

Papers

Neural cell adhesion molecule L1 in gliomas: correlation with TGF- β and p53

T Tsuzuki, S Izumoto, T Ohnishi, S Hiraga, N Arita, T Hayakawa

Abstract

Aims—To assess immunohistochemically whether the neural cell adhesion molecule L1, which is a member of the immunoglobulin superfamily and has been shown recently to be a stimulating factor for glioma migration, is expressed in glioma tissues, and to investigate factors that can regulate this expression.

Methods—Twenty seven glioma tissue specimens including 13 glioblastomas, seven anaplastic astrocytomas, and seven astrocytomas were examined. Immunohistochemical analyses of L1, p53, and transforming growth cell factor β (TGF- β) were performed on each tumour using both polyclonal and monoclonal antibodies.

Results—Nine (33%) specimens (six glioblastomas and three anaplastic astrocytomas) had L1 positive immunostaining. p53 positive staining was detected in 10 (43%) of 23 glioma specimens (seven glioblastomas and three anaplastic astrocytomas). TGF- β positive immunostaining was observed in 12 (52%) of the 23 glioma specimens (six glioblastomas, four anaplastic astrocytomas, and two astrocytomas). There was a statistical correlation between both p53 and L1 expression and TGF- β and L1 expression. No such correlation was found between p53 and TGF- β expression.

Conclusions—These results suggest that mutation of the p53 gene or expression of TGF- β may upregulate the expression of the L1 gene, thus resulting in high grade migration of glioma cells.

(J Clin Pathol 1998;51:13–17)

Keywords: cell adhesion molecules; glioma; p53; transforming growth factor β

The motility of glioma cells, clinically characterised by their high invasiveness into brain tissues, is thought to be mediated by various factors including cell adhesion molecules and extracellular matrices.^{1–3} Several adhesion molecules have been reported in the central nervous system that can regulate neural cell adhesion and motility. Among them, L1 has been identified as a membrane bound glycoprotein that belongs to the immunoglobulin superfamily

and consists of six immunoglobulin C2 (Ig C2) like domains, five fibronectin type III (FN type III) like domains, a transmembrane segment, and a relatively small cytoplasmic region.^{4–6} In vitro studies have demonstrated that the L1 molecule can guide cell migration in primary reaggregation cultures of mouse cerebellar cells.^{7–9} In addition, L1 may function in neuron–neuron adhesion, neurite fasciculation, and neurite outgrowth.^{10–12} Short-type L1, which is specific to non-neuronal-type cells, functions to aggregate cells and promote neurite outgrowth, as does full length L1.^{13–15} In neuronal growth cones, the homophilic binding of L1 molecules expressed on the surface of different cells, with or without the assistance of neural cell adhesion molecule (NCAM), can promote axonal growth.^{16 17} The heterophilic binding of neuronal L1 to substrate associated TAG1 (transiently expressed axonal surface glycoprotein 1) or its chicken homologue, axonin-1, also stimulates neurite outgrowth.^{18 19} Furthermore, L1 has been found to be a cellular ligand for integrin $\alpha_v\beta_3$.²⁰

Recently, using reverse transcriptase-polymerase chain reaction (RT-PCR) and fluorescence activated cell sorter (FACScan) analysis, we have demonstrated that glioma cells in culture express the L1 gene and that glioma cells are stimulated to migrate by soluble L1 molecules, released from L1 transfected fibroblast cells.²¹ Our results suggest that short-type L1 expressed on glioma cells may play an important role in the adhesion and migration of glioma cells through homophilic binding (probably through the extracellular immunoglobulin C2 domain of L1) and that short-type L1 participates in tumour invasion along neuronal fibres. It has been reported that transforming growth cell factor β (TGF- β) can upregulate the expression of NCAM and L1 in cultured Schwann cells.²² In cultures of immature astrocytes, TGF- β 1 and TGF- β 2 increased the expression of L1, which led to an L1 specific increase in neurite outgrowth of dorsal root ganglion neurons of the astrocyte substrate.²³ Osteogenic protein 1 (OP-1), a member of the TGF- β superfamily, has been reported to induce L1 expression in neuroblastoma \times glioma hybrid cells via a transcriptional mechanism of gene regulation.²⁴ In addition to these findings, it has been reported recently

Department of
Neurosurgery, Osaka
University Medical
School, 2-2,
Yamadaoka, Suita,
Osaka 565, Japan

T Tsuzuki
S Izumoto
T Ohnishi
S Hiraga
N Arita
T Hayakawa

Correspondence to:
Dr Izumoto.

Accepted for publication
10 October 1997

that retroviral mediated transduction of the p53 gene increases TGF- β expression in human glioblastoma cell lines.²⁵

Although the expression of L1 has been reported in neuronal tumours,²⁶ there have been no published studies on L1 expression in human glioma tissues. In the present study, we used immunohistochemical analysis to investigate the expression of L1 in human glioma tissues. We also examined possible correlations between both L1 and TGF- β expression and L1 and p53 expression and discuss here the biological significance of these factors for glioma invasion.

Methods

Twenty seven glioma specimens (13 glioblastomas, seven anaplastic astrocytomas, and seven astrocytomas) and a neuroblastoma specimen (used as a positive control for L1 immunohistochemistry) were obtained during surgery. The specimens were embedded in Tissue-Tek OCT compound medium (Miles Inc, Elkhart, Indiana, USA) and kept frozen at -80°C until use. For immunohistochemical analysis of the p53 and TGF- β proteins, 23 of these specimens comprising 10 glioblastomas, six anaplastic astrocytomas, and seven astrocytomas were used.

A rabbit polyclonal antibody against rat L1, which recognises an intracellular domain of L1 was used (a gift from Dr Asou, Keio University). In addition, a mouse monoclonal anti-human p53 antibody (pAb 1801; Oncogene Science, Uniondale, New York, USA) and a chicken polyclonal antihuman TGF- β 1 antibody (King Brewing Co, Kakogawa, Japan) which crossreacts with TGF- β 2 were used.

Frozen sections of 8 μm thickness were prepared on a cryostat, mounted on silane coated glass slides, and air dried for 30 minutes at room temperature. The sections were then fixed in acetone for 10 minutes at 4°C . After washing with 0.05 M Tris HCl buffer (pH 7.6) (Tris buffer), the sections were incubated in 0.3% H_2O_2 /Tris buffer for 30 minutes. After washing again, they were immersed in Tris buffer containing 1.5% normal horse serum and 0.2% Triton X 100, at room temperature. The anti-L1 antibody (1/100 dilution), anti-p53 antibody (1/50 dilution), or anti-TGF- β 1 antibody (1/50 dilution) in Tris buffer with 0.2% Triton X 100 was added to the sections and they were incubated overnight at 4°C . After washing with Tris buffer, the sections were reacted with 1/100 dilution of biotinylated antirabbit IgG (Vector Laboratories, Burlingame, California, USA) for L1, antimouse IgG (Vector Laboratories; 1/200 dilution) for p53, or antichickn IgG (Vector; 1/200 dilution) for TGF- β , for 30 minutes at room temperature. After another wash, the sections were reacted with avidin-biotin peroxidase complex (Vector Laboratories; Vectastain ABC kit, diluted 1/50) for 60 minutes. For visualisation of the immunoreaction, the sections were immersed in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 in Tris buffer. The sections were then washed in tap water, dehydrated, and analysed under a light micro-

Table 1 Results of immunohistochemical analyses

Case	L1	p53	TGF- β
GB1	+	+	+
GB2	+	+	+
GB3	+	+	+
GB4	+	+	-
GB5	+	-	+
GB6	+	+	+
GB7	-	+	-
GB8	-	+	+
GB9	-	-	+
GB10	-	-	-
GB11	-	-	-
GB12	-	-	-
GB13	-	-	-
AA1	+	+	+
AA2	+	+	+
AA3	+	+	+
AA4	-	-	+
AA5	-	-	-
AA6	-	-	-
AA7	-	-	-
AS1	-	-	+
AS2	-	-	+
AS3	-	-	-
AS4	-	-	-
AS5	-	-	-
AS6	-	-	-
AS7	-	-	-

Blanks indicate experiment not done as tumour sections not available. GB, glioblastoma; AA, anaplastic astrocytoma; AS, astrocytoma.

scope. Immunostaining was determined as positive (+) or negative (-) for each of the three proteins (L1, TGF- β , and p53), with faint staining assigned to (-). Statistical analysis to determine the relation between gliomal expression of the three proteins was performed with the χ^2 test.

Results

Immunopositive staining for L1 was detected with the antibody directed against the intracellular segment of L1 in nine (33%) of the 27 glioma specimens (six of the 13 glioblastomas, three of the seven anaplastic astrocytomas, and none of the seven astrocytomas) (table 1). A representative section is shown in fig 1B. In contrast to the section of neuroblastoma used as a positive control (fig 1A), immunopositive staining was not seen in all the cells in the sections of glioma but only in small aggregated cells.

Immunohistochemical examination with the anti-p53 antibody showed that 10 (43%) of 23 cases (seven of the 10 glioblastomas, three of the six anaplastic astrocytomas, and none of the seven astrocytomas) were immunopositive for the p53 protein (p53 (+)) (table 1). Representative p53 immunopositive sections shown in fig 1C show that the nucleus was stained mainly. As shown in fig 2, eight of the 10 p53 (+) cases were L1 (+), while 12 of the 13 p53 (-) cases were L1 (-). This is reflected in the statistically significant correlation between p53 and L1 immunopositive cases (χ^2 test; $p < 0.05$).

Discussion

In the present study, we have demonstrated that the neural cell adhesion molecule L1 is expressed to various degrees in human gliomas and is related strongly to the expression of

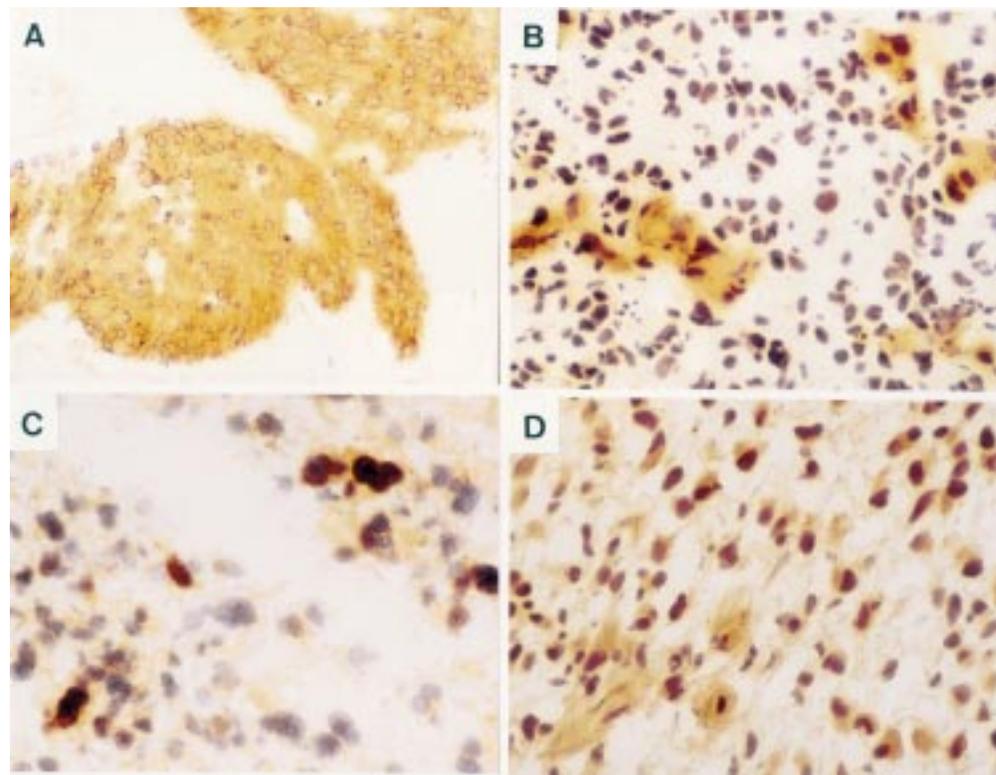


Figure 1 (A) Section of neuroblastoma specimen stained with the anti-L1 intracellular domain antibody as a positive control. L1 expression is seen in the majority of tumour cells. (B) Representative section of a glioma (glioblastoma 1 in table 1) positively immunostained with anti-L1 antibody; L1 is expressed only in small aggregated cells. (C) Shows positive immunostaining with the anti-p53 antibody in glioblastoma 1; positive staining is found mainly in the nucleus. (D) Shows positive immunostaining with the anti-TGF- β antibody in glioblastoma 1; the entire cell body of each cell is immunopositive. (Original magnifications: A, $\times 100$; B–D, $\times 200$.)

TGF- β and p53. This is the first report on immunohistochemical analyses of L1 expression in human glioma tissues.

Our immunohistochemical study showed that 33% of gliomas tested stained positively with the anti-L1 antibody, while 52% of the gliomas were positive for the anti-TGF- β antibody, and 43% were positive for the anti-p53 antibody. Our finding of L1 immunopositivity in more than 40% of both the glioblastomas and anaplastic astrocytomas but in none of the astrocytomas suggests that expression of L1 may be stimulated at a relatively early stage of glioma malignancy. There appeared to be a statistically significant correlation between positive immunostaining for TGF- β and L1, and between that of p53 and L1, whereas no such correlation was detected between positive immunostaining for p53 and TGF- β . These results prompted us to speculate that mutant p53, which can be detected by immunopositive staining with anti-p53 antibody, may cause overexpression of the TGF- β protein, so that the overexpressed TGF- β may regulate the expression of L1 in glioma cells. Fujiwara *et al* reported that expression of either wild-type p53 or mutant p53 can cause increased TGF- β expression,²⁵ while Reiss *et al* reported that mutant p53 inhibits the antiproliferative effect of TGF- β 1 by interfering with its signalling pathway.²⁷ Our immunohistochemical results could not statistically demonstrate a positive relation between p53 and TGF- β but showed only a tendency for correlation. On the other hand, p53 showed a clearly positive correlation

with expression of the L1 protein, suggesting that mutant p53 may upregulate the expression of L1 directly, and not through TGF- β , although there are no other reports on the relation between p53 and L1. The fact that TGF- β itself showed a positive correlation with L1, as also reported by Perides *et al*,²³ may indicate that there are different pathways for regulating the expression of the L1 protein. It is not well known how TGF- β regulates L1, but it seems possible that the TGF- β superfamily produces morphogens that act on mesenchymal and brain tissue by inducing the expression of L1 at critical periods in development and tissue repair.²³

In this report, faint staining was assigned to the TGF- β (-) group, while obviously positive staining, indicating increased expression of TGF- β , was assigned to the TGF- β (+) group. In relation to TGF- β , Paulus *et al* reported that the secretion of endogenous TGF- β 1 by glioma cells is functionally involved in the adhesion and invasion of gliomas.²⁸ In addition, Merzak *et al* stated that in human gliomas TGF- β exhibits a strong stimulatory effect on the migration and invasion of glioma cells *in vitro* as well as a mitogenic or inhibitory effect on cell proliferation.²⁹ Some of the stimulatory effect of TGF- β on the migration and invasion of glioma cells could be mediated by L1, the expression of which is regulated by TGF- β expression.

Sequence analyses described in our previous study revealed that short-type L1, generated by alternative splicing, was expressed in all

LI	p53	Positive	Negative
		Positive	○ ○ ○ ○ ○ □ □ □
Negative	○ ○	○ ○ □ □ △ △ △ △ △ △ △ △	

LI	TGF-β	Positive	Negative
		Positive	○ ○ ○ ○ ○ □ □ □
Negative	○ □ △ △	○ ○ ○ □ △ △ △ △ △ △	

TGF-β	p53	Positive	Negative
		Positive	○ ○ ○ ○ □ □ □
Negative	○ ○ ○	○ □ □ △ △ △ △ △	

Figure 2 Correlation between p53 and L1, TGF-β and L1, and p53 and TGF-β. Immunohistochemical examination with the anti-TGF-β antibody showed that 12 (52%) of 23 cases (six of the 10 glioblastomas, four of the six anaplastic astrocytomas, and two of the seven astrocytomas) were immunopositive for the TGF-β protein (TGF-β (+)). (Representative sections of TGF-β immunopositive staining are shown in fig 1D.) The entire cell body of each of the cells was immunopositive in the TGF-β (+) glioma specimens. Eight of the 12 TGF-β (+) cases were L1 (+), while 10 of the 11 TGF-β (-) cases were L1 (-). A statistically significant correlation was obtained between TGF-β and L1 immunopositive cases (χ^2 test; $p < 0.05$). Whereas seven of the 10 p53 (+) cases were TGF-β (+), and eight of the 13 p53 (-) cases were TGF-β (-); these results were not significant. Circles, glioblastomas; squares, anaplastic astrocytomas; triangles, astrocytomas.

gliomas tested.²¹ Short-type L1 transfected fibroblast cells are reported to function in adhesion and neurite outgrowth but only weakly in migration.¹⁵ To our knowledge, the function of short-type L1 in glioma cells remains unclear. However, we have shown the biological significance of L1 in glioma invasion along neuronal fibres. The immunohistochemical studies presented here suggest a hypothetical process in which mutant p53 directly (or, in part, indirectly through TGF-β) induces expression of L1, and TGF-β directly regulates the expression of L1, while both pathways affect glioma invasion in the central nervous system.

The extensive invasion of glioma cells into the brain creates great problems for tumour treatment. Recently, adhesion molecules other than L1, such as CD44 and integrins, have been shown to be implicated in glioma invasion.^{30,31} Glioma derived motility factor (GMF) promotes the migration of glioma cells by an autocrine mechanism³²; however, the

infiltration of glioma cells along nerve fibres is not fully explained by the function of GMF. Firstly, L1 expressed on glioma cells may contribute to invasion by homophilic interaction with other L1 molecules that are present on neurons or the surrounding oligodendrocytes. Furthermore, L1 in soluble form secreted by neural cell components around the tumour may also contribute to the spread of cells. It is also possible that glioma cells themselves release a soluble form of L1 that stimulates their motility and then accelerates the migration of glioma cells far from the original tumour site through binding to L1, which is expressed constitutively on neuronal fibres. Thus, inhibiting the function of L1 on glioma cells may represent a breakthrough in the treatment of highly invasive gliomas.

Although there have been no reports concerning the relation between p53 and glioma cell migration, p53 immunostaining is reported to correlate with clinical metastasis in breast cancer and colorectal cancer,^{33,34} while a tumour autocrine motility factor receptor revealed a significant homology with the p53 protein.³⁵ The motility of glioma cells that contain mutant p53 and overexpress p53 may be stimulated through the strong expression of L1 and other adhesion molecules, either directly or indirectly via TGF-β expression, resulting in the migration of tumour cells to areas of normal brain tissue.

In our study, we could not determine whether p53 positive gliomas were of a clinically invasive phenotype because it was difficult to identify clearly which cases were invasive or non-invasive. Thus, further studies, using biological and biochemical methods, are needed to investigate the relation between these proteins in gliomas and to validate the present results.

We thank Dr Asou, Keio University, Tokyo Japan, for providing the anti-L1 antibody. This study was supported in part by a Grant in aid from the Ministry of Education, Science, and Culture, Japan.

- Noble M, Albrechtsten M, Moller C, et al. Glial cells express N-CAM/D2-CAM-like polypeptides in vitro. *Nature* 1985;316:725-8.
- Poltorak M, Sadoul R, Keilhauer G, et al. Myelin-associated glycoprotein, a member of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte interaction. *J Cell Biol* 1987;105:1893-9.
- Takeichi M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development* 1988;102:639-55.
- Moos M, Tacke R, Scherer H, et al. Neural cell adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature* 1988;334:701-3.
- Miura M, Kobayashi M, Asou H, et al. Molecular cloning of cDNA encoding the rat neural cell adhesion molecule L1. *FEBS* 1991;289:91-5.
- Hlavin ML, Lemmon V. Molecular structure and functional testing of human L1CAM: an interspecies comparison. *Genomics* 1991;11:416-23.
- Asou H, Miura M, Kobayashi M, et al. Cell adhesion molecule L1 guides cell migration in primary reaggregation cultures of mouse cerebellar cells. *Neurosci Lett* 1992;144:221-4.
- Asou H, Miura M, Kobayashi M, et al. The cell adhesion molecule L1 has a specific role in neural cell migration. *NeuroReport* 1992;3:481-4.
- Miura M, Asou H, Kobayashi M, et al. Functional expression of a full-length cDNA coding for rat neural cell adhesion molecule L1 mediates homophilic intercellular adhesion and migration of cerebellar neurons. *J Biol Chem* 1992;267:10752-8.
- Lindner J, Rathjen FG, Schachner M. L1 mono- polyclonal antibodies modify cell migration in early postnatal mouse cerebellum. *Nature* 1983;305:427-30.

- 11 Appel F, Holm J, Conscience JF, *et al.* Several extracellular domains of the neural cell adhesion molecule L1 are involved in neurite outgrowth and cell body adhesion. *J Neurosci* 1993;13:4764–75.
- 12 Sonderegger P, Rathjen FG. Regulation of axonal growth in the vertebrate nervous system by interactions between glycoproteins belonging to two subgroups of the immunoglobulin superfamily. *J Cell Biol* 1992;119:1387–94.
- 13 Reid RA, Hemperly JJ. Variants of human L1 cell adhesion molecule arise through alternate splicing of RNA. *J Mol Neurosci* 1992;3:127–35.
- 14 Wong EV, Cheng G, Payne HR, *et al.* The cytoplasmic domain of the cell adhesion molecule L1 is not required for homophilic adhesion. *Neurosci Lett* 1995;200:155–8.
- 15 Takeda Y, Asou H, Murakami Y, *et al.* A nonneural isoform of cell adhesion molecule L1: tissue-specific expression and functional analysis. *J Neurochem* 1996;66:2338–49.
- 16 Lemmon V, Farr KL, Lagenaur C. L1-mediated axon outgrowth occurs via a homophilic binding mechanism. *Neuron* 1989;2:1597–603.
- 17 Kadmon G, Kowitz A, Altevogt P, *et al.* The neural cell adhesion molecule N-CAM enhances L1-dependent cell-cell interactions. *J Cell Biol* 1990;110:193–208.
- 18 Kuhn TB, Stoeckli ET, Condrau MA, *et al.* Neurite outgrowth on immobilized axonin-1 is mediated by heterophilic interaction with L1. *J Cell Biol* 1991;115:1113–26.
- 19 Felsenfeld DP, Hynes MA, Skoler KM, *et al.* TAG-1 can mediate homophilic binding, but neurite outgrowth on TAG-1 requires an L1-like molecule and $\beta 1$ integrins. *Neuron* 1994;12:675–90.
- 20 Montgomery AMP, Becker JC, Siu CH, *et al.* Human neural cell adhesion molecule L1 and rat homologue NILE are ligands for Integrin $\alpha_5\beta_3$. *J Cell Biol* 1996;132:475–85.
- 21 Izumoto S, Ohnishi T, Arita N, *et al.* Gene expression of neural cell adhesion molecule L1 in malignant gliomas and biological significance of L1 in glioma invasion. *Cancer Res* 1996;56:1440–4.
- 22 Stewart HJS, Rougon G, Dong Z, *et al.* TGF- β s upregulate NCAM and L1 expression in cultured Schwann cells, suppress cyclic AMP-induced expression of O4 and galactocerebroside, and are widely expressed in cells of the Schwann cell lineage in vivo. *Glia* 1995;15:419–36.
- 23 Perides G, Safran RM, Downing LA, *et al.* Regulation of neural cell adhesion molecule and L1 by the transforming growth factor- β superfamily. *J Biol Chem* 1994;269:765–70.
- 24 Perides G, Hu G, Rueger DC, *et al.* Osteogenic protein-1 regulates L1 and neural cell adhesion molecule gene expression in neural cells. *J Biol Chem* 1993;268:25197–205.
- 25 Fujiwara T, Mukhopadhyay T, Cai DW, *et al.* Retroviral-mediated transduction of p53 gene increases TGF- β expression in a human glioblastoma cell line. *Int J Cancer* 1994;56:834–9.
- 26 Figarella-Branger DF, Durbec PL, Rougon GN. Differential spectrum of expression of neural cell adhesion molecule isoforms and L1 adhesion molecules on human neuroectodermal tumors. *Cancer Res* 1990;50:6364–70.
- 27 Reiss M, Vellucci VF, Zhou Z. Mutant p53 tumor suppressor gene causes resistance to transforming growth factor $\beta 1$ in murine keratinocytes. *Cancer Res* 1993;53:899–904.
- 28 Paulus W, Baur I, Huettnner C, *et al.* Effects of transforming growth factor- $\beta 1$ on collagen synthesis, integrin expression, adhesion and invasion of glioma cells. *J Neuropath Exp Neurol* 1995;54:236–44.
- 29 Merzak A, McCrea S, Koocheckpour S, *et al.* Control of human glioma cell growth migration and invasion in vitro by transforming growth factor $\beta 1$. *Br J Cancer* 1994;70:199–203.
- 30 Okada H, Yoshida J, Sokabe M, *et al.* Suppression of CD44 expression decreases migration and invasion of human glioma cells. *Int J Cancer* 1996;66:255–60.
- 31 Paulus W, Baur I, Beutler AS, *et al.* Diffuse brain invasion of glioma cells requires $\beta 1$ integrins. *Lab Invest* 1996;75:819–26.
- 32 Ohnishi T, Arita N, Hayakawa T, *et al.* Purification of motility factor (GMF) from human malignant glioma cells and its biological significance in tumor invasion. *Biochem Biophys Res Commun* 1993;193:518–25.
- 33 Faille A, De Cremoux P, Extra JM, *et al.* p53 mutations and overexpression in locally advanced breast cancers. *Br J Cancer* 1994;69:1145–50.
- 34 Bertorelle R, Esposito G, Del Mistro A, *et al.* Association of p53 gene and protein alterations with metastases in colorectal cancer. *Am J Surg Pathol* 1995;19:463–71.
- 35 Watanabe H, Carmi P, Hogan V, *et al.* Purification of human tumor cell autocrine motility factor and molecular cloning of its receptor. *J Biol Chem* 1991;266:13442–8.