonas *et al*., 1999). The released and activated COOH-terminal fragment of Notch translocates to the nucleus where it interacts with the transcription factor CBF1 (RBPjk) to transactivate target genes including Hairy and enhancer of Split 1 (*HES1*) (Weinmaster, 1997; Artavanis-Tsakonas *et al*., 1999). The ability of Notch ligands to activate Notch receptor is further modulated by glycosylation of Notch by fringe proteins (Moloney *et al*., 2000).

We present multiple evidences for inhibition of Notch signaling pathway during the development of DA, which ultimately progresses to secondary GBM. Firstly, the level of ASCL1 transcript is found to be significantly high in the majority of grade II/III astrocytoma as well as secondary GBMs. Notch signaling causes transactivation of *HES* genes, which in turn repress *ASCL1* expression through transcriptional mechanisms (Chen *et al*., 1997). A similar regulation is seen in HES1$^{-/-}$ mice, where the level of ASCL1 is found to be elevated (Ito *et al*., 2000). Mash1 and Math3, a murine *ato* homolog, have been shown to play a major role in neuronal versus glial fate determination in the CNS and it is possible that downregulation of the *Mash1* and *Math3* is one of the mechanisms to initiate gliogenesis (Tomita *et al*., 2000). Thus, the increased level of ASCL1 is suggestive of inhibition of Notch signaling in progressive astrocytoma. Secondly, the transcript levels of Notch target *HES1*, an inhibitor of *ASCL1* expression, is not induced in the majority of grade II/III astrocytomas and secondary GBMs in comparison to normal brain samples. Thirdly, the levels of HES6 transcripts, a dominant-negative inhibitor of Notch signaling, is increased several fold in the majority of grade II/III astrocytomas and secondary GBMs. HES6 is a dominant-negative inhibitor of HES1 and it inhibits the function of HES1 by associating with it and abolishing its ability to repress transcription (Bae *et al*., 2000; Gibert and Simpson, 2003). Finally, we found high levels of Delta1 transcripts in most samples analysed belonging to grade II/III astrocytoma and secondary GBM. The reason for increased levels of Delta1 can be explained by the fact that Delta1 is shown to be transcriptionally activated by ASCL1 (Heitzler *et al*., 1996). In fact, the expression of Delta1 appears to be under the control of Mash1 (Post *et al*., 2000). Mash1 knockout is associated with a total loss of Delta1 expression in the lung (Apelqvist *et al*., 1999). Similarly, MASH1 mutants fail to express Delta1 transcripts (Casarosa *et al*., 1999). High levels of Notch ligand Delta1 is capable of inhibiting Notch signaling by forming intracellular cell-autonomus Notch : Delta1 associations (Sakamoto *et al*., 2002). Thus, our data clearly demonstrate the downregulation of Notch signaling during progressive astrocytoma development.

Our study also provides evidence for the fact that inhibition of Notch signaling occurs only in the secondary GBM, which is a progressive type, but not in primary GBM, which arises by a *de novo* process. While ASCL1 levels are upregulated in the majority of grade II/III astrocytoma and secondary GBMs, its levels remain unchanged in the majority of primary GBMs. The expression levels of other genes associated with Notch signaling correlate with the levels of ASCL1 expression. There are two important conclusions, we derive from this data: (1) Inhibition of Notch signaling pathway and the consequent upregulation of ASCL1 seem to be an early event in the development of progressive astrocytoma as this change is seen as early as in grade II DA. In addition, we see a continued inhibition of Notch signaling and upregulation of ASCL1 during the progression from grade II DA to grade III AA and subsequently to secondary GBM. Indeed, ASCL1 expression is reported to be tightly linked to the NE phenotype in lung cancer. Human *ASH1* (*ASCL1*) was found to be selectively expressed in normal fetal pulmonary NE cells as well as in the diverse range of lung cancer with NE features (Ball *et al*., 1993; Borges *et al*., 1997). However, we see an active Notch signaling with low levels of ASCL1 in most of primary GBMs. Thus, we conclude that inhibition of Notch signaling is an early and perhaps also an important event for the low-grade astrocytoma development, which may also play a major role in further progression into secondary GBMs. However, Notch signaling may have no role to play in the development of primary GBM as no change in Notch signaling is observed. There are other pathways involved in the development of primary GBM. For example, amplification of *EGFR*

gene is found in 40% of primary GBMs, but it is rare in secondary GBMs (Frederick *et al*., 2000). (2) Since ASCL1 upregulation is seen in the majority of secondary, but not in primary, GBMs. ASCL1 status could be used as a marker to differentiate secondary GBM from primary GBM. Mutations in *p53* gene are associated with about 50% of grade II/III astrocytomas and secondary glioblastomas, but are seen only in 10–20% of primary glioblastoma (Campomenosi *et al*., 1996; Watanabe *et al*., 1997; Schmidt *et al*., 2002). With our finding that ASCL1 is upregulated in secondary GBMs, combined use of ASCL1, p53 and EGFR immunohistochemical staining to differentiate secondary GBMs from primary could be considered.

From our data showing a correlation between the inhibition of Notch signaling and progressive astrocytoma development, we conclude that Notch signaling may have tumor suppressor or growth inhibitory role in astroglial cell type. Although the human Notch1 was originally isolated as an oncogene in acute lymphoblastic leukemia (Ellisen *et al*., 1991), this pathway has been shown to have distinctive roles in cancers arising from different tissues. For example, while the Notch signals are oncogenic in pre-T cells (Ellisen *et al*., 1991) and cervical epithelium (Nair *et al*., 2003), it suppresses tumor development in keratinocytes (Rangarajan *et al*., 2001; Nicolas *et al*., 2003). Since Notch signaling promotes differentiation of neural stem cells to astroglial cells (Qian *et al*., 2000), Notch expression is likely to be growth inhibitory rather than oncogenic in normal astroglial cells.

Another interesting finding from this study is the upregulation of HES6 in majority of grade II/III astrocytoma and secondary GBMs. *HES6* has been found to be overexpressed in human primary tumors derived from breast, lung and kidney, suggesting that HES6 overexpression may have an oncogenic role (Swearingen *et al*., 2003). Indeed, *HES6* has been located in chromosome 2q37, a region known to be amplified in common adenocarcinomas such as that of the lung, breast, prostate, kidney and ovary (Mitelman *et al*., 2002). The ability of HES6 to inhibit HES1 activity could be significant in that Notch signaling has tumor suppressor role in certain tissues (Rangarajan *et al*., 2001; Nicolas *et al*., 2003) and HES1 has been shown to play a role of tumor suppressor in mammary gland carcinoma cells (Strom *et al*., 2000; Muller *et al*., 2002). Taken together, our data suggest that Notch signaling has a tumor suppressor role in astroglial cell type and is inhibited early during the development of low-grade astrocytoma, which may provide a suitable environment for further development to AA and then to secondary GBM.

Materials and methods

Tissue collection

Astrocytoma tissue samples were collected from patients who were operated at Sri Satya Sai Institute of Higher Medical Sciences and Manipal Hospital, Bangalore, India, at the time of surgical resection. Control samples comprised nontumorous brain tissue samples (temporal lobe) collected from patients who underwent surgery for intractable epilepsy. A total of 37 astrocytoma samples of different grades were used in this study. Tissues were bisected and one-half was snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation. The other half was fixed in formalin and processed for paraffin sections and these were used to identify the histopathological grade and the type of astrocytoma and further immunohistochemical staining.

RNA isolation, cDNA labeling and microarray analysis

Total RNA was extracted from the frozen tissue by a combination of the TRIzol method (Life Technologies Inc.). and RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. The RNA samples were quantified by measuring the absorbance using a spectrophotometer and visualized on a MOPS–formaldehyde gel for quality assurance. cDNA synthesis and labeling of total RNA were carried out using the Micromax direct labeling kit (Perkin-Elmer Life Sciences, Inc.). RNA derived from normal brain tissue was labeled with Cyanine 3-dUTP, while that of tumor was labeled with Cyanine 5-dUTP. Total RNA ($20\,\mu$g) was used for each labeling reaction. The quality of cDNA labeling was monitored by separating small amounts of Cy3- and Cy5-labeled cDNA in an agarose gel made on a microscopic slide and scanning the gel using the laser scanner LSIV (Genomic Solutions, USA). Cy3- and Cy5-labeled cDNAs were added to $100\,\mu$l of $1\times$ hybridization buffer (Ultrahyb, Sigma) incubated at 75°C for 5 min before adding to the GeneMap Human Cancer Array (Genomic Solutions). Hybridization was carried out in a GeneTAC Hyb Station (Genomic Solutions) at 65°C for 4 h, 60°C for 4 h and 55°C for 10 h. The slides were washed using medium stringency, high stringency and postwash buffers (Genomic solutions) for 5 min each, dried and scanned using the GeneTAC LS IV scanner (Genomic Solutions).

Microarray image and data analysis

Image analysis was carried out with the GeneTAC Analyzer software, Version 3.3 (Genomic Solutions. Filtering and assembling) of data were carried out using MS Excel and MS Access. To begin with, the image was visually inspected and spots of questionable quality were flagged and eliminated from further consideration. In the next step, spots having a signal-to-noise ratio less than 1.1 in both channels and total intensity values below a threshold value were excluded. We first computed arithmetic mean and s.d. for background subtracted total intensities of all negative control spots ($3\times$ SSC) on the slide and then computed threshold value as arithmetic mean plus 2 s.d.'s. If the coefficient of variation of expression ratios of duplicate spots of a given gene is greater than 20%, then the gene was excluded from further analysis. Normalization was carried out by median log ratio method. The Cy5 : Cy3 normalized expression ratio was determined for each spot and these values from the duplicate spots within each array were averaged and log 2 transformed. All the subsequent analysis is carried out using log 2-transformed data.

For the purpose of data analysis, the grade II and III samples were classified into one group, while the grade IV GBMs formed the second group. We have analysed three grade II/III samples and four GBM samples by microarray hybridization. Only the genes having values in at least two samples in each group were used for further analysis. To determine the suitability of the data for further supervised analyses, an unsupervised, two-way, average-linkage hierarchical cluster

analysis with uncentered correlation coefficient as similarity metric, using the Gene Cluster software, Version 2.20 (available at http://rana.lbl.gov/EisenSoftware.htm) was carried out (Eisen *et al*., 1998). The Java TreeView software, Version 0.9.4 (available at http://genetics.stanford.edu/~alok/) was used to display and analyse the results of the Gene Cluster program. The objective was to see if samples of same pathological grade cluster together. Clustering was also carried out to visualize the structure of the data and different patterns of gene expression.

Real-time RT–PCR

The relative quantitation of expression levels of selected genes was carried out using two step strategy: in the first step, cDNA was generated from RNA derived from different tissue samples using cDNA Archive kit (ABI PRISM); subsequently, real-time quantitative PCR was carried out in ABI PRISM 7000/7900 (Applied Biosystems) sequence detection system with the cDNA as template using gene-specific primer sets and DyNAmo™ HS SYBR® Green qPCR kit (Finnzymes, Finland). Data were analysed as per the relative quantification model proposed by Pfaffl, which includes efficiency correction (Pfaffl, 2001). All measurements were made in duplicate, and for each qRT–PCR primer set, reaction efficiency estimates were derived from standard curves that were generated using serial dilutions of the pool of cDNA set used for the study. Ribosomal protein L35a was used as internal control as its expression level was found to be unaltered in the array experiments. Normal brain tissue samples from four different epilepsy patients were used as reference. An increase or decrease in gene expression by two fold or more over its mean expression in reference samples, unless stated otherwise, was considered significant. For certain samples, data were obtained by semiquantitative RT–PCR. PCR primer sequences, and conditions used will be provided on request.

Immunohistochemistry

Paraffin sections (5 μm) from the tumor and control tissues were collected on chrome–alum coated slides and subjected for immunohistochemistry using the streptavidin–biotin complex/immunoperoxidase method using the following monoclonal/polyclonal antibodies: MIB-1 (Ki-67 monoclonal antibody, DAKO, Denmark; 1 : 50); p53 (DO-1, Oncogene; 1 : 100); EGFR (Oncogene, 1 : 25); ASCL1 (Polyclonal, SIGMA; 1 : 50). Briefly, 5 μm paraffin sections were deparaffinized in xylene, dehydrated in graded alcohol series and rinsed in Tris buffer (50 mM, pH 7.6) for 15 min. The sections were then microwaved for 15–20 min at 700 W in sodium citrate buffer (10 mM, pH 6.0) to retrieve antigenicity from paraffin sections. For EGFR staining, the sections were pretreated with 0.05% trypsin at 37°C for 30 min. All sections were further treated with methanol and 3% hydrogen peroxide to block endogenous peroxidase followed by washes with Tris buffer. Milk powder (3%) or bovine serum albumin was used to block background staining for 30 min. The sections were then incubated with the primary antibody for 2 h followed by the linked streptavidin-biotinylated secondary antibody (Universal LSAB, DAKO). 3′3-diaminobenzidine (Sigma) was used as the chromogenic substrate.

Brain tumor samples previously characterized for overexpression of p53 and EGFR were used as positive controls. For ASCL1, the tumor sample, which showed marked upregulation by RT–PCR, was taken as the positive control. A negative control slide in which the primary antibody was excluded was used with each batch of slides. For MIB-1 and p53 immunostaining only nuclear staining was regarded as positive, whereas with EGFR, positive sample showed cytoplasmic and cell surface membrane staining.

For ASCL1 immunostaining, only nuclear staining was considered as positive signal. Tumors were considered ASCL1 positive when more than 5% of tumor cells showed nuclear staining. Regarding p53 and EGFR also, specimens with less than 5% immunopositive tumor cells were scored as negative. The MIB-1 labeling index (LI) was expressed as the percentage of tumor cell nuclei stained, in areas of maximum staining and calculated in at least 1000 tumor cells.

MIB-1 LI was used for accurate grading of astrocytomas. The mean cutoff LI for grade II astrocytomas was $2.14 \pm 1.042\%$; $7.68 \pm 1.786\%$ for grade III AA; and $19.6 \pm 7.578\%$ for GBM, which more or less corresponded to mean values laid down by the WHO grading scheme (Kleihues *et al*., 2000).

GBMs were classified as primary or secondary taking into consideration the clinical profile of patients, expression of p53 and EGFR. The mean age of patients with primary GBMs was 50.6 years and mean duration of symptoms was 2.7 months. All tumors showed highly pleomorphic, histomorphological features and evidence of 'field necrosis'. Uniform staining for EGFR by immunohistochemistry was evident in all cases and five revealed additionally p53 expression. Among secondary GBMs, the mean age of the patients was 33.8 years and mean duration of symptoms was 5.3 months. p53 immunoreactivity was uniformly evident in all cases and two revealed additionally EGFR overexpression. Histological evidence of progression from grades II or III astrocytoma was clearly seen in 5/8 cases. The details are given in Table 1.

Abbreviations

ASCL1, Achaete-scute complex-like 1 (*Drosophila*); GBM, glioblastoma multiforme; HES1, Hairy and Enhancer of Split 1; HES6, Hairy and Enhancer of Split 6; DA, diffuse astrocytoma; AA, anaplastic astrocytoma.

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