学　位　論　文

Doctor's Thesis

Analysis for p53 Mutations in Glioma
(神経膠腫における p53 遺伝子変異の解析)

白石　昭司

Shoji Shiraishi

指導教官

生塩　之敬　教授

熊本大学大学院医学研究科　脳神経外科学

2002 年 3 月

熊本大学博士（医学）論文

論文名： Analysis for p53 Mutations in Glioma
（神経膠腫における p53 遺伝子変異の解析）

著者名： Shoji Shiraishi M.D.（白石　昭司）

指導教授： 脳神経外科学講座教授　生塩之敬

審査員名： 1）　発生医学研究センター 器官形成部門
組織制御分野教授　山泉　克
2）　外科学第二講座教授 小川　道雄
3）　発生医学研究センター 胚形成部門
転写制御分野教授　田賀　哲也

2002 年 3 月

Table of Contents

1. Summary

BACKGROUND.

The effect of p53 mutations on the biology of astrocytic tumors is controversial. p53 is thought to be inactivated in the early stage of gliomagenesis, however, the role of its inactivation in the malignancy of gliomas remains unknown. To understand the significance of p53 inactivation, we identified the locus of p53 gene mutations in samples from gliomas at different stages of progression, and studied the correlation between the mutation and clinical behavior.

METHODS.

Samples from newly diagnosed gliomas, including pure and mixed astrocytomas, were analyzed for p53 mutations, using a yeast functional assay. To determine the locus of the gene mutations, DNA sequencing was performed.

RESULTS.

The incidence of p53 mutations was higher in anaplastic astrocytomas (AA, 48%) than glioblastomas (GBM, 31%). There was no significant difference in the average age of GBM patients with and without p53 mutations (54.9 ± 2.3 and 53.2 ± 4.6, respectively). In GBM patients, the mutation did not affect progression-free survival (PFS) or overall survival (OS). AA and GBM differed in the distribution of p53 mutation locus.

CONCLUSIONS.

The p53 gene mutation does not markedly affect the survival of GBM patients. The difference in the location of p53 mutations between AA and GBM suggests that in gliomas, it may contribute not only to tumorigenesis (as an early event) but also

to progression to malignancy (as a late event).

2. List of published papers

1. Influence of p53 Mutations on Prognosis of Patients with Glioblastoma

Shoji Shiraishi, Kenji Tada, Hideo Nakamura, Keishi Makino, Masato Kochi, Hideyuki Saya, Jun-ichi Kuratsu, Yukitaka Ushio

Cancer 2002 July;95(2):249-257.

3. Acknowledgements

I gratefully acknowledge my excellent advisers, Dr. Hideo Nakamura, Dr. Keishi Makino, Dr. Masato Kochi, Dr. Jun-ichiro Hamada, Dr. Yukitaka Ushio (Department of Neurosurgery, Kumamoto University), Dr. Hideyuki Saya (Department of Tumor Genetics and Biology, Kumamoto University) for their invaluable help in the study design and manuscript preparation. I also thank Dr. Mitsuhiro Tada (Division of Cell Biology, Cancer Institute, Hokkaido University School of Medicine, Sapporo) for excellent technical instruction and for providing the expression plasmid vector pss16 and the reporter yeast strain yIG397.

This study has been conducted under the guidance of Professor and Chairman Yukitaka Ushio at the Department of Neurosurgery, Kumamoto University Graduate School of Medical Sciences.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

4. Abbreviations and Acronyms

AA = Anaplastic Astrocytoma

GBM = Glioblastoma Multiforme

PFS = Progression Free Survival

OS = Overall Survival

CNS = Central Nervous System

PA = Pilocytic Astrocytoma

DA = Diffuse Astrocytoma

KPS = Karnofsky Performance Status

MDM2 = Murine Double Minute

NLS = Nuclear Localization Signal

ARF = Alternative Reading Frame

CDKN2A/2B = Cyclin Dependent Kinase Inhibitor 2A/B

INK4 = Inhibitor of Cyclin Dependent Kinase Inhibitor 4

RB = Retinoblastoma

LOH = Loss of Heterozygosity

PTEN = Phosphatase and Tensin Homolog deleted from chromosome 10

DMBT1 = Deleted in Malignant Brain Tumours 1

MMAC1 = mutated in multiple advanced cancers

TEP-1 = TGFβ–regulated and epithelial cell-enriched phosphatase

FAK = Focal Adhesion Kinase

PI3-K = Phosphoinositide 3-kinase

PKB = Protein Kinase B

Ser/Thr = Serine/Threonine

EGFR = Epidermal Growth Factor Receptor

TGF-α = Transforming Factor alpha

EGFRvIII = EGFR variant three

JNK = C-Jun N-terminal Kinase

WHO = World Health Organization

BS = Brain Stem

GGB = Giant Cell Glioblastoma

OA = Oligoastrocytoma

AOA = Anaplastic Oligoastrocytoma

MRI = Magnetic Resonance Imaging

RT-PCR = Reverse Transcription Polymerase Chain Reaction

SSCP = Single Strand Conformational Polymorphism

5. Background and purpose

5.1. Brain tumors

Brain tumors comprised of various histologic types and categorized as astrocytic tumors, oligodendroglial tumors and mixed gliomas, ependymal tumors, choroid plexus tumors, neuroepithelial tumors of uncertain origin, neuronal and mixed neuronal-glial tumors, pineal parenchymal tumors, embryonal tumors, peripheral neuroblastic tumors, tumors of cranial nerves, meningeal tumors, tumors of the haemopoietic system, germ cell tumors, familial tumor syndromes, tumors of the sellar region, and metastatic tumors. A term "glioma" is used for all tumors of glial cell origin (astrocytes, oligodendrocytes, and ependymal cells).

5.2. Astrocytic tumors

Astrocytic tumors are primary neoplasms that differ in their location within the central nervous system (CNS), age and gender distribution, growth potential, extent of invasiveness, morphological features, tendency for progression and clinical course. There is increasing evidence that these differences reflect the type and sequence of genetic alterations acquired during the process of transformation. The following clinicopathological entities can be distinguished: diffusely infiltrating astrocytomas, pilocytic astrocytomas (PA), pleomorphic xanthoastrocytomas (PXA), desmoplastic cerebral astrocytomas of infancy, subependymal giant cell astrocytoma[1].

5.2.1. Diffusely infiltrating astrocytomas

Diffusely infiltrating astrocytomas are the most common astrocytic tumors and account for more than 60% of all primary brain tumors. They have been classified into 3 clinico-pathologic groups, i.e. diffuse astrocytoma (DA), anaplastic astrocytoma (AA),

and glioblastoma multiforme (GBM) including its variants, giant cell glioblastoma (GGB) and gliosarcoma. They commonly have an inherent tendency for malignant progression, with the GBM as the most malignant phenotypic endpoint. GBM is subclassified into two categories according to its clinical course, i.e. primary and secondary GBMs [2]. While the primary GBMs arise de nove without clinical or histologic evidence of a less malignant precursor lesion, the secondary GBMs develop more slowly by malignant progression from DAs or AAs. Both GBM subclasses are regarded as histologically indistinguishable but they occur in different age groups. PA is thought to be a different entity from diffusely infiltrating astrocytomas with respect to the progression pathway[3-9].

5.2.2. Grading for astrocytomas

While neurological symptoms resulting from astrocytic tumor development depend primarily on the site of the tumors within the CNS, the length of the patient's survival time and the chance for a long progression-free survival are more closely associated with the intrinsic biology of the neoplasm itself. Significant indicators of anaplasia in astrocytomas include nuclear atypia (coarse nuclear chromatin, nuclear pleomorphism, multinucleation), mitotic activity, cellularity, vascular proliferation, and necrosis. As a general rule, grading is based on areas exhibiting the highest degree of anaplasia. The malignancy scale of the WHO classification is widely accepted: PA is classified as grade 1, DA as grade 2, AA as grade 3, and GBM as grade 4[1,2].

5.2.3. Patient survival

Patient survival also depends on a variety of clinical parameters including the patient's age and condition, as reflected in the Karnofsky performance status (KPS) score, and the tumor location and treatment, e.g. extent of surgical resection,

radiotherapy, and chemotherapy[10, 11]. Despite these variables, typical ranges of survival are more than 5 years for DA (grade 2), 2-5 years for AA (grade 3), and around 1 year for the majority of patients with GBM (grade 4). Despite the introduction of multimodal treatment regimens, the prognosis of patients with GBM has been improved little over the last two decades.

5.2.4. Molecular genetics

The formation of human tumors is a complex process that involves the accumulation of various genetic alterations. Two types of such genes have been identified, i.e. oncogenes and tumor suppressor genes. Dominantly acting oncogenes whose protein products serve to accelerate cell growth are typically altered by increased gene dosage (amplification) or by activating mutations. On the other hand, tumor suppressor genes whose protein products serve as brakes on cell growth are typically altered by physical elimination or by inactivating mutations.

The molecular genetic alterations associated with the development and progression of human astrocytomas have been intensively studied[12-28]. Among the many genetic alterations detected in astrocytomas, loss of loci on 9p, 10, 17p, and 19q and epidermal growth factor receptor (EGFR) oncogene are the most common. Within regions that show loss of heterozygosity (LOH), the suppressor genes CDKN2A (p16), PTEN, and p53 map to 9p21, 10q23, and 17p13, respectively. They are deleted or mutated in a varying percentage of astrocytomas.

Recent molecular genetic analyses have revealed that diverse genetic alterations are involved in gliomagenesis and progression of each clinical subclass of GBM, primary and secondary GBMs[29-33].

5.3. Oligodendroglial tumors

Oligodendroglial tumors are well-characterized clinico-pathological entities. Their origin from differentiated oligodendrocytes or progenitor cells committed to oligodendroglial differentiation is difficult to prove because there are currently no reliable immunohistochemical markers. Genetic alterations in oligodendrogliomas differ significantly from those commonly found in diffuse astrocytomas[34-44]. The following types of tumors are distinguished.

5.3.1. Oligodendrogliomas (WHO grade 2)

These slowly growing neoplasms often manifest after several years of preoperative epileptic seizures and have a favorable prognosis with respect to time until recurrence.

5.3.2. Anaplastic oligodendrogliomas (WHO grade 3 or 4)

These neoplasms often respond favorably to chemotherapy but the criteria for the grading of oligodendrogliomas are less well defined than are those indicating the stage of progression of astrocytomas. Some of these tumors may develop histological features commonly found in GBMs.

5.4. Mixed gliomas

The diagnosis of mixed gliomas requires the identification of at least two unequivocally neoplastic components resembling different macroglial lineages, i.e. astrocytic, oligodendroglial, and/or ependymal differentiation.

5.4.1. Oligoastrocytomas (OA, WHO grade 2)

This is by far the most common type of mixed glioma. While recent studies have suggested at least two distinct genetic pathways, there is currently no indication of

a polyclonal origin.

5.4.2. Anaplastic oligoastrocytomas (AOA, WHO grade 3 or 4)

This malignant variant manifests histological features of anaplasia and mitotic activity. The prognosis is generally poor although some of these tumors respond well to chemotherapy.

5.5. p53/MDM2/p14 pathway

5.5.1. p53

The p53 gene, located on chromosome 17p13.1, encodes a 53kDa protein that plays a role in several cellular processes including the cell cycle, response of cells to DNA damage, cell death, cell proliferation, and neovascularization. The gene product has several regions that mediate these processes and perhaps those in the central portion, which are responsible for DNA binding, are the most important. This capacity allows the p53 protein to function as a positive or negative regulator of the transcription of other genes. The regions of the p53 gene encoding these protein motifs are most often the targets for mutation. These mutations diminish the ability of the mutant protein to carry out its activities and in some cases can bestow new dominant-negative or gain-of-function properties.

Evidence for causal involvement of p53 in glial tumorigenesis (gliomagenesis) comes from the finding that expression of exogenous wild-type p53 activity in glioblastoma cell lines suppresses their growth[45-50]. Moreover, cortical astrocytes from mice without functional p53 appear to be immortalized when grown in vitro and rapidly acquire a transformed phenotype.

Loss or mutation of the p53 tumor suppressor gene has been detected in many types of glioma[34, 36, 51-58] and represents an early genetic event in a subset of

astrocytomas. Allelic loss of chromosome 17p and p53 mutations are observed in approximately one third of all three grades of adult astrocytomas, suggesting that inactivation of p53 is important in the formation of the grade 2 astrocytoma. Moreover, high-grade astrocytomas with homologous p53 mutations can evolve clonally from subpopulations of similarly mutated cells present in tumors that are initially of low grade[59-62]. An important consequence of the loss of wild-type p53 activity is increased genomic instability which appears to accelerate neoplastic progression. Thus, p53 appears to play a role both in the formation of low-grade disease and in progression towards secondary GBM. In contrast, p53 mutations are rare in primary GBMs. This is corroborated by the observation that younger astrocytoma patients not only had higher incidences of p53 mutation, but also survived longer than did those without mutations. Therefore, GBMs may develop by either one of at least two distinct pathways: one that requires p53 inactivation and one that does not[30].

5.5.2. mdm2

The mdm2 gene lies in the 12q14.3-q15 chromosomal region. It encodes a protein with a predicted molecular weight of 54kDa. This protein has a nuclear localization signal (NLS), putative metal-chelating domains and an acidic region, all hallmarks of a transcription factor. The mdm2 gene appears to code for at least 5 proteins by using a combination of different promoter start sites. Only 2 of the 5 forms can interact with p53. The mdm2 protein binds to mutant and wild-type p53 proteins, thereby inhibiting the ability of wild-type p53 to activate transcription from minimal promoter sequences. Conversely, the transcription of the mdm2 gene is induced by wild-type P53. In normal cells, this autoregulatory feedback loop regulates both the activity of the p53 protein and the expression of mdm2. In addition, mdm2 promotes the degradation of p53[63-67]. Therefore, amplification or overexpression of mdm2 is an

alternative mechanism for escaping p53-regulated control of cell growth[57, 58, 68-70]. Amplification is observed in about 10% of glioblastomas without p53 mutations, i.e. primary glioblastoma. Overexpression of mdm2 was observed immunohistochemically in more than 50% of primary glioblastomas, but the fraction of immunoreactive cells varied considerably[68, 71].

5.5.3. p14 ARF

p14 ARF (human homologue of mouse p19ARF) protein is encoded by the CDKN2A/INK4 locus but is distinct from the p16INK4A protein. P14 ARF is encoded by the unique exon 1α and exon 2 and 3 of p16 INK4, using an alternative reading frame. Exon 1β is located between exon 1α of CDKN2A and exon 2 of CDKN2B on 9p21. It has been shown that in p14ARF protein binds to the p53/mdm2 complex and inhibits mdm2-mediated degradation of p53, indicating that p14ARF is an upstream regulator of p53 via mdm2[72]. There is also evidence suggesting that p53 down-regulates expression of p14ARF; this would establish an autoregulatory feedback loop between p53, mdm2, and p14ARF.

5.6. Rb pathway

The transition from low grade to anaplastic astrocytoma is accompanied by allelic losses on chromosome 9p, 13q and, less frequently, by 12q amplification. As discussed in several reviews, these abnormalities now appear to converge on one critical cell cycle regulatory complex.

The genes encoding p16 and p15 – CDKN2A and CDKN2B, respectively – map to chromosome 9p21, a site that is significantly associated with interstitial and homozygous deletions in high-grade astrocytomas and in two thirds of glioma cell lines. The protein products of these genes act as inhibitors of cyclin dependent kinases (CDK,

especially CDK4 and CDK6) and of their ability to phosphorylate the Rb protein in conjunction with cyclin D. This activity is essential for the G1/S phase transition of the cell cycle and removal of p16 and p15 activities can allow uncontrolled proliferation. There is now direct experimental evidence that CDKN2A and/or CDKN2B represent the tumor suppressorgenes targeted by these structural abnormalities. For example, transfection and expression of the CDKN2A in glioblastoma cell lines that lack the gene results in growth suppression, while there is no effect in similar non-mutated glioblastoma cells. While genetic analyses of primary tumor tissues have shown high frequencies of homozygous deletion[20, 21, 27, 73-81], there are several mechanisms other than mutation/deletion by which p15/p16 function can be overcome. A proportion of malignant gliomas with intact CDKN2A genes do not express the protein, and it has been shown that hypermethylation of the CpG island in the 5` region of the gene results in structural changes of the chromatin, thereby silencing gene transcription[38, 41]. This alternative mechanism for inactivation has also been shown for the CDKN2B gene in gliomas.

5.6.1. CDK4 and CDK6

The gene encoding the 33kDa CDK4 and 38kDa CDK6 protein maps to chromosome 12q13-14 and 7q21-22, respectively. Both are proteins with catalytic kinase activities, can form complexes with members of the cyclin D family, and are inhibited by p16 and p15. Thus overexpression of either CDK can be expected to mimic mutation of the p16/p15 inhibitors and to override their function.

The CDK4 gene is amplified in nearly 15% of high grade gliomas, particularly in those without CDKN2A/CDKN2B alterations[73, 76, 82, 83]. Additionally, a few tumors without CDKN2A/CDKN2B mutations or CDK4 amplification have been shown to

manidest CDK6 amplification[84]. suggesting that the two proteins can functionally compensate for each other.

5.6.2. Rb

The ultimate target of the kinase activities of the CDK4/CDK6-cyclin D complexes is the 107kDa retinoblastoma (Rb) protein. Phosphorylation of Rb allows the release of the E2F transcription factor it complexes with and this in turn activates genes necessary for cell proliferation. The Rb1 gene maps to chromosome 13q14, a site which is altered in about one third of high-grade astrocytic tumors.

Mutations in the Rb1 gene can have the same functional consequences as CDK4/CDK6 amplification of CDKN2A/CDKN2B mutation, and in astrocytic tumors these events are almost exclusive of one another. Therefore, it appears that a considerable proportion of AA and nearly all GBMs have alterations in one or another component of the pathway[77, 85].

5.7. LOH 10

Loss of one copy of chromosome 10 is a frequent event in glioblastoma and is rare in lower grades of astrocytic tumors[33, 44, 86-89]. While PTEN appears to be a bona fide tumor suppressor involved in glioma progression, there is a discrepancy between LOH for the chromosomal region containing PTEN (75-95%) and the mutation frequency of the gene (30-44%)[90-99]. It remains to be determined whether other genes on chromosome 10q such as DMBT1 are also involved in gliomagenesis.

5.8. PTEN

The PTEN gene also known as MMAC1 or TEP-1 was identified as a candidate tumor suppressor gene located at chromosome 10q23.3. It is mutated in 30-44% of high grade gliomas, particularly primary GBM and a variety of extraneural neoplasms, including prostate, endometrial, renal and small cell carcinoma, and melanoma. The protein product of the PTEN gene has been demonstrated to possess protein phosphatase activities and 3'phosphoinositol phosphatase activities. The former are important in regulating cell migration and invasion by direct dephosphorylation of focal adhesion kinase (FAK). The latter are directed against the product of phosphoinositide 3-kinase (PI3-K), PtdIns-3,4,5-P3, a lipid second messenger required for activation of the AKT/PKB Ser/Thr kinase, which in turn modulates the activity of a variety of downstream proteins that play important roles in cell proliferation and survival. Loss of PTEN function in tumor cells and in cells derived from PTEN deficient mice correlates with an increase in cellular levels of PtdIns-3,4,5-P3 and led to enhanced activation of AKT/PKB. Introduction of wild type PTEN into glioma cells containing endogenous wild type mutant alleles induced in vitro and in vivo growth suppression, but had no effect in cells containing endogenous wild type PTEN[92]. In GBM cells, this growth suppression is mediated by a G1 cell cycle block, however, sensitivity to anoikis and detachment-induced apoptosis, can also be detected. Furthermore, although some mutants of PTEN that lack growth-suppressive activity are invariably defective in 3' phosphoinositol phosphatase activity, some retain activity against protein substrates, indicating that the lipid phosphatase activity of PTEN is essential for the control of glioma growth.

5.9. EGFR

The majority of gene amplification events in high-grade astrocytomas involves the gene for the receptor tyrocine kinase, epidermal growth factor receptor (EGFR). EGFR is a transmembrane receptor responsible for sensing its extracellular ligands, EGF and transforming growth factor alpha (TGF-α) , and for transducing this signal to the cell. EGFR has been associated with cancer for 3 main reasons: (1) it is the cellular homologue of the v-erbB oncogene found in the acutely transforming avian erythroblastosis virus, (2) when expressed ectopically in cells, it can set up a transforming autocrine loop so that cellular transformation is ligand-dependent, and (3) it has been shown to be amplified in several tumor types, with an increased copy number that is directly correlated with an increase in the number of receptors on the cell surface. While the gene encoding EGFR maps to chromosome 7, the amplified genes are typically present as double-minute extra-chromosomal elements. The wild-type EGFR protein is 70kDa, and is composed of 4 major domains: the ligand binding extracellular domain, the transmembrane anchoring domain, the catalytic tyrosine kinase domain, and the carboxyl terminus, which contains 5 tyrosines that are target substrates for the kinase, and the motifs responsible for ligand-activated endocytosis. The EGFR gene is the most frequently amplified oncogene in astrocytic tumors; it is amplified in about one third of GBMs and in a few AAs[91, 99-101]. Moreover, there is evidence that gliomas express the EGFR ligands, EGF and TGF-α, suggesting the possibility that these tumor cells have autocrine growth stimulatory loop[102].

In about half of GBMs with receptor amplification, the event is coupled with gene rearrangement. The most common rearrangement results in a variant form called EGFRvIII, delta EGFR, or de2-7EGFR. This mutant lacks a portion of the extracellular ligand-bindin domain as the result of genomic deletions that precisely eliminate exons 2-7 in the EGFR mRNA. These mutant receptors are expressed on the cell surface and

are constitutively autophosphorylated, but at a significantly lower level than is wild type EGFR activated by ligand exposure. Unlike wild-type EGFR, the constitutively active mutants are not downregulated, suggesting that their altered conformation does not result in exposure of the receptor sequence motifs required for endocytosis, lysosomal degradation、 and signal attenuation. Consequently, mutant receptors are only internalized at the same low rate as unoccupied wild-type EGFR.

Amplification and overexpression of the EGFR gene occurs in approximately one third of GBMs. Recent studies have shown that this constitutes a hallmark of primary glioblastomas, more than 60% of which show upregulated EGFR expression. This is in contrast to TP53 mutations, which are common in secondary GBMs that evolved through progression from DAs or AAs; thus TP53 inactivation and EGFR amplification are mutually exclusive events[29]. All GBMs with EGFR amplification show simultaneous loss of chromosome 10. However, EGFR amplification occurs at similar frequencies among glioblastomas with of without homozygous deletions or mutations of the PTEN gene which is located on chromosome 10q23, although about 20% of primary GBMs show both EGFR amplification and PTEN mutations.

Numerous genetic alterations have been revealed to contribute to gliomagenesis and/or its progression. In this study, clinical materials were analysed with special focus on the p53 gene mutation in efforts to elucidate the role of p53 mutation in gliomagenesis.

6. Methods

6.1. Patients and Tissue Specimens

The samples analyzed in this study were obtained from the Department of Neurosurgery at Kumamoto University Hospital (Kumamoto, Japan) and its affiliated hospitals. They were from newly diagnosed, consecutive patients treated between 1995 and 2000. There were 73 males and 50 females ranging in age from 0 to 78 years (mean 45 years). The patients and/or their legal guardians had given written informed consent for use of their specimens. Tumor specimens were obtained by surgical resection (including biopsy), quickly frozen, and kept at –80 °C until use. Formalin-fixed, paraffin-embedded specimens were subjected to histopathological examinations. Each specimen was classified according to established World Health Organization (WHO) criteria. The presence or absence of high cellularity, nuclear atypia, mitoses, microvascular proliferation, and necrosis were recorded. The presence of necrosis and/or microvascular proliferation was used as major criteria to distinguish between GBMs and AAs. There were 11 PAs, 15 DAs, 31 AAs, 73 supratentorial GBMs, 4 brain stem (BS) GBMs, 2 giant cell glioblastomas (GGB), 1 oligoastrocytoma (OA) and 14 anaplastic oligoastrocytomas (AOA). All patients underwent surgical resection (including biopsy) with or without postoperative radiotherapy and/or nitrosourea-based chemotherapy. Most GBM patients younger than 70 years received both radio- and chemotherapy; older patients usually underwent only radiotherapy. To determine the extent of surgical resection we performed postoperative Magnetic Resonance Imaging (MRI) study. Total resection was recorded when there were no residual lesions; subtotal resection when less than 10% of the preoperative mass remained, and partial resection when more than 10% of the mass was left. For analysis, subtotal and partial resections were subsumed into the "subtotal" resection group. All

patients were re-evaluated after receiving initial adjuvant therapy; at periodical follow-up visit, MRI was performed. Clinical details, including the Karnofsky Performance Status (KPS) at the time of diagnosis, the extent of surgery, date of recurrence (or regrowth) on MRI, and date of death were recorded.

6.2. Yeast Functional Assay

To investigate the p53 gene status in various glioma subtypes, we performed yeast functional assay as previously described[103-106] (Fig.1, 2).

6.2.1. mRNA Extraction and RT-PCR

The mRNA was extracted from the frozen tissue samples using the Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ), and random hexamer-primed single-strand cDNA was synthesized using the SUPERSCRIPT Preamplification System (Life Technologies, Rockville, MD) according to the manufacturer's instructions. To amplify the p53 cDNA, PCR was performed in 25 µl of reaction mixture containing 2.5 µl of 10 X Pfu buffer (Stratagene, La jolla, CA), 1.25 units of Pfu polymerase (Stratagene), 100 ng of each primer, 50 µM of dNTPs, and 10% (vol/vol) dimethyl sulfoxide (DMSO) using a Thermal Cycler (Perkin-Elmer, Norwalk, CT) for 5 minutes at 94 °C; 35 cycles of 40 seconds at 94 °C, 70 seconds at 65 °C, 90 seconds at 78 °C; then for 8 minutes at 78 °C. The p53 specific primers for a 1kb fragment encompassing codons 53-364 (exons 4-10) were P3 (5'-ATT TGA TGC TGT CCC CGG ACG ATA TTG AAsC-3', where s represents a phosphorothioate linkage) and P4 (5'-ACC CTT TTT GGA CTT CAG GTG GCT GGA GTsG-3').

6.2.2. Yeast Transfection

The p53 PCR product and linearized p53-expression vector pSS16 were co-transfected into the reporter yeast strain yIG397, using the lithium acetate procedure. The transformed yeast cells were plated, incubated at 30 °C for two days to generate colonies, and stored at 4 °C overnight to develop color. At least 200 colonies were examined for each plate. When more than 15% of the colonies were red, we judged the sample positive for p53 functional loss and proceeded to sequencing analysis.

6.2.3. DNA sequencing

To examine possible effects of p53 mutations in gliomas, we performed DNA sequencing on samples judged positive for the mutation. Eight red colonies from each positive plate were randomly picked and p53 fragments were amplified by direct PCR with P3 and P4 primers, using rTth polymerase (PE Applied Biosystems, Foster City, CA). The PCR products were separated on 0.8% agarose, then the DNA bands were excised and purified using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Sequencing reactions were performed with P3 and P4 primers using a Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems). The sequencing instrument was an ABI377 automated sequencer (PE Applied Biosystems).

6.3. Statistical Analysis

Few patients with GBM, the most malignant glioma subtype, are treated successfully. To clarify whether P53 gene mutations contribute to the malignancy of GBM, we analyzed the association between the p53 status and clinical parameters in adult patients with cerebral GBM. Progression-free survival (PFS) and overall

survival (OS) were calculated from the day of surgery to the day of recurrence or death. We used student's t-test to evaluate the relationship between the p53 status and age and the chi-square test to determine the relationship between the p53 status and gender. The effect of different parameters, i.e. age (54 vs. 55), gender, preoperative KPS (60 vs. 70), extent of surgical resection (total vs. subtotal/biopsy), and p53 mutation status (intact vs. mutated), on the Kaplan-Meier survival curve of PFS or OS was analyzed using the log rank (Mantel-Cox) test. The independent effect of each parameter on PFS and OS was analyzed using the multivariate Cox proportional hazards regression model. Also, to make clear the overall effect of p53 status on clinical behavior regardless of tumor grade, we performed the same survival analyses on the entire patient population, including DA, AA, and GBM. All calculations were performed using Statview statistical software (Version 5.0; Abacus Concepts, Inc., Berkeley, CA). P-values lower than 0.05 were considered significant.

7. Results

7.1. p53 mutations and Statistical Analyses on GBM Patients

Clinical data and the results of mutation analysis are summarized in Table 1 to 5. p53 mutations were present in 48 % of AAs and 31 % of supratentorial GBMs; they were less frequent, or absent, in the other subtypes. The age of GBM patients with and without p53 mutations was 53.2 ± 4.6 and 54.9 ± 2.3 years, respectively; there was no significant difference in age or gender between these 2 groups. The median survival of GBM patients carrying the mutation was 16.0 ± 3.5 months; it was 16.3 ± 0.9 months for GBM patients with normal p53 and the difference was not statistically significant (Table 6). p53 mutations had no influence on PFS or OS not only in GBM (Figure 3 and Table6. 7). OS was longer in younger patients (≤ 54) and those with a higher KPS (70 ≤) ($p = 0.02$ and $p = 0.01$, respectively; Figure 4, 5).

7.2. Sequencing Analysis

Mutation positive cases with DA, AA, and GBM are extracted in Table 8 and 9. Of an overall 43 mutations, 36 (83.7%) were missense mutations. There was only one nonsense mutation (patient no. 28 , Table 9). Of these point mutations, 23 (62.1%) were located on hot spots. Of the 37 point mutations, 27 (72.9%) were transition mutations. Of the 43 mutations, 6 (13.9%) were base deletions; one was a base insertion mutation. With respect to mutation location, 10 of 15 (66%) p53 mutations in AA and 7 of 24 (29.1%) in GBM were located on exon 8 (Tables 8, 9); the difference between AA and GBM was significant at $p = 0.02$; other characteristics of the mutation had no differences between these tumors (Table 10).

8. Discussion

Mutation of tumor suppressor gene p53 is a genetic alteration often seen in astrocytic tumors, especially malignant gliomas including AA and GBM. The mutation has been documented by single-strand conformational polymorphism (SSCP)- and immunohistochemical study and by yeast functional assays.

The aim of the current study was to categorize gliomas genetically to promote the development of evidence-based cancer therapies. If such genetics-based treatment becomes available, a patient's p53 status must be known. While the yeast functional assay employed here can detect only loss-of-function mutations, it is simple and its results are reproducible, and it is as sensitive as other assays for the detection of mutations in the p53 gene. In fact, the incidence of the mutation in the current series is equivalent to that reported by others (Table 11). Therefore, the yeast functional assay was judged to be best suited for the current studies.

The influence of p53 mutations in gliomas has been discussed[11, 23, 27, 36, 56, 57, 106-114]. Tumor cells carrying p53 mutations are resistant to apoptosis induced by DNA damage, and an overexpression of wild-type p53 enhances the radiosensitivity of glioma cells. However, the effect of p53 mutations on the radio- and chemosensitivity of human gliomas, especially GBMs, remains controversial. Some have reported that the p53 status of GBM patients did not affect their survival or sensitivity to radiotherapy[107, 108], while others have shown that the presence of p53 gene mutations in GBM was associated with longer survival and a better radiation response[23, 106]. Ishii et al. have presented evidence showing that in low-grade gliomas, the p53 mutation was correlated with a worse prognosis[110].

Our data show that the time to tumor progression after surgery in patients receiving radiochemotherapy was not affected by the presence of p53 mutation. Therefore, the p53 gene mutation alone does not account for the radiochemoresistance

of GBM. In fact, there is some evidence that p21 overexpression due to wild-type p53 overexpression results in the radio- and chemoresistance of glioma[115-117], and that drug-resistance gene expressions renders glioma cells chemoresistant[118-122].

Diverse gene alterations are involved in glioblastoma progression. The MDM2 oncogene, whose product degrades and inactivates p53 protein, is amplified and overexpressed in approximately 10% of GBMs, particularly primary GBMs with intact p53. Amplification of MDM2 leads to inhibition of the tumor-suppressive effects of p53. In gliomas, transcription of a short alternative splice variant of MDM2 is frequently observed[123, 124] and the variant lacks the ability to bind p53 protein for its degradation. This may be one explanation for the observation that in 30% of primary GBMs there is accumulation of wild-type p53 protein. It is accepted that the p53 mutation occurs early in the progression from low-grade diffuse astrocytoma to glioblastoma. We posit that in secondary GBMs that manifest the mutation, it may be carried over from earlier stages in tumor progression. We found that in 73% of AAs and 24% of GBMs, the p53 mutation was localized to exon 8 (Tables 8, 9). This suggests that AAs containing the p53 mutation on exon 8 have a lower tendency for malignant progression. If this were not the case, GBMs could be expected to have acquired new p53 mutations on exons other than exon 8 as a later event in progression to GBM. On the other hand, if the propensity for malignant progression is indeed lower in AAs with the mutation on exon 8, one could expect a lower incidence of p53 mutations in GBMs. In fact, this was not the case in our study where no less than 31% of GBMs manifested the p53 mutation. Besides, the result that the rate of exon 8 mutation in DA was lower than in AA is inconsistent with the first hypothesis. Therefore, we concluded that some of mutations in GBM were acquired de novo.

We also found that mixed gliomas (OA and AOA) contained much fewer p53 mutations (Table 5). This suggests that in these tumors, the pathway for tumorigenesis

or progression is different from that in pure astrocytomas. To classify gliomas genetically and to elucidate factors involved in their tumorigenesis and progression to malignancy, large-scale studies must be performed that also address the status of other genes, for example, MDM2, p16, p14, EGFR, and PTEN.

Mechanisms upstream and downstream from the p53 tumor suppressive pathway are being revealed. TP53 transactivates many target genes that are involved in various biological functions[125-130]. Each gene has its own p53-binding sequence in its promoter region, and is under the control of p53 in a promoter-specific manner. As we screened for the functional loss of p53 by RGC, one of the binding sequences, we were unable to detect all possible mutations and may have overlooked some mutations that result in the loss of important unknown promoter activities. Studies are underway to determine whether such mechanisms are impaired in gliomas.

Although we found that in AA the p53 mutation tended to be located on exon 8, this tendency was observed in past literatures (Table 12). Our finding may reflect promoter-selective transactivation. In breast cancer, p53 mutations on exon 4 were associated with a particularly poor prognosis, while mutations on exons 6 and 7, and on hot spot regions were not, suggesting that different p53 domains may affect patient survival differently[131]. Further investigations may yield information on whether this is also the case in patients with glioma.

9. Conclusions

The current study confirmed that the p53 mutation is not a significant prognostic factor in patients with GBM and points to the existence of still unknown pathways for gliomagenesis and progression.

10. References

1. Kleihues P, Cavenee WK. Pathology and genetics of tumors of the nervous system. In: Kleihues P, Sobn LH. eds. World Health Organization Classification of Tumours Lyon: IARC press, 2000.

2. Kleihues P, Ohgaki H. Primary and secondary glioblastomas: from concept to clinical diagnosis. *Neuro-oncol* 1999:1:44-51.

3. Lang FF, Miller DC, Pisharody S, Koslow M, Newcomb EW. High frequency of p53 protein accumulation without p53 gene mutation in human juvenile pilocytic, low grade and anaplastic astrocytomas. *Oncogene* 1994;9:949-54.

4. Willert JR, Daneshvar L, Sheffield VC, Cogen PH. Deletion of chromosome arm 17p DNA sequences in pediatric high-grade and juvenile pilocytic astrocytomas. *Genes Chromosomes Cancer* 1995:12:165-72.

5. Patt S, Gries H, Giraldo M, Cervos-Navarro J, Martin H, Janisch W, et al. p53 gene mutations in human astrocytic brain tumors including pilocytic astrocytomas. *Hum Pathol* 1996;27:586-9.

6. Raffel C. Molecular biology of pediatric gliomas. *J Neurooncol* 1996;28:121-8.

7. Ishii N, Sawamura Y, Tada M, Daub DM, Janzer RC, Meagher-Villemure M, et al. Absence of p53 gene mutations in a tumor panel representative of pilocytic astrocytoma diversity using a p53 functional assay. *Int J Cancer*

1998;76:797-800.

8. Hayes VM, Dirven CM, Dam A, Verlind E, Molenaar WM, Mooij JJ, et al. High frequency of TP53 mutations in juvenile pilocytic astrocytomas indicates role of TP53 in the development of these tumors. *Brain Pathol* 1999;9:463-7.

9. Nakamizo A, Inamura T, Ikezaki K, Yoshimoto K, Inoha S, Mizoguchi M, et al. Enhanced apoptosis in pilocytic astrocytoma: a comparative study of apoptosis and proliferation in astrocytic tumors. *J Neurooncol* 2002;57:105-14.

10. Ino Y, Zlatescu MC, Sasaki H, Macdonald DR, Stemmer-Rachamimov AO, Jhung S, et al. Long survival and therapeutic responses in patients with histologically disparate high-grade gliomas demonstrating chromosome 1p loss. *J Neurosurg* 2000;92:983-90.

11. Schmidt MC, Antweiler S, Urban N, Mueller W, Kuklik A, Meyer-Puttlitz B, et al. Impact of genotype and morphology on the prognosis of glioblastoma. *J Neuropathol Exp Neurol* 2002;61:321-8.

12. Steck PA, Saya H. Pathways of oncogenesis in primary brain tumors. *Curr Opin Oncol* 1991;3:476-84.

13. Schlegel U. [Mechanisms of molecular development of human glioma]. *Nervenarzt* 1993;64:485-93.

14. von Deimling A, von Ammon K, Schoenfeld D, Wiestler OD, Seizinger BR, Louis

DN. Subsets of glioblastoma multiforme defined by molecular genetic analysis. *Brain Pathol* 1993:3:19-26.

15. Lang FF, Miller DC, Koslow M, Newcomb EW. Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alterations in 65 astrocytic tumors. *J Neurosurg* 1994;81:427-36.

16. Kleihues P, Aguzzi A, Ohgaki H. Genetic and environmental factors in the etiology of human brain tumors. *Toxicol Lett* 1995;82-83:601-5.

17. von Deimling A, Louis DN, Wiestler OD. Molecular pathways in the formation of gliomas. *Glia* 1995;15:328-38.

18. Dirven CM, Tuerlings J, Molenaar WM, Go KG, Louis DN. Glioblastoma multiforme in four siblings: a cytogenetic and molecular genetic study. *J Neurooncol* 1995;24:251-8.

19. Ohgaki H, Schauble B, zur Hausen A, von Ammon K, Kleihues P. Genetic alterations associated with the evolution and progression of astrocytic brain tumours. *Virchows Arch* 1995;427:113-8.

20. Zhang S, Endo S, Koga H, Ichikawa T, Feng X, Onda K, et al. A comparative study of glioma cell lines for p16, p15, p53 and p21 gene alterations. *Jpn J Cancer Res* 1996;87:900-7.

21. Tsuzuki T, Tsunoda S, Sakaki T, Konishi N, Hiasa Y, Nakamura M. Alterations of

retinoblastoma, p53, p16(CDKN2), and p15 genes in human astrocytomas. *Cancer* 1996;78:287-93.

22. Louis DN. A molecular genetic model of astrocytoma histopathology. *Brain Pathol* 1997;7:755-64.

23. Leenstra S, Oskam NT, Bijleveld EH, Bosch DA, Troost D, Hulsebos TJ. Genetic sub-types of human malignant astrocytoma correlate with survival. *Int J Cancer* 1998;79:159-65.

24. Rasheed BK, Wiltshire RN, Bigner SH, Bigner DD. Molecular pathogenesis of malignant gliomas. *Curr Opin Oncol* 1999;11:162-7.

25. Saxena A, Shriml LM, Dean M, Ali IU. Comparative molecular genetic profiles of anaplastic astrocytomas/glioblastomas multiforme and their subsequent recurrences. *Oncogene* 1999;18:1385-90.

26. Weiss WA. Genetics of brain tumors. *Curr Opin Pediatr* 2000;12:543-8.

27. Kraus JA, Glesmann N, Beck M, Krex D, Klockgether T, Schackert G, et al. Molecular analysis of the PTEN, TP53 and CDKN2A tumor suppressor genes in long-term survivors of glioblastoma multiforme. *J Neurooncol* 2000;48:89-94.

28. Kraus JA, Felsberg J, Tonn JC, Reifenberger G, Pietsch T. Molecular genetic analysis of the TP53, PTEN, CDKN2A, EGFR, CDK4 and MDM2 tumour-associated genes in supratentorial primitive neuroectodermal tumours and

glioblastomas of childhood. *Neuropathol Appl Neurobiol* 2002;28:325-33.

29. Watanabe K, Tachibana O, Sata K, Yonekawa Y, Kleihues P, Ohgaki H. Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. *Brain Pathol* 1996;6:217-23; discussion 23-4.

30. Biernat W, Tohma Y, Yonekawa Y, Kleihues P, Ohgaki H. Alterations of cell cycle regulatory genes in primary (de novo) and secondary glioblastomas. *Acta Neuropathol (Berl)* 1997;94:303-9.

31. Biernat W, Kleihues P, Yonekawa Y, Ohgaki H. Amplification and overexpression of MDM2 in primary (de novo) glioblastomas. *J Neuropathol Exp Neurol* 1997;56:180-5.

32. Tohma Y, Gratas C, Biernat W, Peraud A, Fukuda M, Yonekawa Y, et al. PTEN (MMAC1) mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas. *J Neuropathol Exp Neurol* 1998;57:684-9.

33. Fujisawa H, Reis RM, Nakamura M, Colella S, Yonekawa Y, Kleihues P, et al. Loss of heterozygosity on chromosome 10 is more extensive in primary (de novo) than in secondary glioblastomas. *Lab Invest* 2000;80:65-72.

34. Chung R, Whaley J, Kley N, Anderson K, Louis D, Menon A, et al. TP53 gene mutations and 17p deletions in human astrocytomas. *Genes Chromosomes Cancer* 1991;3:323-31.

35. Hagel C, Laking G, Laas R, Scheil S, Jung R, Milde-Langosch K, et al. Demonstration of p53 protein and TP53 gene mutations in oligodendrogliomas. *Eur J Cancer* 1996;32A:2242-8.

36. Hagel C, Krog B, Laas R, Stavrou DK. Prognostic relevance of TP53 mutations, p53 protein, Ki-67 index and conventional histological grading in oligodendrogliomas. *J Exp Clin Cancer Res* 1999;18:305-9.

37. Watanabe T, Yokoo H, Yokoo M, Yonekawa Y, Kleihues P, Ohgaki H. Concurrent inactivation of RB1 and TP53 pathways in anaplastic oligodendrogliomas. *J Neuropathol Exp Neurol* 2001;60:1181-9.

38. Wolter M, Reifenberger J, Blaschke B, Ichimura K, Schmidt EE, Collins VP, et al. Oligodendroglial tumors frequently demonstrate hypermethylation of the CDKN2A (MTS1, p16INK4a), p14ARF, and CDKN2B (MTS2, p15INK4b) tumor suppressor genes. *J Neuropathol Exp Neurol* 2001;60:1170-80.

39. Hoang-Xuan K, He J, Huguet S, Mokhtari K, Marie Y, Kujas M, et al. Molecular heterogeneity of oligodendrogliomas suggests alternative pathways in tumor progression. *Neurology* 2001;57:1278-81.

40. Burger PC, Minn AY, Smith JS, Borell TJ, Jedlicka AE, Huntley BK, et al. Losses of chromosomal arms 1p and 19q in the diagnosis of oligodendroglioma. A study of paraffin-embedded sections. *Mod Pathol* 2001;14:842-53.

41. Watanabe T, Nakamura M, Yonekawa Y, Kleihues P, Ohgaki H. Promoter hypermethylation and homozygous deletion of the p14ARF and p16INK4a genes in oligodendrogliomas. *Acta Neuropathol (Berl)* 2001;101:185-9.

42. Kraus JA, Lamszus K, Glesmann N, Beck M, Wolter M, Sabel M, et al. Molecular genetic alterations in glioblastomas with oligodendroglial component. *Acta Neuropathol (Berl)* 2001:101:311-20.

43. Mueller W, Hartmann C, Hoffmann A, Lanksch W, Kiwit J, Tonn J, et al. Genetic signature of oligoastrocytomas correlates with tumor location and denotes distinct molecular subsets. *Am J Pathol* 2002:161:313-9.

44. Ueki K, Nishikawa R, Nakazato Y, Hirose T, Hirato J, Funada N, et al. Correlation of histology and molecular genetic analysis of 1p, 19q, 10q, TP53, EGFR, CDK4, and CDKN2A in 91 astrocytic and oligodendroglial tumors. *Clin Cancer Res* 2002;8:196-201.

45. Gjerset RA, Turla ST, Sobol RE, Scalise JJ, Mercola D, Collins H, et al. Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. *Mol Carcinog* 1995:14:275-85.

46. Kock H, Harris MP, Anderson SC, Machemer T, Hancock W, Sutjipto S, et al. Adenovirus-mediated p53 gene transfer suppresses growth of human glioblastoma cells in vitro and in vivo. *Int J Cancer* 1996;67:808-15.

47. Blagosklonny MV, el-Deiry WS. In vitro evaluation of a p53-expressing

adenovirus as an anti-cancer drug. *Int J Cancer* 1996;67:386-92.

48. Gomez-Manzano C, Fueyo J, Kyritsis AP, Steck PA, Roth JA, McDonnell TJ, et al. Adenovirus-mediated transfer of the p53 gene produces rapid and generalized death of human glioma cells via apoptosis. *Cancer Res* 1996;56:694-9.

49. Li H, Lochmuller H, Yong VW, Karpati G, Nalbantoglu J. Adenovirus-mediated wild-type p53 gene transfer and overexpression induces apoptosis of human glioma cells independent of endogenous p53 status. *J Neuropathol Exp Neurol* 1997;56:872-8.

50. Lang FF, Yung WK, Sawaya R, Tofilon PJ. Adenovirus-mediated p53 gene therapy for human gliomas. *Neurosurgery* 1999;45:1093-104.

51. Mashiyama S, Murakami Y, Yoshimoto T, Sekiya T, Hayashi K. Detection of p53 gene mutations in human brain tumors by single-strand conformation polymorphism analysis of polymerase chain reaction products. *Oncogene* 1991;6:1313-8.

52. Ohgaki H, Eibl RH, Schwab M, Reichel MB, Mariani L, Gehring M, et al. Mutations of the p53 tumor suppressor gene in neoplasms of the human nervous system. *Mol Carcinog* 1993;8:74-80.

53. Rasheed BK, McLendon RE, Herndon JE, Friedman HS, Friedman AH, Bigner DD, et al. Alterations of the TP53 gene in human gliomas. *Cancer Res* 1994;54:1324-30.

54. Van Meir EG, Kikuchi T, Tada M, Li H, Diserens AC, Wojcik BE, et al. Analysis of the p53 gene and its expression in human glioblastoma cells. *Cancer Res* 1994;54:649-52.

55. Koga H, Zhang S, Washiyama K, Ichikawa T, Onda K, Kumanishi T. Analysis of p53 gene mutations in human glioma cell lines. *Noshuyo Byori* 1996;13:1-10.

56. Pollack IF, Hamilton RL, Finkelstein SD, Campbell JW, Martinez AJ, Sherwin RN, et al. The relationship between TP53 mutations and overexpression of p53 and prognosis in malignant gliomas of childhood. *Cancer Res* 1997;57:304-9.

57. Schiebe M, Ohneseit P, Hoffmann W, Meyermann R, Rodemann HP, Bamberg M. Analysis of mdm2 and p53 gene alterations in glioblastomas and its correlation with clinical factors. *J Neurooncol* 2000;49:197-203.

58. Fulci G, Labuhn M, Maier D, Lachat Y, Hausmann O, Hegi ME, et al. p53 gene mutation and ink4a-arf deletion appear to be two mutually exclusive events in human glioblastoma. *Oncogene* 2000;19:3816-22.

59. Hayashi Y, Yamashita J, Yamaguchi K. Timing and role of p53 gene mutation in the recurrence of glioma. *Biochem Biophys Res Commun* 1991;180:1145-50.

60. del Arco A, Garcia J, Arribas C, Barrio R, Blazquez MG, Izquierdo JM, et al. Timing of p53 mutations during astrocytoma tumorigenesis. *Hum Mol Genet* 1993;2:1687-90.

61. Kraus JA, Bolln C, Wolf HK, Neumann J, Kindermann D, Fimmers R, et al. TP53 alterations and clinical outcome in low grade astrocytomas. *Genes Chromosomes Cancer* 1994;10:143-9.

62. Watanabe K, Sato K, Biernat W, Tachibana O, von Ammon K, Ogata N, et al. Incidence and timing of p53 mutations during astrocytoma progression in patients with multiple biopsies. *Clin Cancer Res* 1997;3:523-30.

63. Honda R, Yasuda H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *Embo J* 1999;18:22-7.

64. Rodriguez MS, Desterro JM, Lain S, Midgley CA, Lane DP, Hay RT. SUMO-1 modification activates the transcriptional response of p53. *Embo J* 1999;18:6455-61.

65. Buschmann T, Fuchs SY, Lee CG, Pan ZQ, Ronai Z. SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. *Cell* 2000;101:753-62.

66. Weber JD, Kuo ML, Bothner B, DiGiammarino EL, Kriwacki RW, Roussel MF, et al. Cooperative signals governing ARF-mdm2 interaction and nucleolar localization of the complex. *Mol Cell Biol* 2000;20:2517-28.

67. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat Cell Biol*

2001;3:973-82.

68. Reifenberger G, Liu L, Ichimura K, Schmidt EE, Collins VP. Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. *Cancer Res* 1993;53:2736-9.

69. Korkolopoulou P, Christodoulou P, Kouzelis K, Hadjiyannakis M, Priftis A, Stamoulis G, et al. MDM2 and p53 expression in gliomas: a multivariate survival analysis including proliferation markers and epidermal growth factor receptor. *Br J Cancer* 1997;75:1269-78.

70. Ichimura K, Bolin MB, Goike HM, Schmidt EE, Moshref A, Collins VP. Deregulation of the p14ARF/MDM2/p53 pathway is a prerequisite for human astrocytic gliomas with G1-S transition control gene abnormalities. *Cancer Res* 2000;60:417-24.

71. He J, Reifenberger G, Liu L, Collins VP, James CD. Analysis of glioma cell lines for amplification and overexpression of MDM2. *Genes Chromosomes Cancer* 1994;11:91-6.

72. Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, et al. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* 1998;92:713-23.

73. He J, Allen JR, Collins VP, Allalunis-Turner MJ, Godbout R, Day RS, 3rd, et al. CDK4 amplification is an alternative mechanism to p16 gene homozygous

deletion in glioma cell lines. *Cancer Res* 1994;54:5804-7.

74. Jen J, Harper JW, Bigner SH, Bigner DD, Papadopoulos N, Markowitz S, et al. Deletion of p16 and p15 genes in brain tumors. *Cancer Res* 1994;54:6353-8.

75. Schmidt EE, Ichimura K, Reifenberger G, Collins VP. CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. *Cancer Res* 1994;54:6321-4.

76. Sonoda Y, Yoshimoto T, Sekiya T. Homozygous deletion of the MTS1/p16 and MTS2/p15 genes and amplification of the CDK4 gene in glioma. *Oncogene* 1995;11:2145-9.

77. Ueki K, Ono Y, Henson JW, Efird JT, von Deimling A, Louis DN. CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. *Cancer Res* 1996;56:150-3.

78. Hayashi Y, Ueki K, Waha A, Wiestler OD, Louis DN, von Deimling A. Association of EGFR gene amplification and CDKN2 (p16/MTS1) gene deletion in glioblastoma multiforme. *Brain Pathol* 1997;7:871-5.

79. Simon M, Koster G, Menon AG, Schramm J. Functional evidence for a role of combined CDKN2A (p16-p14(ARF))/CDKN2B (p15) gene inactivation in malignant gliomas. *Acta Neuropathol (Berl)* 1999;98:444-52.

80. Mochizuki S, Iwadate Y, Namba H, Yoshida Y, Yamaura A, Sakiyama S, et al.

Homozygous deletion of the p16/MTS-1/CDKN2 gene in malignant gliomas is infrequent among Japanese patients. *Int J Oncol* 1999;15:983-9.

81. Steilen-Gimbel H, Steudel WI, Feiden W, Moringlane JR, Henn W, Zang KD. Genetic heterogeneity in human astrocytomas: spatial distribution of P16 and TP53 deletions in biopsies. *Cancer Genet Cytogenet* 1999;113:115-9.

82. Rollbrocker B, Waha A, Louis DN, Wiestler OD, von Deimling A. Amplification of the cyclin-dependent kinase 4 (CDK4) gene is associated with high cdk4 protein levels in glioblastoma multiforme. *Acta Neuropathol (Berl)* 1996;92:70-4.

83. Holland EC, Hively WP, Gallo V, Varmus HE. Modeling mutations in the G1 arrest pathway in human gliomas: overexpression of CDK4 but not loss of INK4a-ARF induces hyperploidy in cultured mouse astrocytes. *Genes Dev* 1998;12:3644-9.

84. Besson A, Yong VW. Mitogenic signaling and the relationship to cell cycle regulation in astrocytomas. *J Neurooncol* 2001;51:245-64.

85. Dirks PB, Rutka JT. Current concepts in neuro-oncology: the cell cycle--a review. *Neurosurgery* 1997;40:1000-13; discussion 13-5.

86. Fults D, Brockmeyer D, Tullous MW, Pedone CA, Cawthon RM. p53 mutation and loss of heterozygosity on chromosomes 17 and 10 during human astrocytoma progression. *Cancer Res* 1992;52:674-9.

87. Lee SH, Kim JH, Rhee CH, Kang YS, Lee JH, Hong SI, et al. Loss of heterozygosity on chromosome 10, 13q(Rb), 17p, and p53 gene mutations in human brain gliomas. *J Korean Med Sci* 1995;10:442-8.

88. Fujisawa H, Kurrer M, Reis RM, Yonekawa Y, Kleihues P, Ohgaki H. Acquisition of the glioblastoma phenotype during astrocytoma progression is associated with loss of heterozygosity on 10q25-qter. *Am J Pathol* 1999;155:387-94.

89. Tada K, Shiraishi S, Kamiryo T, Nakamura H, Hirano H, Kuratsu J, et al. Analysis of loss of heterozygosity on chromosome 10 in patients with malignant astrocytic tumors: correlation with patient age and survival. *J Neurosurg* 2001;95:651-9.

90. Rasheed BK, Stenzel TT, McLendon RE, Parsons R, Friedman AH, Friedman HS, et al. PTEN gene mutations are seen in high-grade but not in low-grade gliomas. *Cancer Res* 1997;57:4187-90.

91. Liu W, James CD, Frederick L, Alderete BE, Jenkins RB. PTEN/MMAC1 mutations and EGFR amplification in glioblastomas. *Cancer Res* 1997;57:5254-7.

92. Teng DH, Hu R, Lin H, Davis T, Iliev D, Frye C, et al. MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. *Cancer Res* 1997;57:5221-5.

93. Somerville RP, Shoshan Y, Eng C, Barnett G, Miller D, Cowell JK. Molecular analysis of two putative tumour suppressor genes, PTEN and DMBT, which have been implicated in glioblastoma multiforme disease progression. *Oncogene*

1998;17:1755-7.

94. Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC, et al. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. *Brain Pathol* 1999;9:469-79.

95. Zhou XP, Li YJ, Hoang-Xuan K, Laurent-Puig P, Mokhtari K, Longy M, et al. Mutational analysis of the PTEN gene in gliomas: molecular and pathological correlations. *Int J Cancer* 1999;84:150-4.

96. Davies MP, Gibbs FE, Halliwell N, Joyce KA, Roebuck MM, Rossi ML, et al. Mutation in the PTEN/MMAC1 gene in archival low grade and high grade gliomas. *Br J Cancer* 1999;79:1542-8.

97. Kato H, Kato S, Kumabe T, Sonoda Y, Yoshimoto T, Han SY, et al. Functional evaluation of p53 and PTEN gene mutations in gliomas. *Clin Cancer Res* 2000;6:3937-43.

98. Tachibana I, Smith JS, Sato K, Hosek SM, Kimmel DW, Jenkins RB. Investigation of germline PTEN, p53, p16(INK4A)/p14(ARF), and CDK4 alterations in familial glioma. *Am J Med Genet* 2000;92:136-41.

99. Smith JS, Tachibana I, Passe SM, Huntley BK, Borell TJ, Iturria N, et al. PTEN mutation, EGFR amplification, and outcome in patients with anaplastic astrocytoma and glioblastoma multiforme. *J Natl Cancer Inst* 2001;93:1246-56.

100. Kordek R, Biernat W, Alwasiak J, Maculewicz R, Yanagihara R, Liberski PP. p53 protein and epidermal growth factor receptor expression in human astrocytomas. *J Neurooncol* 1995;26:11-6.

101. Feldkamp MM, Lala P, Lau N, Roncari L, Guha A. Expression of activated epidermal growth factor receptors, Ras-guanosine triphosphate, and mitogen-activated protein kinase in human glioblastoma multiforme specimens. *Neurosurgery* 1999;45:1442-53.

102. Tang P, Steck PA, Yung WK. The autocrine loop of TGF-alpha/EGFR and brain tumors. *J Neurooncol* 1997;35:303-14.

103. Flaman JM, Frebourg T, Moreau V, Charbonnier F, Martin C, Chappuis P, et al. A simple p53 functional assay for screening cell lines, blood, and tumors. *Proc Natl Acad Sci U S A* 1995;92:3963-7.

104. Tada M, Iggo RD, Ishii N, Shinohe Y, Sakuma S, Estreicher A, et al. Clonality and stability of the p53 gene in human astrocytic tumor cells: quantitative analysis of p53 gene mutations by yeast functional assay. *Int J Cancer* 1996;67:447-50.

105. Tada M, Iggo RD, Waridel F, Nozaki M, Matsumoto R, Sawamura Y, et al. Reappraisal of p53 mutations in human malignant astrocytic neoplasms by p53 functional assay: comparison with conventional structural analyses. *Mol Carcinog* 1997;18:171-6.

106. Tada M, Matsumoto R, Iggo RD, Onimaru R, Shirato H, Sawamura Y, et al. Selective sensitivity to radiation of cerebral glioblastomas harboring p53 mutations. *Cancer Res* 1998;58:1793-7.

107. Danks RA, Chopra G, Gonzales MF, Orian JM, Kaye AH. Aberrant p53 expression does not correlate with the prognosis in anaplastic astrocytoma. *Neurosurgery* 1995:37:246-54.

108. Baxendine-Jones J, Campbell I, Ellison D. p53 status has no prognostic significance in glioblastomas treated with radiotherapy. *Clin Neuropathol* 1997;16:332-6.

109. Rainov NG, Dobberstein KU, Bahn H, Holzhausen HJ, Lautenschlager C, Heidecke V, et al. Prognostic factors in malignant glioma: influence of the overexpression of oncogene and tumor-suppressor gene products on survival. *J Neurooncol* 1997;35:13-28.

110. Ishii N, Tada M, Hamou MF, Janzer RC, Meagher-Villemure K, Wiestler OD, et al. Cells with TP53 mutations in low grade astrocytic tumors evolve clonally to malignancy and are an unfavorable prognostic factor. *Oncogene* 1999;18:5870-8.

111. Simmons ML, Lamborn KR, Takahashi M, Chen P, Israel MA, Berger MS, et al. Analysis of complex relationships between age, p53, epidermal growth factor receptor, and survival in glioblastoma patients. *Cancer Res* 2001;61:1122-8.

112. Peraud A, Kreth FW, Wiestler OD, Kleihues P, Reulen HJ. Prognostic impact of

TP53 mutations and P53 protein overexpression in supratentorial WHO grade II astrocytomas and oligoastrocytomas. *Clin Cancer Res* 2002;8:1117-24.

113. Pollack IF, Finkelstein SD, Woods J, Burnham J, Holmes EJ, Hamilton RL, et al. Expression of p53 and prognosis in children with malignant gliomas. *N Engl J Med* 2002;346:420-7.

114. Rasheed A, Herndon JE, Stenzel TT, Raetz JG, Kendelhardt J, Friedman HS, et al. Molecular markers of prognosis in astrocytic tumors. *Cancer* 2002;94:2688-97.

115. Jung JM, Bruner JM, Ruan S, Langford LA, Kyritsis AP, Kobayashi T, et al. Increased levels of p21WAF1/Cip1 in human brain tumors. *Oncogene* 1995;11:2021-8.

116. Kokunai T, Tamaki N. Relationship between expression of p21WAF1/CIP1 and radioresistance in human gliomas. *Jpn J Cancer Res* 1999;90:638-46.

117. Kraus A, Gross MW, Knuechel R, Munkel K, Neff F, Schlegel J. Aberrant p21 regulation in radioresistant primary glioblastoma multiforme cells bearing wild-type p53. *J Neurosurg* 2000;93:863-72.

118. Harris LC, Remack JS, Houghton PJ, Brent TP. Wild-type p53 suppresses transcription of the human O6-methylguanine-DNA methyltransferase gene. *Cancer Res* 1996;56:2029-32.

119. Grombacher T, Eichhorn U, Kaina B. p53 is involved in regulation of the DNA

repair gene O6-methylguanine-DNA methyltransferase (MGMT) by DNA damaging agents. *Oncogene* 1998;17:845-51.

120. Silber JR, Bobola MS, Ghatan S, Blank A, Kolstoe DD, Berger MS. O6-methylguanine-DNA methyltransferase activity in adult gliomas: relation to patient and tumor characteristics. *Cancer Res* 1998;58:1068-73.

121. Rolhion C, Penault-Llorca F, Kemeny JL, Kwiatkowski F, Lemaire JJ, Chollet P, et al. O(6)-methylguanine-DNA methyltransferase gene (MGMT) expression in human glioblastomas in relation to patient characteristics and p53 accumulation. *Int J Cancer* 1999;84:416-20.

122. Nakamura M, Watanabe T, Yonekawa Y, Kleihues P, Ohgaki H. Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G:C --> A:T mutations of the TP53 tumor suppressor gene. *Carcinogenesis* 2001;22:1715-9.

123. Matsumoto R, Tada M, Nozaki M, Zhang CL, Sawamura Y, Abe H. Short alternative splice transcripts of the mdm2 oncogene correlate to malignancy in human astrocytic neoplasms. *Cancer Res* 1998;58:609-13.

124. Kraus A, Neff F, Behn M, Schuermann M, Muenkel K, Schlegel J. Expression of alternatively spliced mdm2 transcripts correlates with stabilized wild-type p53 protein in human glioblastoma cells. *Int J Cancer* 1999;80:930-4.

125. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator

of the human bax gene. *Cell* 1995;80:293-9.

126. Nishimori H, Shiratsuchi T, Urano T, Kimura Y, Kiyono K, Tatsumi K, et al. A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. *Oncogene* 1997;15:2145-50.

127. Nakano K, Balint E, Ashcroft M, Vousden KH. A ribonucleotide reductase gene is a transcriptional target of p53 and p73. *Oncogene* 2000;19:4283-9.

128. Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, et al. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000;288:1053-8.

129. Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, et al. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 2000;102:849-62.

130. Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, et al. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* 2000;404:42-9.

131. Powell B, Soong R, Iacopetta B, Seshadri R, Smith DR. Prognostic significance of mutations to different structural and functional regions of the p53 gene in breast cancer. *Clin Cancer Res* 2000;6:443-51.

Figure 1 Schema of Yeast Functional Assay

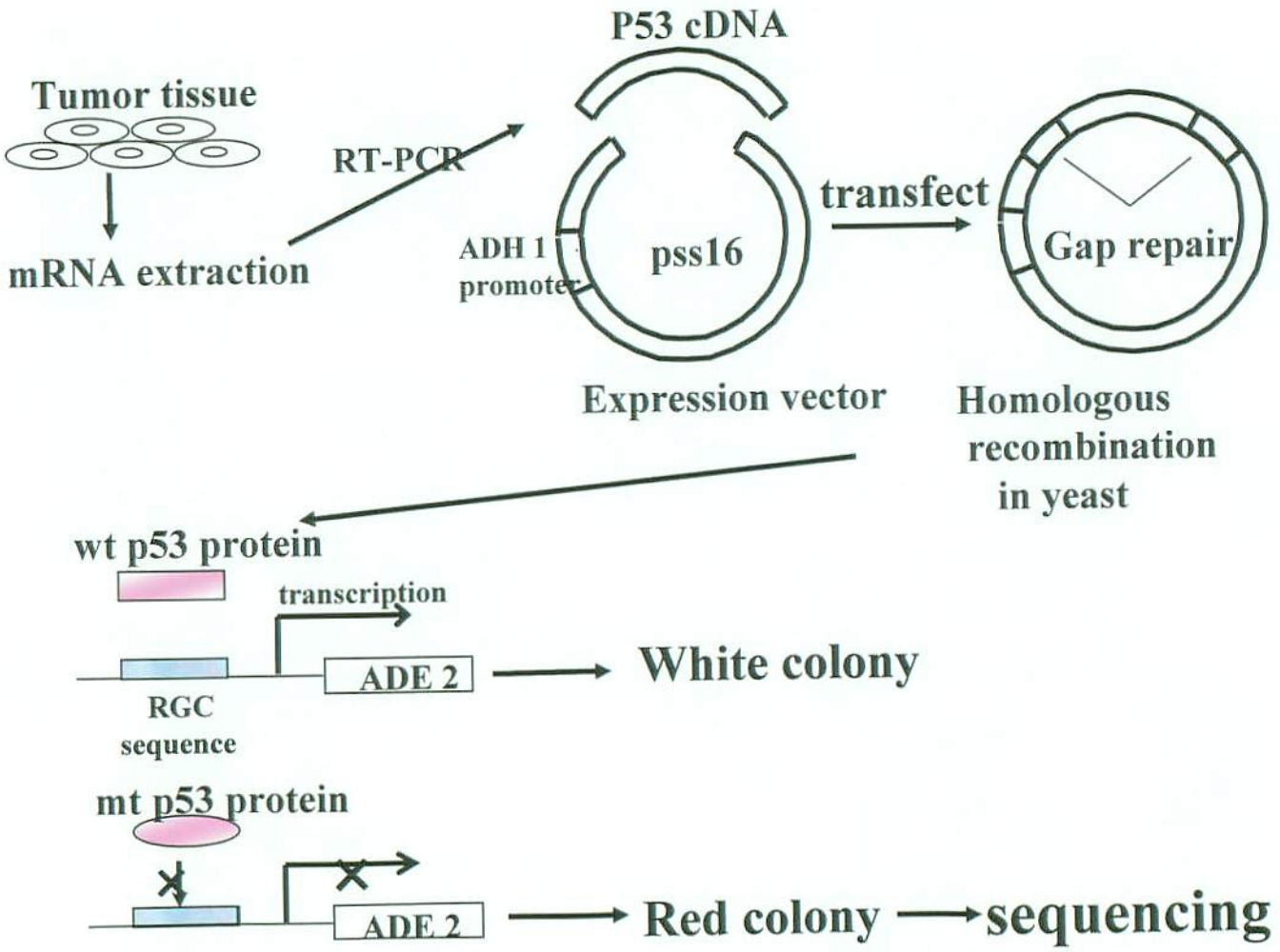


Figure 2-A
Yeast Colonies

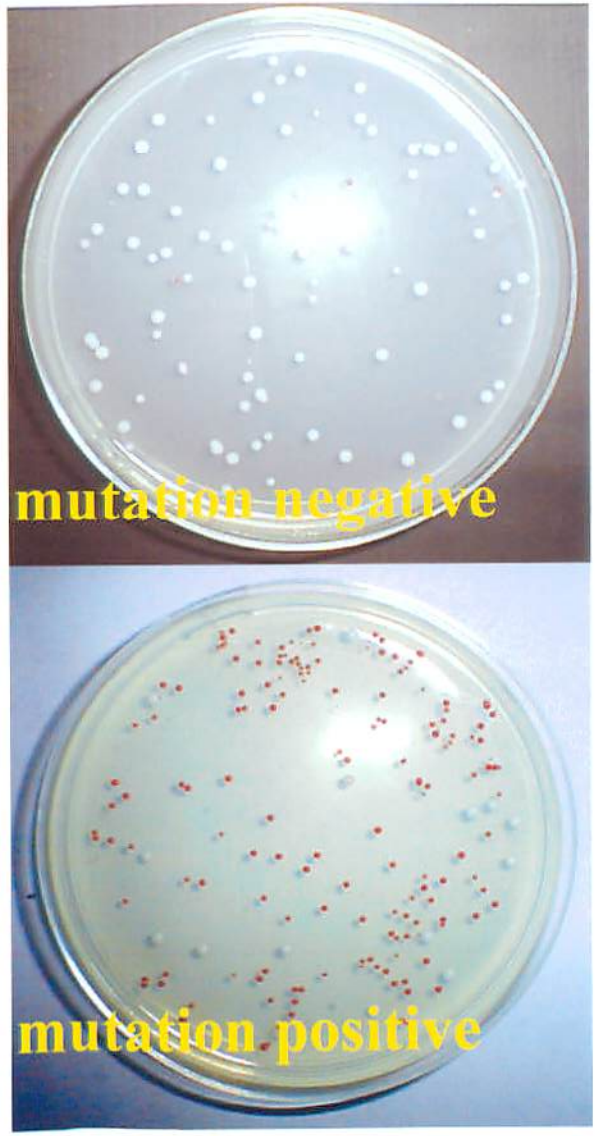


Figure 2-B
Electropherograms

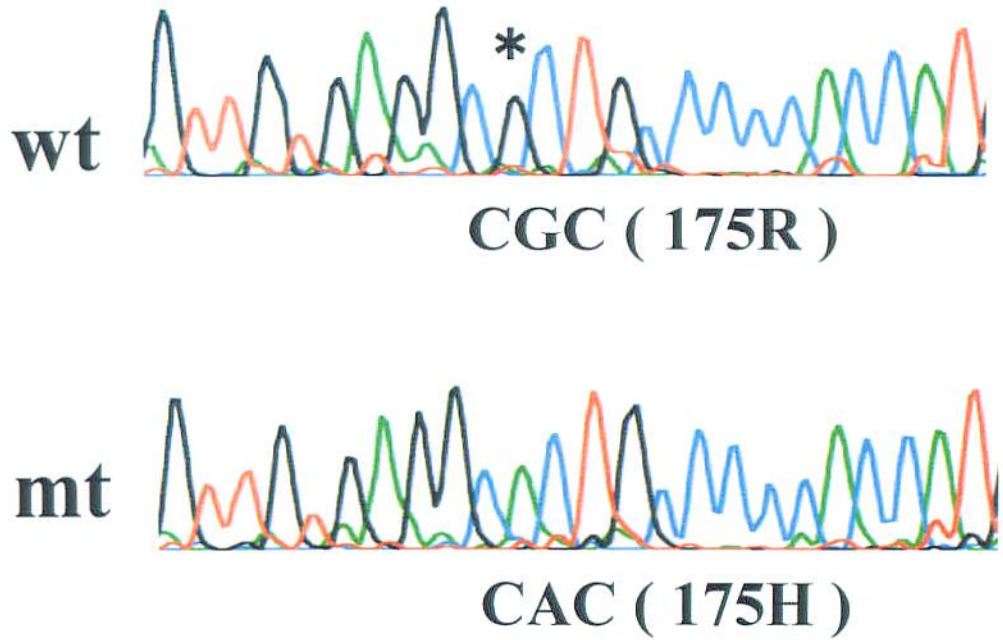
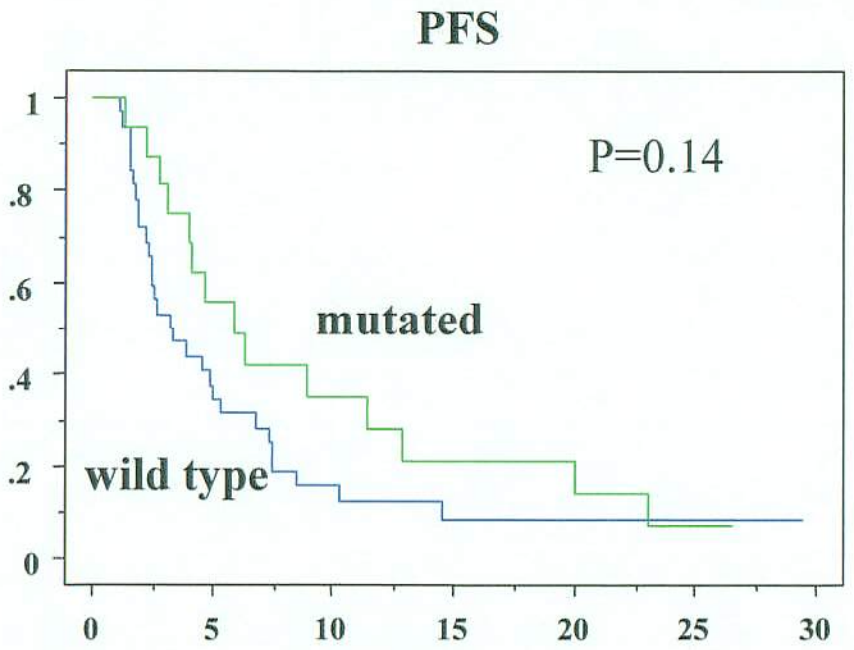
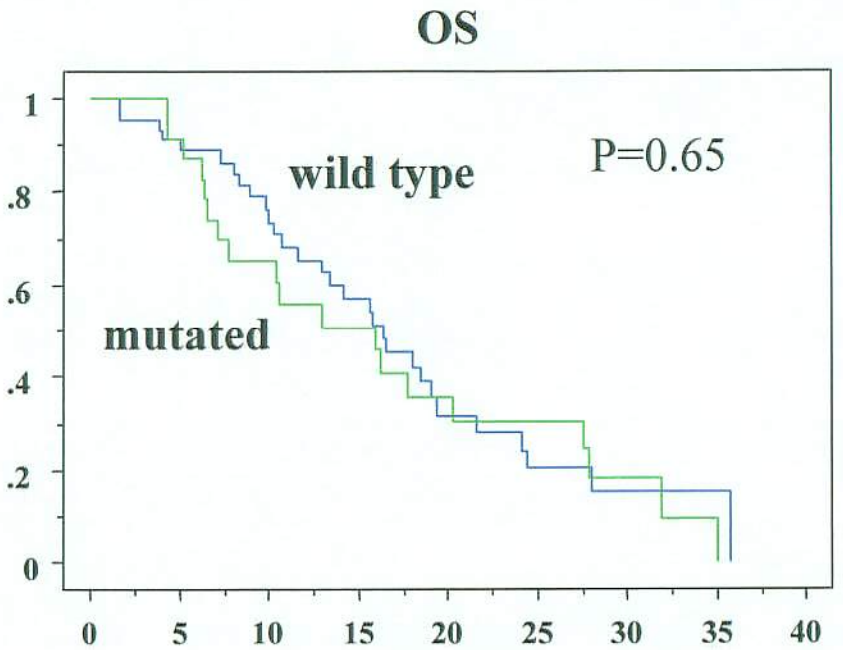


Figure 3
Correlation of P53 mutation and patients' survival





PFS: progression free survival

OS: overall survival

Figure 4
Survival Curves in Patients with Glioblastoma

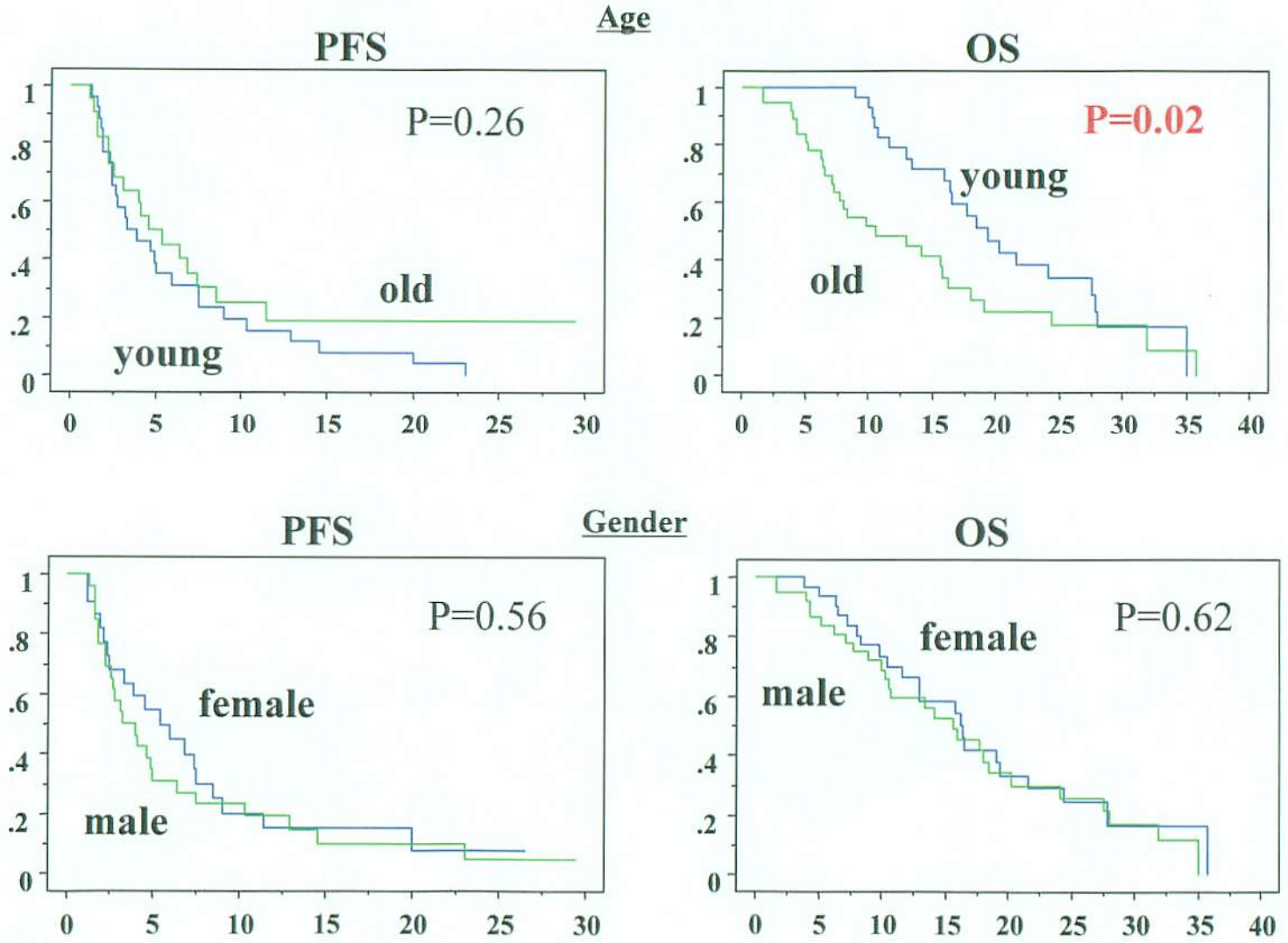


Figure 5
Survival Curves in Patients with Glioblastoma

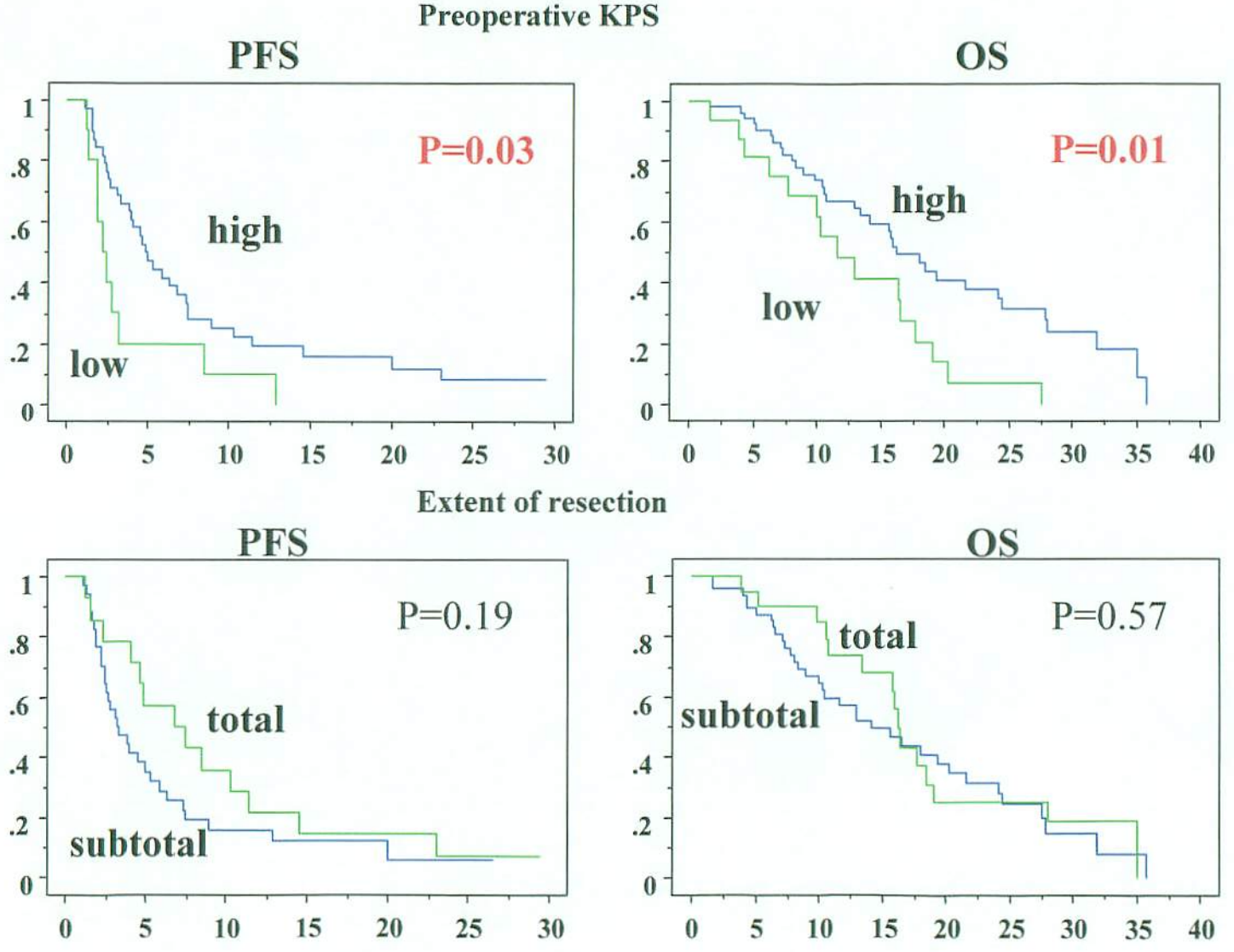


Table 1

Genetic Status and Clinical Features of Patients with Pilocytic Astrocytoma

No.	Age	Gender	WHO Class	WHO Grade	P53 Mutation					KPS	Resect.
					Mut.	Exon	Codon	Base Change	AA Change		
1	2	M	PA	I	N					90	*total*
2	9	F	PA	I	N					60	*total*
3	20	M	PA	I	N					80	*subtotal*
4	8	M	PA	I	N					40	*total*
5	6	M	PA	I	N					70	*subtotal*
6	9	F	PA	I	N					80	*subtotal*
7	6	M	PA	I	N					40	*subtotal*
8	6	M	PA	I	N					100	*subtotal*
9	10	M	PA	I	N					80	*total*
10	7	F	PA	I	N					*70*	*subtotal*

AA change: amino acid change: KPS: Karnofsky Performance Status; Resect.: extent of resection; M: male; F: female; PA: pilocytic astrocytoma; N: negative; P: positive; del.: deletion; ins.: insertion; stop: stop codon

Table 2

Genetic Status and Clinical Features of Patients with Diffuse Astrocytoma

No.	Age	Gender	WHO Class	WHO Grade	P53 Mutation					KPS	Resect.
					Mut.	Exon	Codon	Base Change	AA Change		
11	43	F	DA	2	N					*90*	*total*
12	48	M	DA	2	N					*100*	*subtotal*
13	62	M	DA	2	N					*100*	*subtotal*
14	4	M	DA	2	N					*90*	*total*
15	43	F	DA	2	N					*100*	*subtotal*
16	50	M	DA	2	N					*80*	*subtotal*
17	78	M	DA	2	N					*80*	*biopsy*
18	70	M	DA	2	N					*90*	*biopsy*
19	35	M	DA	2	N					*80*	*subtotal*
20	23	M	DA	2	N					*80*	*biopsy*
21	0	F	DA	2	N					*90*	*total*
22	60	F	DA	2	P	6	220	tat to tgt	Y to C	*100*	*total*
23	62	M	DA	2	P	8	273	cgt to tgt	R to C	*100*	*subtotal*
24	39	M	DA	2	P	8	273	cgt to tgt	R to C	*100*	*total*
25	41	F	DA	2	P	8	273	cgt to tgt	R to C	*100*	*subtotal*

AA change: amino acid change; KPS: Karnofsky Performance Status; Resect.: extent of resection; M: male; F: female; DA: diffuse astrocytoma: N: negative; P: positive; del.: deletion; ins.: insertion; stop: stop codon;

Table 3

Genetic Status and Clinical Features of Patients with Anaplastic Astrocytoma

No.	Age	Gender	WHO Class	WHO Grade	P53 Mutation					KPS	Resect.
					Mut.	Exon	Codon	Base Change	AA Change		
26	38	F	AA	3	N					*100*	*subtotal*
27	62	M	AA	3	N					*70*	*biopsy*
28	26	M	AA	3	N					*70*	*subtotal*
29	43	M	AA	3	N					*100*	*biopsy*
30	43	M	AA	3	N					*100*	*biopsy*
31	72	M	AA	3	N					*100*	*subtotal*
32	50	F	AA	3	N					*70*	*subtotal*
33	68	F	AA	3	N					*90*	*total*
35	60	F	AA	3	N					*70*	*biopsy*
36	43	F	AA	3	N					*100*	*total*
37	68	M	AA	3	N					*100*	*total*
38	50	F	AA	3	N					*80*	*subtotal*
39	65	M	AA	3	N					*70*	*total*
40	49	M	AA	3	N					*100*	*biopsy*
41	65	F	AA	3	P	5	152-153	gccc	del.	*80*	*total*
42	54	F	AA	3	P	6	213	cga to caa	R to E	*70*	*subtotal*
43	71	M	AA	3	P	7	241	tcc to ttc	S to F	*60*	*biopsy*
44	41	M	AA	3	P	7	248	cgg to cag	R to Q	*100*	*total*
45	43	F	AA	3	P	7	261	agt to agg	S to R+ ins.	*100*	*subtotal*
46	40	M	AA	3	P	8	264	cta del.	L del.	*100*	*subtotal*
47	18	F	AA	3	P	8	273	cgt to cat	R to H	*70*	*total*
48	31	M	AA	3	P	8	273	cgt to tgt	R to C	*100*	*subtotal*
49	42	F	AA	3	P	8	273	cgt to tgt	R to C	*90*	*biopsy*
50	29	M	AA	3	P	8	273	cgt to cat	R to H	*100*	*subtotal*
51	56	F	AA	3	P	8	273	cgt to tgt	R to C	*100*	*total*
52	37	F	AA	3	P	8	280	aga to gga	R to G	*20*	*subtotal*
53	48	M	AA	3	P	8	280	aga to aaa	R to K	*100*	*subtotal*
54	35	M	AA	3	P	8	273	cgt to cat	R to H	*100*	*subtotal*
55	26	M	AA	3	P	8	273	cgt to tgt	R to C	*90*	*biopsy*

AA change: amino acid change; KPS: Karnofsky Performance Status; Resect.: extent of resection; M: male; F: female; AA: anaplastic astrocytoma; N: negative; P: positive; del.: deletion; ins.: insertion; stop: stop codon

Table 4

Genetic Status and Clinical Features of Patients with Glioblastoma

No.	Age	Gender	WHO Class	WHO Grade	P53 Mutation					KPS	Resect.
					Mut.	Exon	Codon	Base Change	AA Change		
56	54	M	GBM	4	N					70	total
57	51	F	GBM	4	N					90	subtotal
58	64	M	GBM	4	N					90	subtotal
59	45	M	GBM	4	N					100	total
60	69	F	GBM	4	N					80	subtotal
61	38	F	GBM	4	N					10	subtotal
62	74	F	GBM	4	N					60	total
63	61	F	GBM	4	N					90	biopsy
64	56	M	GBM	4	N					90	total
65	47	M	GBM	4	N					90	total
66	53	M	GBM	4	N					60	subtotal
67	71	M	GBM	4	N					70	biopsy
68	54	M	GBM	4	N					70	subtotal
69	29	F	GBM	4	N					70	biopsy
70	53	F	GBM	4	N					70	subtotal
71	64	M	GBM	4	N					90	subtotal
72	54	F	GBM	4	N					50	subtotal
73	75	F	GBM	4	N					60	total
74	71	F	GBM	4	N					70	subtotal
75	72	M	GBM	4	N					90	subtotal
76	66	F	GBM	4	N					100	subtotal
77	45	M	GBM	4	N					70	total
78	53	M	GBM	4	N					90	biopsy
79	63	F	GBM	4	N					70	total
80	59	M	GBM	4	N					100	subtotal
81	51	F	GBM	4	N					50	subtotal
82	38	F	GBM	4	N					70	subtotal
83	72	M	GBM	4	N					100	subtotal
84	20	F	GBM	4	N					60	total
85	52	F	GBM	4	N					70	subtotal

AA change: amino acid change; KPS: Karnofsky Performance Status; Resect.: extent of resection; M: male; F: female; GBM: glioblastoma multiforme; BS: brain stem; GGB: giant cell glioblastoma; N: negative: P: positive: del.: deletion: ins.: insertion; stop: stop codon; NR: not recorded

Table 4 Continued

Genetic Status and Clinical Features of Patients with Glioblastoma

No.	Age	Gender	WHO Class	WHO Grade	P53 Mutation					KPS	Resect.
					Mut.	Exon	Codon	Base Change	AA Change		
86		F	GBM	4	N					NR	NR
87	40	M	GBM	4	N					80	total
88	68	M	GBM	4	N					60	subtotal
89	60	F	GBM	4	N					100	subtotal
90	73	F	GBM	4	N					50	total
91	68	F	GBM	4	N					70	subtotal
92	71	F	GBM	4	N					100	subtotal
93	21	M	GBM	4	N					80	biopsy
94	48	M	GBM	4	N					100	total
95	25	M	GBM	4	N					60	subtotal
96	64	F	GBM	4	N					70	total
97	59	M	GBM	4	N					90	biopsy
98	65	M	GBM	4	N					90	subtotal
99	70	M	GBM	4	N					NR	NR
100	54	M	GBM	4	N					70	subtotal
101	21	M	GBM	4	N					100	biopsy
102	70	F	GBM	4	N					NR	NR
103	63	F	GBM	4	N					100	subtotal
104	54	M	GBM	4	N					70	total
105	59	M	GBM	4	P	6	205	Tat to cat	Y to H	70	NR
106	54	M	GBM	4	P	6	190	Cct to ctt	P to L	60	total
107	61	M	GBM	4	P	7	248	cgg to cag	R to Q	70	total
108	45	M	GBM	4	P	6	196	cga to tga	R to stop	100	total
109	74	M	GBM	4	P	7	248	cgg to tgg	R to W	80	subtotal
110	32	F	GBM	4	P	9	307	gca to gat	A to D	70	subtotal
111	69	F	GBM	4	P	8	282	cgg to tgg	R to W	80	subtotal
112	66	M	GBM	4	P	8	273	cgt to cat	R to H	90	subtotal
113	73	F	GBM	4	P	10	333	Cgtg to gtg	frame shift	100	subtotal
114	60	M	GBM	4	P	5	131	aac to del	N to del	70	subtotal
115	47	M	GBM	4	P	7	245	ggc to agc	G to S	60	subtotal

AA change: amino acid change; KPS: Karnofsky Performance Status; Resect.: extent of resection: M: male: F: female; GBM: glioblastoma multiforme; BS: brain stem; GGB: giant cell glioblastoma; N: negative; P: positive: del.: deletion; ins.: insertion; stop: stop codon; NR: not recorded

Table 4 Continued

Genetic Status and Clinical Features of Patients with Glioblastoma

No.	Age	Gender	WHO Class	WHO Grade	P53 Mutation					KPS	Resect.
					Mut.	Exon	Codon	Base Change	AA Change		
116	33	M	GBM	4	P	5	158	cgc to ctc	R to L	70	total
117	71	M	GBM	4	P	5	175	cgc to cac	R to H	60	subtotal
118	64	M	GBM	4	P	8	273	cgt to ggt	R to G	70	total
119	67	F	GBM	4	P	7	251	atc to ttc	I to F	90	total
120	77	F	GBM	4	P	7	246	atg to gtg	M to V	70	subtotal
121	55	M	GBM	4	P	8	273	cgt to tgt	R to C	60	biopsy
122	23	F	GBM	4	P	6	195	atc to acc	I to T	100	subtotal
123	23	M	GBM	4	P	5	179	cat to ctt	H to L	50	biopsy
124	56	F	GBM	4	P	8	267	cgg to tgg	R to W	80	subtotal
125	71	M	GBM	4	P	5	179	cat to tat	H to Y	50	subtotal
126	17	F	GBM	4	P	4	90—105	del		100	subtotal
127	49	M	GBM	4	P	8	273	cgt to tgt	R to C	70	total
128	44	F	GBM	4	P	8	273	cgt to tgt	R to C	100	subtotal
129	71	M	GBM, BS	4	N						
130	53	M	GBM, BS	4	N						
131	4	F	GBM, BS	4	N						
132	3	M	GBM, BS	4	P	8	277	tgt to ttt	C to F		
133	22	F	GGB	4	N						
134	32	F	GGB	4	P	5	148	cgg to cag	R to Q		

AA change: amino acid change; KPS: Karnofsky Performance Status; Resect.: extent of resection; M: male; F: female; GBM: glioblastoma multiforme; BS: brain stem; GGB: giant cell glioblastoma; N: negative; P: positive; del.: deletion; ins.: insertion; stop: stop codon; NR: not recorded

Table 5

Genetic Status and Clinical Features of Patients with Mixed Glioma

No.	Age	Gender	WHO Class	WHO Grade	P53 Mutation					KPS	Resect.
					Mut.	Exon	Codon	Base Change	AA Change		
135	1	M	OA	2	N					NR	total
136	49	F	AOA	3	N					90	subtotal
137	44	F	AOA	3	N					100	total
138	67	M	AOA	3	N					80	subtotal
139	52	F	AOA	3	N					100	total
140	63	F	AOA	3	N					90	total
141	52	M	AOA	3	N					100	total
142	19	M	AOA	3	N					100	total
143	52	M	AOA	3	N					100	total
144	53	M	AOA	3	N					90	total
145	39	M	AOA	3	N					100	total
146	68	F	AOA	3	N					50	biopsy
147	70	F	AOA	3	N					70	subtotal
148	54	M	AOA	3	N					100	subtotal
149	42	F	AOA	3	N					100	subtotal
150	62	M	AOA	3	N					100	subtotal
151	51	M	AOA	3	N					70	total
152	47	M	AOA	3	N					100	total
153	45	M	AOA	3	N					60	total
154	55	M	AOA	3	P	4	101	aaa to gaa	K to E	NR	NR
155	36	M	AOA	3	P	5	167-172	del	del	100	subtotal
156	55	F	AOA	3	P	7	248	cgg to cag	R to Q	100	subtotal
157	53	M	AOA	3	P	8	273	cgt to tgt	R to C	100	subtotal
158	37	M	AOA	4	N					NR	NR
159	58	M	AOA	4	N					100	total
160	65	F	AOA	4	N					80	total

AA change: amino acid change: KPS: Karnofsky Performance Status; Resect.: extent of resection; M: male; F: female; AOA: anaplastic oligoastrocytoma; OA: oligoastrocytoma; N: negative; P: positive; del.: deletion; ins.: insertion; stop: stop codon; NR: not recorded

Table 6

Univariate Analysis for Prognosis Factors in GBM

Factor		No. of cases	PFS		OS	
			Median ± S.E. (month)	p-Values	Median ± S.E. (month)	p-Values
Age	54≥	32	3.3 ± 0.8	0.26	19.4 ± 2.1	0.02
	55≤	41	4.5 ± 1.4		10.6 ± 3.9	
Gender	male	40	3.2 ± 1.1	0.56	15.6 ± 2.4	0.62
	female	33	5.4 ± 1.4		16.3 ± 0.1	
KPS	60≥	16	2.2 ± 0.4	0.03	11.5 ± 2.5	0.01
	70≤	54	4.8 ± 0.4		16.3 ± 2.8	
	not recorded	3				
Extent of resection	subtotal	49	3.2 ± 0.2	0.19	14.1 ± 3.8	0.57
	total	20	6.8 ± 2.4		16.3 ± 0.3	
	not recorded	4				
P53 status	intact	48	3.2 ± 0.8	0.14	16.3 ± 0.9	0.65
	mutated	25	5.9 ± 1.6		16.0 ± 3.5	

p-value lower than 0.05 were considered significant

PFS: progression-free survival; OS: overall survival: S.E.: standard error

Table 7

Multivariate Analysis for Prognosis Factors in GBM

Factor	PFS			OS		
	Hazard ratio	95% Confidence intervals	p-Values	Hazard ratio	95% Confidence intervals	p-Values
Age (54≥)	1.38	0.71 ~ 2.67	0.32	0.41	0.22 ~ 0.76	0.005
Gender (female)	0.79	0.40 ~ 1.55	0.49	0.75	0.41 ~ 1.38	0.36
KPS (70≤)	0.49	0.22 ~ 1.06	0.07	0.36	0.18 ~ 0.70	0.003
Extent of resection (total)	0.52	0.24 ~ 1.09	0.08	0.83	0.44 ~ 1.56	0.57
P53 status (mutated)	0.48	0.24 ~ 0.96	0.03	1.03	0.55 ~ 1.94	0.90

p-value lower than 0.05 were considered significant

PFS: progression-free survival: OS: overall survival

Table 8
Mutations in DAs and AAs

No.	Age (yrs) / gender	WHO classification	Mutation			
			Exon	Codon	Base change	AA change
1	60 /F	DA	6	220	TAT → TGT	Y → C
2	39 /M	DA	8	273*	CGT → TGT	R → C
3	62 /M	DA	8	273*	CGT → TGT	R → C
4	41/F	DA	8	273*	CGT → TGT	R → C
5	65/F	AA	5	152-153	GCCC → del.	frame shift
6	54 /F	AA	6	213*	CGA → CAA	R → E
7	71/M	AA	7	241	TCC → TTC	S → F
8	41 /M	AA	7	248*	CGG → CAG	R → Q
9	43 /F	AA	7	261	AGT → AGG	S → R+Ins.
10	40 /M	AA	8	264	CTA → del.	L → del.
11	56 /F	AA	8	273*	CGT → TGT	R → C
12	29 /M	AA	8	273*	CGT → CAT	R → H
13	42 /F	AA	8	273*	CGT → TGT	R → C
14	31 /M	AA	8	273*	CGT → TGT	R → C
15	18 /F	AA	8	273*	CGT → CAT	R → H
16	35/M	AA	8	273*	CGT → CAT	R → H
17	26/M	AA	8	273*	CGT → TGT	R → C
18	48 /M	AA	8	280	AGA → AAA	R → K
19	37 /F	AA	8	280	AGA → GGA	R → G

Table 9
Mutations in GBMs

No.	Age (yrs) / gender	WHO classification	Mutation			
			Exon	Codon	Base change	AA change
20	17 /F	GBM	4	90∽105	del	In-frame
21	60 /M	GBM	5	131	CAA→del.	N → del.
22	33 /M	GBM	5	158	CGC → CTC	R → L
23	71 /M	GBM	5	175*	CGC → CAC	R → H
24	71 /M	GBM	5	179	CAT → TAT	H → Y
25	23 /M	GBM	5	179	CAT → CTT	H → L
26	54 /M	GBM	6	190	CCT → CTT	P → L
27	23 /F	GBM	6	195	ATC → ACC	I → T
28	45 /M	GBM	6	196*	CGA → TGA	R → stop
29	59/M	GBM	6	205	TAT → CAT	Y → H
30	47 /M	GBM	7	245*	GGC → AGC	G → S
31	77 /F	GBM	7	246	ATG → GTG	M → Y
32	61/M	GBM	7	248*	CGG → CAG	R → Q
33	74 /M	GBM	7	248*	CGG → TGG	R → W
34	67 /F	GBM	7	251	ATC → TTC	I → F
35	56 /F	GBM	8	267	CGG → TGG	R → W
36	66/M	GBM	8	273*	CGT → CAT	R → H
37	64 /M	GBM	8	273*	CGT → GGT	R → G
38	55 /M	GBM	8	273*	CGT → TGT	R → C
39	49/M	GBM	8	273*	CGT → TGT	R → C
40	44/F	GBM	8	273*	CGT → TGT	R → C
41	69 /F	GBM	8	282*	CGG → TGG	R → W
42	32/F	GBM	9	307-331	del.	frame shift
43	73/F	GBM	10	333	CGTG → GTG	frame shift

Table 10
Comparison in Characteristics of p53 Mutations between AA and GBM

WHO class	Mutation	Missense (%)	p-value	Hot spot (%)	p-value	C:G to T:A Transition (%)	p-value	Exon 8 (%)	p-value
AA	15	13 (86)		9 (60)		11 (73)		10 (66)	
			0. 55		0.38		0.23		0.02
GBM	24	19 (79)		11 (45)		13 (54)		7 (29)	

Table 11
Incidence of P53 Mutations in Astrocytic Tumors in Literatures

	Author	Assay	DA	AA	GBM
1)	Fults, 1992	SSCP	0 / 6 (0%)	5 / 14 (36%)	7 / 25 (28%)
2)	von Deimling, 1992	SSCP	4 / 8 (50%)	4 / 14 (28%)	
3)	Frankel, 1992	SSCP			10 / 23 (43%)
4)	Louis, 1993	SSCP	4 / 8 (50%)	4 / 12 (33%)	4 / 14 (28%)
5)	Rasheed, 1994	SSCP	3 / 8 (37%)	5 / 9 (55%)	9 / 46 (20%)
6)	Lang, 1994	SSCP	2 / 8 (25%)	3 / 16 (18%)	
7)	Lang, 1994	SSCP	5 / 8 (63%)	10 / 16 (63%)	23 / 34 (68%)
8)	Rainov, 1997	direct		7 / 22 (31.8%)	25 / 69 (36%)
9)	Leenstra, 1998	DGGE		8 / 12 (66%)	15 / 63 (23%)
10)	Newcomb, 1998	SSCP			37 / 78 (47%)
11)	Tada, 1998	yeast			18 / 42 (43%)
12)	Kato, 2000	yeast		9 / 14 (64%)	7 / 27 (26%)
13)	Ichimura, 2000	SSCP	10 / 15 (67%)	26 / 39 (67%)	49 / 136 (36%)
14)	Fulci, 2000	yeast			6 / 20 (30%)
15)	Kraus, 2001	SSCP			20 / 77 (26%)
	Total		28 / 61 (46%)	81 / 168 (48%)	230 / 654 (35%)
16)	Present data	yeast	3 / 15 (20%)	11 / 23 (48%)	17 / 55 (31%)

Table 12
Mutation Locations (% of exon 8) in Literatures

	Author	AA	GBM
(1)	von Deimling, 1992	1/4 (25%)	
(2)	Rasheed, 1994	1/5 (20%)	1/9 (11%)
(3)	Leenstra, 1998	4/8 (50%)	2/16 (12.5%)
(4)	Fulci, 2000		1/6 (16%)
(5)	Ichimura, 2000	3/5 (60%)	10/38 (26%)
(6)	Pollack, 2001	5/9 (55%)	6/17 (35%)
(7)	Rasheed, 2002	7/15 (46%)	5/19 (26%)
(8)	IARC Data Base		55/175 (31%)
	Total	21/46 (45%)	71/280 (25%)
(9)	Present data	8/11(66%)	4/17 (29%)