Brain Pathology 11: 39-43(2001)

RESEARCH ARTICLE

Clonal Analysis in Glioblastoma with Epithelial Differentiation

Wolf Mueller[1], Ulrike Lass[1], Jochen Herms[2], Klaus Kuchelmeister[3], Markus Bergmann[4], Andreas von Deimling[1]

[1] Institute for Neuropathology, Charité Humboldt University, D-13353 Berlin, Germany
[2] Institute for Neuropathology, University Göttingen, D-37075 Göttingen, Germany
[3] Institute for Neuropathology, University Giessen, D-35392 Giessen, Germany
[4] Institute for Neuropathology, General Hospital Bremen, D-28325 Bremen, Germany

Epithelial differentiation in glioblastomas (GBM) may be associated with circumscribed growth and focal keratin expression resembling carcinoma metastasis. Therefore these rare lesions can pose a diagnostic problem suggesting coincidental occurrence of two separate neoplasms. However molecular analysis should succeed in establishing a common origin of seemingly unrelated tumor samples. Five GBMs exhibiting epithelial differentiation were microdissected and analyzed for mutations in the *TP53* gene. SSCP analysis of exons 5-8 was followed by direct sequencing of aberrantly migrating fragments. *TP53* mutations were identified in tumors from two of five patients. A G→T transversion in codon 176 was detected in a tumor, initially diagnosed as metastases of unknown origin, however, a later autopsy revealed GBM. In this lesion, the mutation was observed in both, areas of astrocytic differentiation and areas of epithelial differentiation. One tumor diagnosed as GBM with epithelial differentiation carried C→T transition in codon 211 in both, areas of astrocytic and epithelial differentiation. Thus, molecular analysis proved clonality in two GBMs with epithelial differentiation, thereby excluding a collision tumor. The present data support the concept of clonal origin of these morphologically heterogeneous lesions.

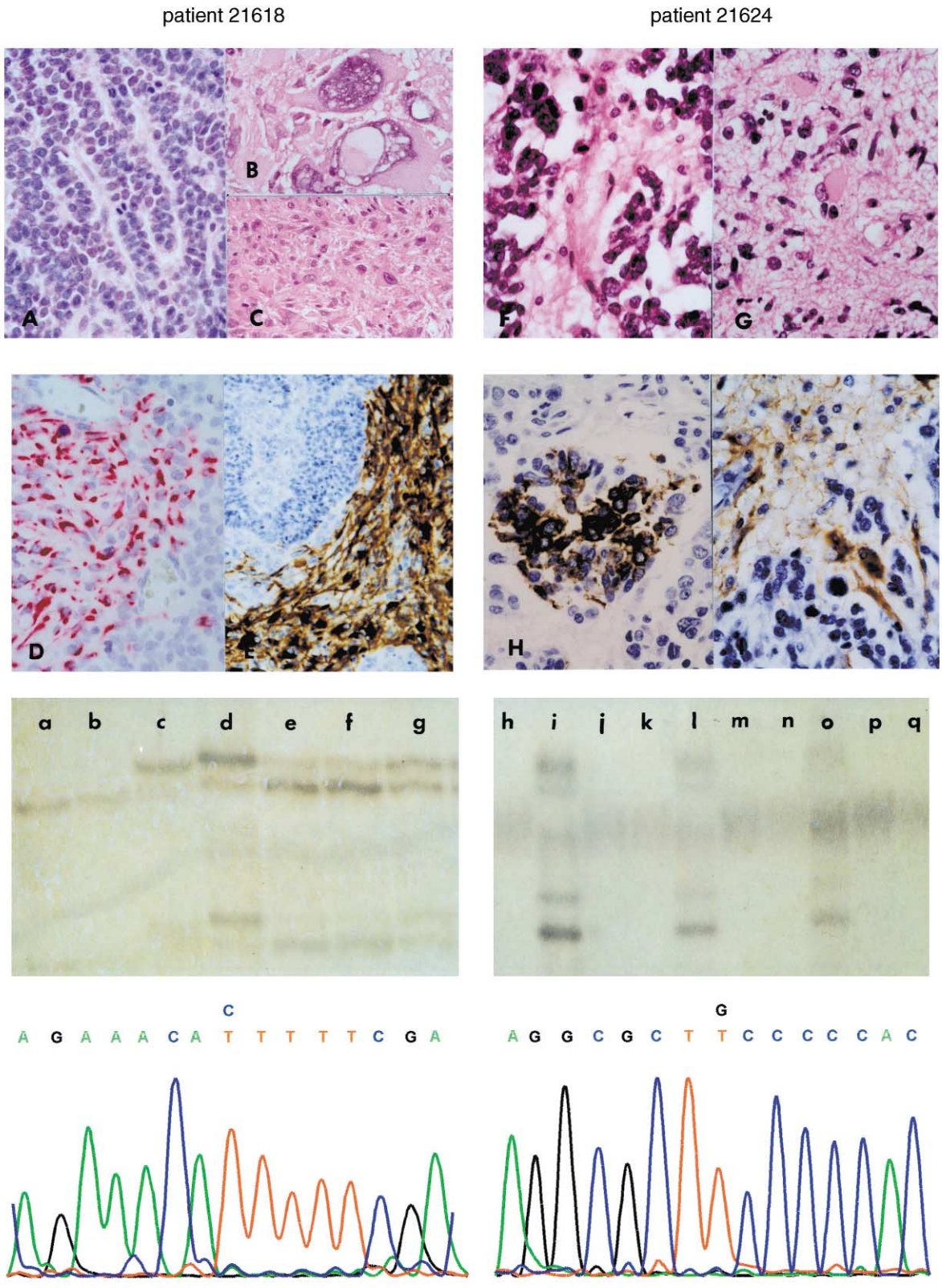
Introduction

Epithelial differentiation is a rare event in malignant gliomas. It is characterized by both morphological and immunohistochemical features typically seen in carcinoma (13, 17). Epithelial differentiation in gliomas therefore poses the problem of distinguishing these gliomas from metastases of carcinoma. In a study of 6 gliomas with epithelial differentiation including four gliosarcomas and two glioblastomas (GBM) five of the tumors initially were diagnosed as or suspected to be carcinoma metastases (17). In gliomas, areas with epithelial differentiation frequently exhibit adenoid or papillary architectures with rounded cells lacking prominent fibrillary processes. Immunohistochemically these cells are frequently devoid of glial fibrillary acidic protein (GFAP) and focally bind antibodies against cytokeratins (13, 17). The interpretation of these lesions as variant of GBM have rested so far on the presence of tumor cells with epithelial, astrocytic and intermediate differentiation as well as focal GFAP expression in at least a fraction of these different tumor portions (13, 17).

GBM is thought to develop by the clonal expansion of a genetically altered precursor cell. Clonality of different tumor portions can be assessed with different methods such as X-chromosome inactivation (12) or detection of identical mutations. *TP53* mutations have been demonstrated in up to half of astrocytomas WHO grade II and anaplastic astrocytomas WHO grade III (4, 6, 7, 21, 18) and up to a third of GBMs (4, 6, 7, 9, 15, 18, 22). *TP53* mutations cluster in the highly conserved domains II–V including exons 5-8 (2). This clustering allows to efficiently detect the majority of mutations in a limited stretch of DNA.

In order to establish clonality and exclude the possibility of a collision tumor we examined a series of 5

Correspondence author:
Andreas von Deimling, M.D., Institut für Neuropathologie, Charité Humboldt University, Campus Virchow Klinikum, Augustenburger Platz 1, D-13353 Berlin, Germany; Tel.: +49-30-450-56042 / Fax: +49-30-450-56940; E-mail: andreas.von_deimling@charite.de


patient 21618
patient 21624
A
B
C
F
G
D
E
H
a b c d e f g
h i j k l m n o p q
C
A G A A A C A T T T T T C G A
G
A G G C G C T T C C C C C A C

GBMS with epithelial differentiation for mutations in *TP53* by single strand conformation polymorphism (SSCP) analysis and direct sequencing of aberrantly migrating DNA fragments.

Material and Methods

Tissue Samples. Paraffin embedded sections were collected from five different patients diagnosed with GBMs with epithelial differentiation. One patient each was treated at the University Hospital of Göttingen, the General Hospital of Bremen and the Charité, Berlin. Tumor samples from two patients were retrieved from the University Hospital of Giessen. The patient from Göttingen was autopsied and therefore, additional material was made available. A corresponding blood sample was available from the patient in Berlin. All tumors were classified and graded as GBMs with focal epithelial differentiation following the guidelines of the World Health Organization (14). Genomic DNAs from paraffin embedded tumor samples and leukocytes were extracted using commercially available kits (InViSorb MaxiBlood Kit, Invitec, Berlin, Germany and QIAamp DNA Mini Kit, Quiagen, Hilden, Germany).

Analysis of the TP53 Gene. In the first round of investigation exons 5 to 8 of the *TP53* gene were analyzed by single stranded conformational polymorphism (SSCP) and direct sequencing without microdissecting areas of distinct differentiation. PCR products were separated electrophoretically on long acrylamide gels followed by silver staining as previously described (1). Aberrantly migrating signals were excised, re-amplified, and sequenced bidirectionally on a semiautomatic sequencer (Applied Biosystems model 377).

Tumors with *TP53* mutations were subjected to a second round of investigation following microdissection of areas with astrocytic and epithelial differentiation. A series of 10 adjacent sections, 10μm each was cut. The first and the last sections were stained with HE in order to delineate the borders of areas with astrocytic and epithelial differentiation. The respective areas were removed from the unstained sections using a small scalpel. Following excision all slides were stained with HE and compared with the first and last section of the respective series. In the tumor sample obtained at our hospital an additional area with giant cell GBM morphology was excised.

Results

Neuropathological examination revealed GBM with focal epithelial differentiation in all five cases. In all instances areas of epithelial differentiation were well circumscribed. Focal cytokeratin expression was detected immunohistochemically in all specimens with epithelial differentiation. In tumor portions from all five patients areas of typical astroglial differentiation were present. The tumor of patient 21618 exhibited both, areas of typical GBM and areas corresponding to giant cell GBM. Representative fields from patients 21618 and 21624 are shown in Figure 1. All five patients were male. With the exception of one patient who was aged 45 all patients were older then 60 years at surgery. None of the patients had a history of a preceding lower grade glioma.

SSCP analysis and consecutive bi-directional sequence analysis revealed mutations in two of the five tumors. Patient 21618 exhibited a C632T transition in codon 211 of exon 6 resulting in an exchange of threonine by isoleucine. This mutation was detected in all, areas of fibrillary astrocytic differentiation, areas resembling giant cell GBM and areas characterized by epithelial differentiation. Corresponding DNA from peripheral white blood cells were wild type.

Patient 21624 was initially diagnosed as suffering from an intracranial metastasis of undetermined origin. However, later autopsied revealed a temporal GBM. In addition multiple malignant tumors of epithelial appearance were removed from an intradural spinal localization. Molecular analysis revealed a point mutation resulting in a G527T transversion in codon 176 of exon 5. This mutation led to the substitution of cysteine by

Figure 1. (Opposing page) *Morphological, immunohistochemical and molecular findings in two glioblastomas with epithelial differentiation.* Left upper panels refer to patient 21618: **A.** epithelial component (HE ×200), **B.** giant cell component (HE ×200), **C.** astrocytic component (HE ×200), **D.** expression of cytokeratin (CKMNF, APAP, DAKO ×200), **E.** expression of GFAP (DAKO, DAB ×200). Right upper panels refer to patient 21624: **F.** epithelial component (HE ×400), **G.** astrocytic component (HE ×200), **H.** expression of cytokeratin (CKMNF, DAB, DAKO ×200), **I.** expression of GFAP (DAKO, DAB ×200). Left lower panel shows aberrant migration pattern of a *TP53* exon 6 fragment in the astrocytic component (lanes c and d), the giant cell component (lanes e and f) and the epithelial component (lane g). Lanes a and b correspond to leukocyte DNA of the same patient. Bottom left panel shows the C to T transition present in all three tumor portions. Right lower panel shows aberrant migration pattern of a *TP53* exon 5 fragment in the astrocytic component (lane i, intracranial lesion) and the epithelial components (lanes l and o, intraspinal lesion). The remaining lanes correspond to leukocyte DNA of unaffected control individuals. Bottom right panel shows the G to T transversion present in both tumor portions.

phenylalanine. Both, areas of astrocytic and epitheloid appearance from intracranial and spinal locations carried this mutation. Again, DNA from non tumorous tissue of this patient exhibited a wild type sequence for *TP53*. SSCP data from both patients and representative sequence data are shown in Figure 1.

Discussion

We present a morphological and molecular analysis on a rare variant of GBM at risk of diagnostic misinterpretation. GBM or astrocytoma with epithelial differentiation has been reported in several independent studies (8, 13, 17, 20). In a considerable fraction of those cases, the initial diagnosis has been metastasis (8, 11, 17). Since the advent of immunohistochemistry the focal expression of glial fibrillary acidic protein has greatly helped to characterize these tumors as GBM. However, GFAP expression is not exclusively restricted to tumors of neuroectodermal origin but has also been described in rare instances of epithelial neoplasms (3, 5).

An X-chromosome inactivation study was not applicable on the material available since all patients were male. Therefore, a mutational analysis of *TP53*, a gene with frequent somatic alterations in GBM, was performed. In two of five GBMs with focal epithelial differentiation point mutations in the *TP53* gene were detected by SSCP analysis and direct sequencing of the exon 5-8. Both mutations resulted in amino acid substitutions. These alterations were seen in all specimens sampled from the respective patients. Constitutional DNA from both patients corresponded to the wild type sequence. Detection of identical somatic mutations in tumor portions of both astrocytic and epithelial differentiation in a single patient strongly argues for clonal origin of the neoplastic lesions. The likelihood of identical mutations arising in tumors from different origins in a patient without a germ line defect is neglectible. However, cleanly separating distinct tumorous areas may pose a problem since minor contamination may result in misleading PCR signals. The specimens of patient 21624 were retrieved from distant sites such as the cerebral hemispheres and the lumbar spinal cord. Both the intracranial glioblastomatous and the spinal epithelial lesions carried the same alteration. Therefore, contamination with adjacent tumor tissue of other differentiation can be excluded in this patient.

TP53 mutations were detected in only two of the five patients. This detection rate corresponds to those of large GBM series examined for *TP53* mutations (10, 22, 24). From this point of view GBM with epithelial differentiation does not differ from unselected GBM. GBMs have been subgrouped on grounds of the presence or absence of LOH17p and *TP53* mutations into GBM type 1 (with LOH17p/*TP53* mutations) or GBM type 2 (without LOH17p/*TP53* mutations) (9, 23). The association of secondary GBMs (prior history of astrocytoma or anaplastic astrocytoma) with a higher incidence of *TP53* than primary GBM has been the basis of an attempt to merge clinical and molecular data (24). However, a significant fraction of primary GBM do also exhibit *TP53* mutations and due to the low incidence of secondary GBM the absolute number of primary GBM with *TP53* mutations exceeds that for secondary GBM (22). Both tumors in the present study with mutations in our series had no report of a previous astrocytoma. Thus, based on the limited number of cases examined, epithelial differentiation does not seem to be associated with one of the genetically or clinically defined GBM subsets.

The tumor of patient 21618 exhibited focally the typical morphological features of giant cell GBM (see Figure 1B). This tumor had a *TP53* mutation. Two recent studies have shown, that giant cell GBMS are characterized by a very high mutation rate in *TP53* compared to typical and unselected GBMs (16, 19). In this context, it is not surprising to find a *TP53* mutation in that GBM with epithelial differentiation, that also exhibits focally a giant cell GBM morphology.

In conclusion, we demonstrated identical *TP53* mutations in morphologically distinct areas of GBMS with epithelial differentiation. The findings proof clonality and point towards the feasibility of molecular analyses in cases not allowing morphological or immunohistochemical distinction between colliding GBM and carcinoma metastases or GBM with epithelial differentiation.

Acknowledgements

Supported by DFG (SFB 507) and Deutsche Krebshilfe.

References

1. Bender B, Wiestler OD, von Deimling A (1994) A device for processing large acrylamide gels. *Biotechniques* 16: 204-206
2. Bögler O, Huang SH-J, Kleihues P, Cavenee WK (1995) The p53 gene and its role in human brain tumors. *Glia* 15: 308-327
3. Chen PC, Chen CK, Nicastri AD, Wait RB (1994) Myoepithelial carcinoma of the breast with distant metastasis and accompanied by adenomyoepitheliomas. *Histopathology* 24: 543-8

4. Chung RY, Whaley J, Kley N, Anderson K, Louis D, Menon A, Hettlich C, Freiman R, Hedley-Whyte ET, Martuza R, Jenkins R, Yandell D, Seizinger BR (1991) TP53 mutation and 17p deletion in human astrocytomas. *Genes Chromosom Cancer* 3: 323-331
5. Doglioni C, Barbareschi M, Balercia G, Bontempini L, Iuzzolino P (1993) Atypical lung carcinoid with GFAP immunoreactive cells. *Pathol Res Pract* 189: 83-9; discussion 90-2
6. Frankel RH, Bayona W, Koslow M, Newcomb EW (1992) p53 mutations in human malignant gliomas: comparison of loss of heterozygosity with mutation frequency. *Cancer Res* 52: 1427-1433
7. Fults D, Brockmeyer D, Tullous MW, Pedone CA, Cawthon RM (1992) p53 mutation and loss of heterozygosity on chromosome 17 and 10 during human astrocytoma progression. *Cancer Res* 52: 674-679
8. Galloway PG, Roessmann U (1986) Anaplastic astrocytoma mimicking metastatic carcinoma. *Am J Surg Pathol* 10: 728-32
9. Hayashi Y, Ueki K, Waha A, Wiestler OD, Louis DN, von Deimling A (1997) Association of EGFR gene amplification and CDKN2 (p16/MTS1) gene deletion in glioblastoma multiforme. *Brain Pathol* 7: 871-875
10. Ichimura K, Bolin MB, Goike HM, Schmidt EE, Moshref A, Collins VP (2000) Deregulation of the p14ARF/MDM2/p53 pathway is a prerequisite for human astrocytic gliomas with G1-S transition control gene abnormalities. *Cancer Res* 60: 417-24
11. Kato K, Watanabe M (1999) Glioblastoma multiforme with epithelial appearance: a case report. *Brain Tumor Pathol* 16: 45-8
12. Kattar MM, Kupsky WJ, Shimoyama RK, Vo TD, Olson MW, Bargar GR, Sarkar FH (1997) Clonal analysis of gliomas. *Hum Pathol* 28: 1166-79
13. Kepes JJ, Fulling KH, Garcia JH (1982) The clinical significance of “adenoid” formations of neoplastic astrocytes, imitating metastatic carcinoma, in gliosarcomas. A review of five cases. *Clin Neuropathol* 1: 139-50
14. Kleihues P, W.K. C (2000) *Pathology and genetics of tumours of the nervous system*, 1, IARC Press: Lyon
15. Louis DN, von Deimling A, Chung RY, Rubio MP, Whaley JH, Eibl R, Ohgaki H, Wiestler OD, Thor AD, Seizinger BR (1993) Comparative study of p53 gene and protein alterations in human astrocytoma. *J Neuropath Exp Neurol* 52: 31-38
16. Meyer-Puttlitz B, Hayashi Y, Waha A, Rollbrocker B, Boström J, Wiestler OD, Louis DN, Reifenberger G, von Deimling A (1997) Molecular genetic analysis of giant cell glioblastomas. *Am J Pathol* 151: 853-857
17. Moork SJ, Rubinstein LJ, Kepes JJ, Perentes E, Uphoff DF (1988) Patterns of epithelial metaplasia in malignant gliomas. II. Squamous differentiation of epithelial-like formations in gliosarcomas and glioblastomas. *J Neuropathol Exp Neurol* 47: 101-18
18. Ohgaki H, Eibl RH, Schwab M, Reichel MB, Mariani L, Gehring M, Petersen I, Höll T, Wiestler OD, Kleihues P (1993) Mutations in the p53 tumor suppressor gene in neoplasms of the human nervous system. *Molecular Carcinogenesis* 8: 74-80
19. Peraud A, Watanabe K, Plate KH, Yonekawa Y, Kleihues P, Ohgaki H (1997) p53 mutations versus EGF receptor expression in giant cell glioblastomas. *J Neuropath Exp Neurol* 56: 1236-1241
20. Rosenblum MK, Erlandson RA, Budzilovich GN (1991) The lipid-rich epithelioid glioblastoma. *Am J Surg Pathol* 15: 925-34
21. von Deimling A, Eibl RH, Ohgaki H, Louis DN, von Ammon K, Petersen I, Kleihues P, Chung RY, Wiestler OD, Seizinger BR (1992) p53 mutations are associated with 17p allelic loss in grade II and grade III astrocytoma. *Cancer Res* 52: 2987-2990
22. von Deimling A, Fimmers R, Schmidt MC, Bender B, Fassbender F, Nagel J, Jahnke R, Kaskel P, Duerr E-M, Koopmann J, Maintz D, Schild S, Vogel Y, Wick W, Platten M, Müller D, Przkora R, Waha A, Rollbrocker B, Wellenreuther R, Meyer-Puttlitz B, Schmidt O, Mollenhauer J, Poustka A, Stangl AP, Lenartz D, von Ammon K, Henson JW, Schramm J, Louis DN, Wiestler OD (2000) Comprehensive allelotype and genetic analysis of 466 human nervous system tumors. *J Neuropathol Exp Neurol* in press
23. von Deimling A, von Ammon K, Schoenfeld D, Wiestler OD, Seizinger BR, Louis DN (1993) Subsets of glioblastoma multiforme defined by molecular genetic analysis. *Brain Pathol* 3: 19-26
24. Watanabe K, Tachibana O, Sato K, Yonekawa Y, Kleihues P, Ohgaki H (1996) Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. *Brain Pathol* 6: 217-223