# Loss of DCC expression in astrocytomas: relation to p53 abnormalities, cell kinetics, and survival

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**Abstract**

***Aims*—Although frequent reduction or loss of DCC (deleted in colorectal carcinomas) has been demonstrated in gliomas, the association with cell kinetics and survival is still unclear.**

***Methods*—A total of 119 astrocytomas, comprising 39 grade IV, 36 grade III, and 44 low grade tumours, were immunohistochemically investigated, along with 26 normal adult brain samples and two fetal brains. The results were compared with p53 abnormalities, Ki-67 labelling index (LI), mitotic index (MI), apoptotic index (AI), and survival.**

***Results*—In normal adult and fetal brain tissues, DCC expression was detected in mature and terminally differentiated neuronal cells but not glial elements. In astrocytomas, whereas DCC expression was still clearly shown with low grade malignancy, DCC scores were significantly decreased in high histological grade malignancy, along with an increase in cell kinetics determined by AI, MI, and Ki-67 LI values. In addition, p53 LI values were significantly increased, although a direct link between DCC scores and p53 LI values was not evident. Univariate analysis revealed that high DCC scores and low p53 LI values were closely related to a favourable outcome for astrocytoma, although only the AI was an independent prognostic factor.**

***Conclusions*—The loss of DCC expression may be closely related to changes in cell kinetics and tumour phenotype in astrocytomas, independent of p53 abnormalities.**

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Keywords: astrocytoma; deleted in colorectal carcinoma gene; p53; cell kinetics

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Gliomas are the most common primary brain tumours, accounting for more than 40% of all central nervous system neoplasms.[1] They range in clinical behaviour and histological appearance from indolent, well differentiated lesions to highly anaplastic, rapidly growing tumours.[2] Although cell kinetic information is an important aspect of the biology of gliomas, it has become clear that neoplastic evolution towards the glioblastoma entity is a multistep process that involves deregulation of several genes related both to proliferation and differentiation.

Several studies have indicated distinct differences in molecular genetic pathways between primary and secondary glioblastomas. For example, primary glioblastomas, without a history of any preceding low grade lesion, exhibit a loss of 10p and 10q, whereas secondary glioblastomas, through progression from low grade glioma, feature losses of 9p, 17p, 13q, 19q, and 22q; overexpression of the genes encoding platelet derived growth factor (PDGF) and the PDGF receptor (PDGFR); and mutation of the gene encoding the retinoblastoma protein (Rb). Moreover, it has been proposed that two major defining characteristics are frequent epidermal growth factor (EGFR) gene amplification in the former and p53 mutations in the latter.[1 3]

The deleted in colorectal carcinomas (DCC) gene, located on human chromosome 18q21, has been identified as a candidate tumour suppressor gene.[4] It encodes a 1447 amino acid transmembrane protein that belongs to the neural cell adhesion molecule family based on its four immunoglobulin-like and six fibronectin type III-like extracellular domains.[5] DCC transcripts and protein are found in low amounts in most tissues but are abundant in the central and peripheral nervous system.[5 6] Although the function of the DCC gene is still poorly understood, some data suggest a role in regulating cell growth and differentiation, particularly in the nervous system.[5 7] In our present study, to investigate the hypothesis that DCC may play a role in glioma formation, we assessed DCC gene expression in astrocytic tumours, the most common type of gliomas, by means of immunohistochemistry, and sought to correlate this with tumour grading, p53 expression, cell kinetics, and prognosis.

## Methods

### CASE SELECTION

A total of 119 cases of astrocytoma, surgically resected at the Kitasato University Hospital during the period from 1978 to 1999, were investigated, along with 26 samples of normal brain tissue adjacent to neoplastic lesions. Two cases of fetal brain tissue (11 and 12 gestational weeks), incidentally obtained as surgical materials, were also examined. All tissues were routinely fixed in 10% formalin and processed for embedding in paraffin wax. Histological diagnoses were made according to the World Health Organisation (WHO) classification of brain tumours (1993). The tumours comprised 75 high grade astrocytomas, including 39 glioblastomas (G IV; age range, 12–38 years; mean, 49.3; SD, 14.8) and 36 anaplastic astrocytomas (G III; age range, 0–72 years; mean, 41.0; SD, 20.2), in addition to 44 low grade astrocytomas (G II; age range, 5–71 years; mean, 34.3; SD, 17.2), which were

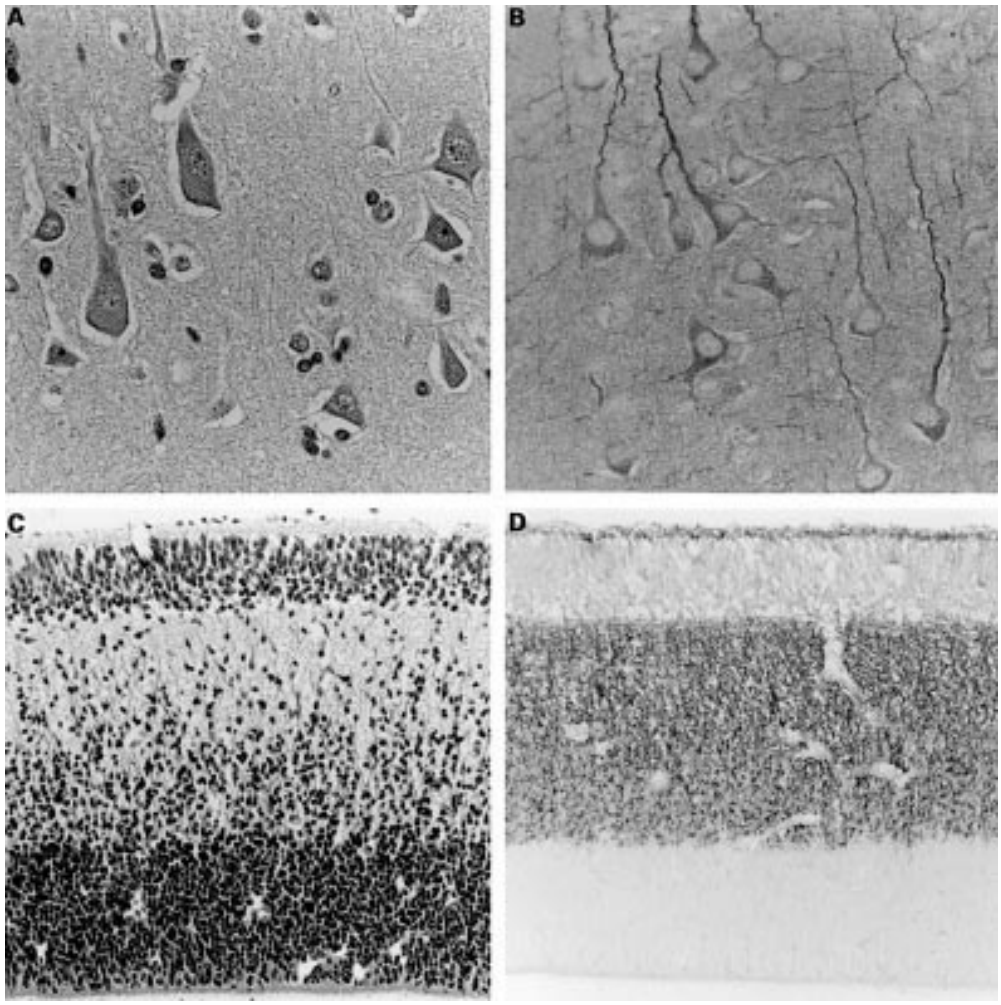


*Figure 1 (A, C) Haematoxylin and eosin staining and (B, D) DCC (deleted in colorectal carcinoma) immunoreactivity in adult (A, B) and fetal (C, D) normal brain tissue. (B) DCC immunopositivity is evident in axons and nerve cell bodies, but not in glial cells. (D) Note that neuronal and glial precursor cells in the ventricular and subventricular zone (lower lamina) are negative. Original magnification (A, B), ×400; (C, D) ×200.*

further subclassified into 42 fibrillary (six cases had a small foci of gemistocytic cell component) and two gemistocytic subtypes. No pilocytic astrocytomas were included. Four of the 39 glioblastomas were categorised as secondary type because the patients had a history of preceding low grade astrocytoma.

Thirty four of the patients with low grade astrocytoma had been treated with postoperative radiation because of incomplete resection, whereas all the patients with high grade astrocytoma had received adjuvant radiation and chemotherapy.

### IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed using a combination of microwave oven heating and standard streptavidin–biotin–peroxidase complex (LSAB kit; Dako, Copenhagen, Denmark) methods. After heat treatment (six five minute cycles for DCC and three five minute cycles for Ki-67 and p53) in 10 mM citrate buffer (pH 6.0), the slides were incubated overnight at 4°C with optimum dilutions of primary antibodies. The antibodies used were a mouse monoclonal antihuman DCC antibody (clone G97–449; 1/1000 dilution; PharMingen, San Diego, California, USA), a rabbit antihuman Ki-67 rabbit polyclonal antibody (1/150 dilution; Dako), and DO-7 (1/1000 dilution; Novocastra, Newcastle upon Tyne, UK) for the p53 protein (this antibody reacts both with wild-type and mutant p53). As a negative control, 0.01M phosphate buffered saline or mouse serum (1/500 dilution) was used instead of primary antibodies. As a positive control for p53, an ovarian carcinoma known to express the protein was used.[8]

### SCORING FOR DCC IMMUNOREACTIVITY

Scoring of the immunohistochemistry results was performed according to the methods described by Sinicrope *et al*,[9] with minor modifications. Briefly, based on the proportions of immunopositive cells, five categories were defined as follows: 0, all negative; 1+, < 2% positive cells; 2+, 25–49%; 3+, 50–74%; and 4+, > 75%. The immunointensity was also subclassified into four groups as follows: 0, negative; 1+, weak; 2+, moderate; 3+, strong. Normal nerve cells were used as internal positive controls, the immunointensity being set as 3+. Immunoreactivity scores for each case were

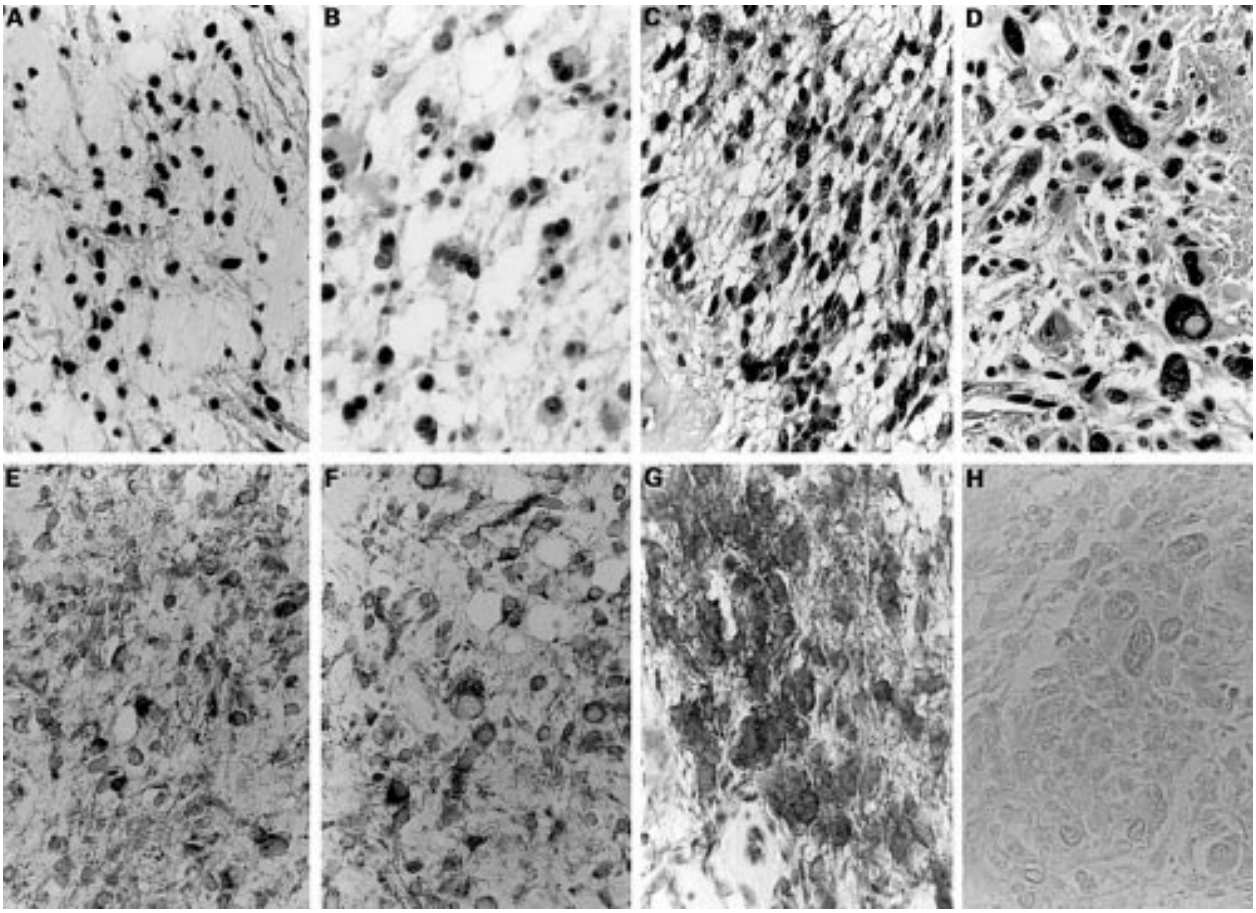


*Figure 2 Haematoxylin and eosin staining (A, B, C, D) and DCC (deleted in colorectal carcinoma) immnoreactivity (E, F, G, H) in low grade astrocytomas (A, E: fibrillary subtype; B, F: gemistocytic subtype), an anaplastic astrocytoma (C, G), and a glioblastoma (D, H). Note strong intensities (E, F, G), and absence of staining (H). Original magnification, ×400.*

produced by multiplication of the values for the two parameters.

### p53 AND KI-67 LABELLING INDICES

p53 and Ki-67 labelling indices (LI) were determined as percentages by counting at least 1000 cells in randomly selected high power fields (×400). Only nuclei with strong diffuse staining were considered positive.

### APOPTOTIC AND MITOTIC INDICES

Apoptotic cells, which generally showed condensed and homogenous chromatin and strongly eosinophilic cytoplasm, with or without small nuclear fragments, were identified according to the criteria of Kerr and colleagues[10] in haematoxylin and eosin stained sections using high power magnification (×400). Apoptotic indices (AI) were calculated after at least 1000 nuclei in randomly selected fields were examined for each sample. Areas of severe inflammatory cell infiltration and necrosis were excluded. Mitotic indices (MI) were calculated in a similar manner, counting mitotic figures.

### STATISTICS

Statistical analysis of data for immunoreactivity scores, p53, Ki-67 LI values, MI values, and AI values was performed using the Mann-Whitney U test, Spearman' s correlation coefficient, and the $\chi^2$ test. Survival was measured from the time of the primary operation and survival curves were generated by the methods of Kaplan and Meier. The log rank test and Cox proportional hazard modelling were performed to compare survival rates. The cut off point for significance was defined as $p < 0.05$.

## Results

### DCC AND p53 EXPRESSION

DCC immunoreactivity was intense in the axons of neuronal cells but faint in nerve cell bodies, in contrast to glial cells, which were consistently negative in normal brain tissue (fig 1B). In fetal brain tissue, DCC immunopositivity was detected in mature neuronal cells in the intermediate zone, cortical plate, and molecular layers, whereas neuronal precursor cells and glial fibrillary acidic protein positive glial cells lacked immunoreactivity (fig 1D). In low grade astrocytomas, moderate to strong diffuse immunoreactivity for DCC was seen in the cytoplasm and neuropil, with similar expression patterns seen in fibrillary and gemistocytic phenotypes, independent of cell type and cell size. Relatively frequent DCC immunostaining was also seen in anaplastic astrocytomas, whereas it was completely lacking or sporadically weak in glioblastomas (fig 2E–H). In addition, progressive loss of DCC immunoreactivity was seen in two of four secondary glioblastomas.

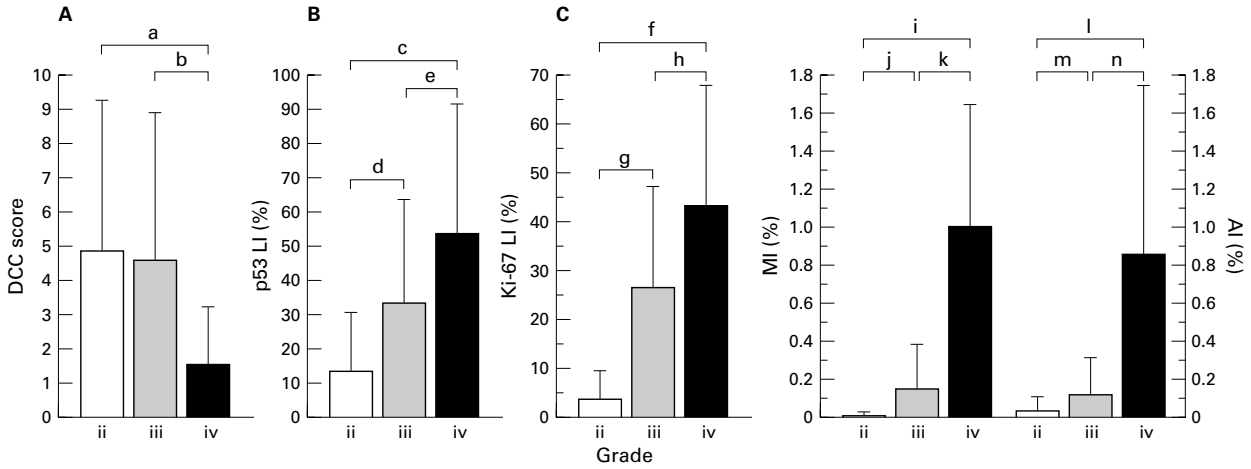


*Figure 3 (A) DCC (deleted in colorectal carcinoma) immunoreactivity scores; (B) p53 labelling index (LI); and (C) Ki-67 LI, mitotic index (MI), and apoptotic index (AI). The data are mean (SD) values. a: p = 0.004; b: p = 0.0032; c, f, g, I, k, l, n: p = < 0.0001; d: p = 0.0006; e: p = 0.017; h: p = 0.005; j: p = 0.0013; n: p = 0.0023.*

A distinct nuclear pattern of p53 immunoreactivity was seen in astrocytomas—sporadic in low and diffuse in high grade tumours—but no immunostaining was seen in normal tissue.

Average DCC scores were significantly lower in grade IV astrocytomas than in other tumours, whereas average p53 LI values showed a stepwise increase from low to high grade astrocytomas, with the difference being significant (fig 3A, B).

### Ki-67 LI, MI, AND AI

Average Ki-67 LI values, in addition to MI and AI values, showed a stepwise increase from grade II to grade IV astrocytomas, with the difference being significant (fig 3C).

Significant correlations among Ki-67 LI, MI, and AI were evident in all categories (Ki-67 LI *v* AI, $r = 0.47$, $p < 0.0001$; MI *v* AI, $r = 0.45$, $p < 0.0001$; Ki-67 LI *v* MI, $r = 0.48$, $p < 0.0001$).

### RELATIONS BETWEEN DCC, p53 LI, Ki-67 LI, MI, AND AI

To examine the relation between DCC or p53 expression and cell kinetics, a subdivision was made into two categories on the basis of average values (mean DCC score, 3.7; SD, 0.4; mean p53 LI, 32.4; SD, 3.1). Overall, high

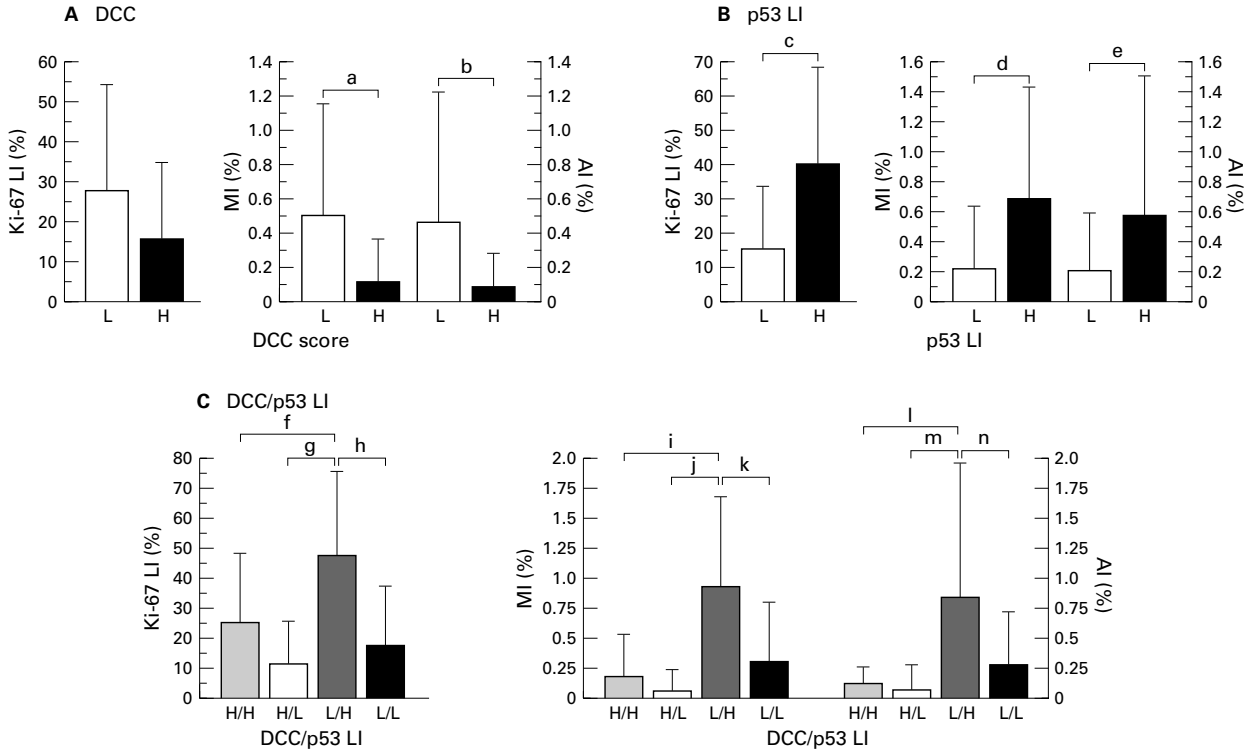


*Figure 4 Correlations among immunoreactive scores for (A) DCC (deleted in colorectal carcinoma), (B) p53 labelling index (LI), or (C) DCC/p53 LI combination and Ki-67 LI, mitotic index (MI), or apoptotic index (AI) overall. Subdivisions were into two categories on the basis of average DCC score (mean, 3.7; SD 0.4) and p53 LI (mean, 3.2; SD, 3.1). a: p = 0.0004; b: p = 0.0013; c: p < 0.0001; d: p = 0.0002; e: p = 0.0004; f: p = 0.0168; g: p < 0.0001; h: p < 0.0001; I: p = 0.0004; j: p < 0.0001; k: p < 0.0001; l: p = 0.0024; m: p < 0.0001; n: p = 0.0015. L, low DCC score; H, high DCC score.*

*Table 1 Univariate analysis of prognostic factors for astrocytomas*

| *Factor* | *Category* | *n* | *Log rank c2* | *p Value* | *Favourable feature* |
|---|---|---|---|---|---|
| Grade | II | 25 | 22.919 | <0.0001 | Grade II |
| | III | 24 | | | |
| | IV | 19 | | | |
| DCC score | ⩾3 | 41 | 4.346 | 0.037 | High score |
| | ⩽4 | 27 | | | |
| p53 LI (%) | >40 | 22 | 5.330 | 0.021 | Low value |
| | ⩽40 | 46 | | | |
| Ki-67 LI (%) | >30 | 21 | 13.004 | 0.0003 | Low value |
| | ⩽30 | 47 | | | |
| MI (%) | >0.40 | 19 | 20.795 | <0.0001 | Low value |
| | ⩽0.40 | 49 | | | |
| AI (%) | >0.40 | 14 | 5.032 | <0.0001 | Low value |
| | ⩽0.40 | 54 | | | |
| DCC score ⩾3 | p53 LI >40 | 31 | 15.105 | 0.002 | DCC score ⩽4 |
| DCC score ⩾3 | p53 LI ⩽40 | 10 | | | p53 LI >40 |
| DCC score ⩽4 | p53 LI >40 | 15 | | | |
| DCC score ⩽4 | p53 LI ⩽40 | 12 | | | |

n, number of cases; DCC, deleted in colorectal cancer; LI, labelling index; MI, mitotic index; AI, apoptotic index.

DCC expression (score = 4) was associated with lower values of MI and AI (fig 4A). Although there was a similar association with Ki-67 LI, it was not significant (p = 0.067). In contrast, high p53 LI values were significantly associated with higher values for the three proliferation indices (fig 4B). Tumours with a combination of low DCC score and high p53 LI showed significantly higher values for Ki-67 LI, MI, and AI than those with a high DCC and a low p53 LI (fig 4C). These associations were also retained after exclusion of low grade tumours (data not shown).

### SURVIVAL ANALYSIS

Overall univariate analysis for prognostic markers by the log rank test revealed prognostic value for histological grade, DCC score, and p53 LI, Ki-67 LI, MI, and AI values, and the combination of DCC and p53 status (table 1). Figure 5 illustrates Kaplan-Meier curves for the relation between DCC score or p53 LI and overall survival time. In the multivariate analysis, AI values had an independent prognostic impact.

In high grade tumour groups, none of the markers investigated had prognostic significance, with the exception of MI (p = 0.02, univariate) and AI (p = 0.02, multivariate).

## Discussion

Our study clearly showed that DCC protein is expressed predominantly in axons and nerve cell bodies in the adult normal brain, limited to mature, terminally differentiated neuronal cells, and is not present in precursor cells in fetal brain. The fact that DCC is a receptor or a component of a receptor that mediates the effects of netrin-1 on commissural axons[11] suggests that it plays a role in controlling and maintaining the differentiation of neurones. In contrast, we could not detect DCC immunoexpression in normal astrocytes, even in fetal brain, although DCC transcripts and protein have been detected in the normal astrocytic lineage using the reverse transcription polymerase chain reaction (RT-PCR) and immunoblot assays.[12]

Under pathological conditions, if DCC is present, increased cell proliferation may be kept under control, whereas a lack of this protein might lead to loss of cell interaction and proliferation control, and eventually cell dispersion.[13] Recent studies have revealed that frequent loss of heterozygosity (LOH), loss of expression, or somatic mutations of the DCC gene are involved in tumorigenesis in various types of human cancers.[5 14–18] We have shown previously that reduction or loss of DCC immunoreactivity correlates positively with impaired mRNA expression but not LOH status in ovarian and endometrial carcinomas.[8 19]

Several investigators have indicated a frequent loss of DCC expression in most high grade glioblastomas. Scheck and Coons suggested that DCC transcripts are reduced in most high grade gliomas,[20] and Ekstrand *et al* showed altered DCC expression in glioblastoma xenografts.[21] Reyes-Mugica *et al* demonstrated that the DCC expression is more likely to be reduced in higher grade gliomas, and that secondary glioblastomas are more often DCC negative (eight of 15) compared with primary cases (six of 26).[12] In our series, the reduction or loss of DCC immunoreactivity was closely related to high histological grades, being significantly associated with cell proliferation, even in high grade astrocytomas. Moreover, progressive reduction of DCC expression was also seen in two of four secondary glioblastomas.

In contrast, we found that p53 LI values were positively linked with histological malignancy and cell kinetics determined by parameters of cell division and apoptosis. Therefore, an inverse association might have been expected between DCC expression and p53 alteration during astrocytoma progression, but our results revealed no direct significant link between the two, suggesting that impaired DCC expression might affect changes in tumour cell kinetics, independent of p53 status.

Nakatani *et al* have reported a significant association between reduced DCC expression and unfavourable prognosis in astrocytomas

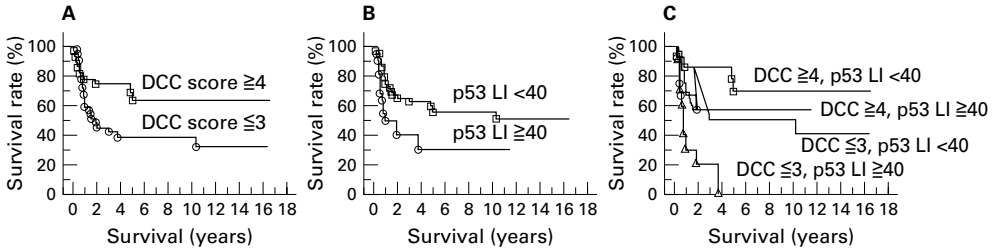


*Figure 5 Kaplan-Meier survival curves according to immunoreactivity scores for (A) DCC (deleted in colorectal carcinoma), (B) p53 labelling index (LI), and (C) a combination of the two parameters. (A), p = 0.0371; (B), p = 0.021; (C), p = 0.0017.*

using the RT-PCR approach,[22] whereas conflicting results have been published regarding the prognostic value of p53 alterations.[2 23 24] In our series, low DCC scores and high p53 LI values were significantly related to poor prognosis in astrocytoma on univariate but not multivariate analysis. Moreover, tumours with low DCC and high p53 expression showed the shortest survival, with higher values for the cell kinetic markers investigated. We therefore concluded that a combined analysis of DCC and p53 might be useful for predicting the behaviour of astrocytomas.

Regarding outcome, cell proliferation determined by Ki-67 immunohistochemistry or mitosis, in addition to tumour grade and patient age, have been widely accepted as important for astrocytomas.[25 26] Our results also revealed a significant correlation between high AI values and poor outcome, in contrast to Korshunov *et al*, who documented a positive association between high AI and favourable outcome in glioblastoma, postulating that a greater proportion of cell loss through cell death should result in slower tumour growth and a longer time to relapse and death of the patient.[27] Although the reason for this discrepancy is unclear, we conclude from our present data that a high AI has an independent poor prognostic impact because we found significant positive correlations between AI, MI, and Ki-67 LI values in astrocytomas. In addition, several investigations have also indicated a significant relation between high AI indexes and a poor prognosis in a variety of human malignancies, such as breast, lung, and ovarian carcinomas.[28–30]

In conclusion, our current study demonstrated that loss of DCC expression might be closely related to changes in cell kinetics and astrocytoma phenotype, independent of p53 abnormalities.

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