# Clonal expansion of p53 mutant cells is associated with brain tumour progression

**David Sidransky*, Tom Mikkelsen†, Karl Schwechheimer‡, Mark L. Rosenblum§, Web Cavanee† & Bert Vogelstein*‖**

* The Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, USA
† Ludwig Institute for Cancer Research, Montreal, Province of Québec, H3A 1A1, Canada
‡ Abteilung Neuropatologi Pathologischer Institut, Albert Ludwigs Universitat, Frieberg, Germany
§ Brain Tumor Research Center, Department of Neurological Surgery, University of California Medical Center, San Francisco, California 94143, USA

**TUMOUR progression is a fundamental feature of the biology of cancer[1]. Cancers do not arise *de novo* in their final form, but begin as small, indolent growths, which gradually acquire characteristics associated with malignancy. In the brain, for example, low-grade tumours (astrocytomas) evolve into faster growing, more dysplastic and invasive high-grade tumours (glioblastomas)[2,3]. To define the genetic events underlying brain tumour progression, we analysed the p53 gene in ten primary brain tumour pairs. Seven pairs consisted of tumours that were high grade both at presentation and recurrence (group A) and three pairs consisted of low-grade tumours that had progressed to higher grade tumours (group B). In group A pairs, four of the recurrent tumours contained a p53 gene mutation; in three of them, the same mutation was found in the primary tumour. In group B pairs, progression to high grade was associated with a p53 gene mutation. A subpopulation of cells were present in the low-grade tumours that contained the same p53 gene mutation predominant in the cells of the recurrent tumours that had progressed to glioblastoma. Thus, the histological progression of brain tumours was associated with a clonal expansion of cells that had previously acquired a mutation in the p53 gene, endowing them with a selective growth advantage. These experimental observations strongly support Nowell's clonal evolution model of tumour progression[4].**

Brain tumours, like most other tumour types, are associated with several genetic changes. Among these, loss or mitotic recombination of chromosome 17p is common[5-7]. Furthermore, in several glioblastomas[8], as in many other tumour types[9,10], loss of one 17p allele correlates with mutation of p53 in the remaining allele. To evaluate the relationship between p53 and astrocytoma progression, tumour pairs were obtained from patients with brain tumours that had been surgically removed. Tumours were carefully dissected to ensure optimal removal of contaminating normal tissue. A portion of the p53 gene, including exons 5-8, was amplified by the polymerase chain reaction (PCR) and subcloned into a phagemid vector; pooled clones were sequenced as previously described[11]. Four group A tumours contained p53 mutations at recurrence and, in three, the identical p53 mutation was seen at initial presentation (Table 1). All were missense mutations resulting in nonconservative amino-acid changes. From the ratio of wild-type to mutant p53 alleles in the sequencing gels, it was clear that all group A tumours with p53 gene mutations had lost the wild-type allele.

All three of the advanced tumours from group B (Fig. 1) had a p53 gene mutation. Again, all mutations were missense mutations resulting in nonconservative amino-acid changes (Table 1). Two of the three high-grade tumours (from patients B8 and B9) had lost the wild-type allele. No evidence of these mutations was observed in the less advanced tumours of the pairs in the sequencing assays. But were these mutations present in a small fraction of cells in the initial tumour, in a proportion not detectable by sequencing? We used a sensitive plaque assay to identify a potential cell minority containing p53 mutations among a majority of cells with normal p53 genes. The p53 gene was amplified through PCR as described above and the PCR products subcloned into a phage vector. Specific oligonucleotides that recognize a mutant base pair in the p53 gene were then used to probe filter lifts of the phage plaques.

Specific oligonucleotides were synthesized that would detect the mutations seen in the tumours of patients B8 and B9 and used to probe the cloned PCR products of the low grade tumours. A small number of positive plaques were seen in the low-grade tumours (Fig. 2). Positive plaques were picked and the phage DNA sequenced to confirm the presence of the mutation. Blots were then stripped and hybridized with a probe detecting all p53 clones (mutant plus wild type) to calculate the percentage of mutant p53 genes in the low-grade tumours. This showed that 8% and 21% of the tumour cells from patients B8 and B9, respectively, contained a mutant p53 gene. The high-grade tumours from both patients consisted of cells in which over 95% contained mutant p53 genes as assessed by the same assay. Histological sections were examined and all tumours were found to be contaminated by $<10\%$ nomal cells (Fig. 1).

The low-grade component of the tumour from patient B10 contained a p53 mutation at codon 253 and a light mutant band at codon 273 when pooled clones were sequenced, whereas the high-grade component was found to contain mutant bands of equal intensity at codons 253 and 273 (Table 1). After subcloning

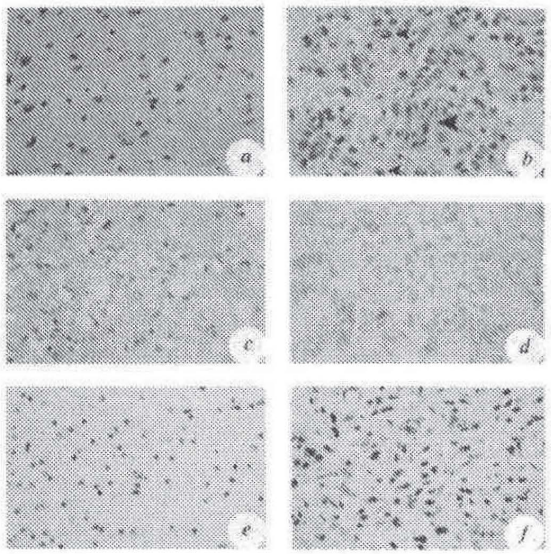

FIG. 1 Micrographs of tumours from patients in group B. The tumour from patient 10 had two distinct patterns (*a* and *b*). Most of the neoplasm was moderately cellular with a dense feltwork of glial processes. The nuclei of the glioma cells in *a* were mildly to moderately pleomorphic. Mitotic figures were rare and no vascular endothelial proliferation was identified. This region was classified as a moderately anaplastic astrocytoma. Sharply demarcated from this region and invading the leptomeninges was a more cellular component of the neoplasm with larger nuclei, nuclear pleomorphism and multiple mitotic figures (*b*, small arrows). The glial feltwork was less extensive and vascular endothelial proliferation (large arrow) was evident. This latter region was classified as glioblastoma multiforme. Both regions were glial fibrillary acid protein (GFAP) immunopositive, an indicator of their glial origin. The tumours in *c* and *e* also had features of low-grade glial tumours, whereas those in *d* and *f* had features of glioblastoma multiforme. *a*, *c*, and *e*, Initial low-grade tumours from patients B8, B9, and B10, respectively; *b*, *d*, and *f*, high-grade tumours from the same patients.

‖ To whom correspondence should be addressed.

TABLE 1 Mutations in each tumour

| Patient | Initial tumour grade | Initial tumour mutation* | Time to progression (months) | Progressed tumour grade | Progressed tumour mutation |
|---|---|---|---|---|---|
| Group A | | | | | |
| B1 | IV | Codon 173 GTG→ATG, Val→Met | 7 | IV | Codon 173 GTG→ATG, Val→Met |
| B2 | IV | Codon 244 GGC→AGC, Gly→Ser | 13 | IV | Codon 244 GGC→AGC, Gly→Ser |
| B3 | IV | Codon 248 CGG→TGG, Arg→Trp | 9 | IV | Codon 248 CGG→TGG, Arg→Trp |
| B4 | IV | None | 11 | IV | Codon 281 GAG→GAA, Asp→Glu |
| B5 | IV | None | 5 | IV | None |
| B6 | IV | None | 9 | IV | None |
| B7 | IV | None | 7 | IV | None |
| Group B | | | | | |
| B8 | II | None | 15 | IV | Codon 273 CGT→TGT, Arg→Cys |
| B9 | I | None | 28 | IV | Codon 270 TTT→Ctt, Phe→Leu |
| B10 | II | Codon 253 ACC→ATC, Thr→Leu | 0 | IV | Codon 253 ACC→ATC, Thr→Leu<br>Codon 273 CGT→TGT, Arg→Cys |

Group A tumours consisted of glioblastoma at both presentation and recurrence, whereas group B tumours were low-grade (I and II) at presentation and high-grade (III and IV) at recurrence. DNA was isolated from tumours and exons 5–8 of the p53 gene were amplified by the PCR, subcloned into lambda Zap, and sequenced as described previously[11]. The tumour from patient B10 contained two components, histologically and mechanically separable, from the same tumour.

* As assessed by sequencing (see text).

amplified DNA, individual clones were sequenced and these two mutations were found to reside on separate alleles. Using the oligonucleotide-specific hybridization method described, phage plaques from low- and high-grade components were probed with an oligonucleotide specific for the codon-253 mutation, an oligonucleotide specific for the 273 mutation, and a normal oligonucleotide detecting all p53 clones (mutant and wild type). This analysis revealed that 60% of the cells in the low-grade component contained one allele which was wild type and one allele that was mutant at codon 253, whereas both alleles were mutant (one at codon 253 and one at codon 273) in 40% of the cells. Virtually all of the cells in the high-grade component contained two mutant alleles. Thus progression appeared to be associated with mutation of the second allele at codon 273 in a cell with a pre-existing mutation in the first allele.

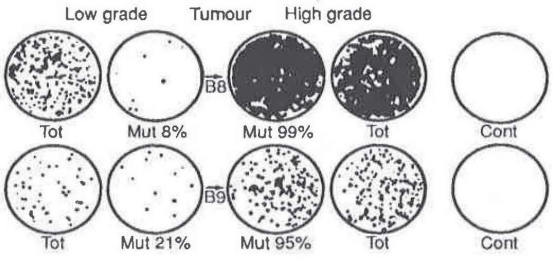


FIG. 2 Clonal expansion of p53 mutant cells. Over 95% of the clones obtained by PCR from low-grade and high-grade tumours in patients B8 (top) and B9 (bottom) hybridized to an oligonucleotide probe detecting all p53 clones (Tot). An oligonucleotide probe specific for mutant p53 at codon 273 hybridized to only a small number of plaques (8%) in the low-grade tumour from patient B8 (top left), but hybridized to the majority of plaques (99%) in the high-grade tumour. A mutant-specific oligonucleotide probe for the high-grade tumour in B9 also hybridized to 21% of clones in the low-grade tumour (bottom left) and 95% in the high-grade tumour. Neither of the mutant-specific probes hybridized to control plaques (far right) from another glioblastoma which contained a p53 mutation at a different codon.

METHODS. Tumour DNAs were amplified using PCR, ligated to lambda Zap arms (Stratagene), packaged, plated at a density of 200–500 plaques per plate, and the plaque DNAs transferred to nylon membranes as described[11]. Mutant-specific oligomers corresponding to the mutant sequences in the high-grade tumours were synthesized to recognize a mutation at codon 273 (5′-TTGAGGTGTGTGTTTGTG-3′) for tumours of patient B8 and B10; a mutation at codon 270 (5′-GGAACAGCCTTGAGGTGC-3′) for tumours in patient B9; and a mutation at codon 253 (5′-CATCCTCATCATCATCAC-3′) for the tumour in patient B10. As a control, the filters were rehybridized to an oligonucleotide probe for the wild-type sequence at codons 243 to 248 (5′-ATGGGCGGCATGAACCGG-3′), which would identify all p53 clones (mutant or wild type, Tot). The oligomers were labelled with [$^{32}$P]ATP and T4 kinase to a specific activity of $5\times10^8$ d.p.m. Hybridization with oligomers was at 45 °C for 1 h. After hybridization, the membranes were rinsed in 3×SSC (450 mM NaCl, 18 mM sodium citrate, 1 mM Tris, pH 7.2), 0.1% SDS at room temperature for 5 min and then washed at 2–5 °C below the calculated melting temperature. The blots were then briefly dried and exposed to film. For the one-allele tumours from patients B8 and B9, the percentage of mutant cells was calculated by counting the mutant clones and dividing this number by the total number of clones that contained p53 sequences. We assumed that the subset of cells in the low-grade tumours which contained a p53 gene mutation also had a concomitant deletion of the wild-type allele, as was found in the high-grade tumours of these patients.

Nowell postulated that a cell acquiring a genetic change might acquire a selective growth advantage[4]. Clonal expansion of this cell, driven by successive mutations, would lead to tumour progression[4,12]. Although the occurrence of several mutations has been observed in human tumours[13,14], evidence for clonal expansion of a subpopulation of cells with a specific endogenous mutation has until now not been available. The data presented here suggest that mutation of p53 leads to a selective growth advantage *in vivo* that seems to be a critical step in transformation from low-grade to high-grade tumours. This provides a direct experimental demonstration of clonal expansion in a human tumour: a rare cell carrying a specific change in a critical gene became the dominant cell type as the tumour progressed. This concept will probably be useful in understanding the role of various oncogenes and suppressor gene mutations in the pathway to malignancy. □

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