Pathol. Oncol. Res. (2013) 19:421–428
DOI 10.1007/s12253-012-9596-7

RESEARCH

# High Frequency of Temperature-Sensitive Mutants of *p53* in Glioblastoma

**Jana Smardova · Kvetoslava Liskova · Barbora Ravcukova · Lenka Kubiczkova · Sabina Sevcikova · Jaroslav Michalek · Miluse Svitakova · Vaclav Vybihal · Leos Kren · Jan Smarda**

Received: 20 April 2012 / Accepted: 21 December 2012 / Published online: 28 March 2013


**Abstract** Glioblastoma is the most common and the most aggressive type of brain cancer. Aberrations of the RTK/RAS/PI3K-, p53-, and RB cell signaling pathways were recognized as a core requirement for pathogenesis of glioblastoma. The p53 tumor suppressor functions as a transcription factor transactivating expression of its target genes in response to various stress stimuli. We determined the p53 status in 36 samples of glioblastoma by functional analyses FASAY and split assay. Seventeen *p53* mutations were detected and further analyzed by cDNA and gDNA sequencing in 17 patients (47.2 %). Fifteen (88.2 %) of the mutations were missense mutations causing amino acid substitutions, seven of them exhibited temperature-sensitivity. Two mutations were determined as short deletions, one of them causing formation of premature termination codon in position 247. Fluorescent *in situ* hybridization revealed the loss of the *p53*-specific 17p13.3 locus in four of 33 analyzed samples (12 %). In 12 out of 30 samples (40 %), the p53 protein accumulation was shown by immunoblotting. There was high (80 %) concordance between the presence of the clonal *p53* mutation and the p53 protein accumulation.

**Keywords** Glioblastoma · p53 tumor suppressor · FASAY · Temperature-sensitive mutations, *p53* gene deletion

J. Smardova (✉) · K. Liskova · M. Svitakova · L. Kren
Department of Pathology, University Hospital Brno, Jihlavska 20, 625 00 Brno, Czech Republic
e-mail: janasmarda@seznam.cz

B. Ravcukova
Molecular Genetics Laboratory, Centre for Cardiovascular Surgery and Transplantation, Brno, Czech Republic

L. Kubiczkova · S. Sevcikova
Babak Myeloma Group, Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

J. Michalek
Advanced Cell Immunotherapy Unit, Department of Pharmacology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

V. Vybihal
Department of Neurosurgery, Faculty Hospital Brno, Brno, Czech Republic

J. Smarda
Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic

## Introduction

High-grade gliomas are the most common primary brain tumors in humans. They account for 31 % of all primary CNS tumors and for 80 % of malignant CNS tumors. Glioblastoma is the most aggressive form of high-grade gliomas characterized by poorly differentiated and highly proliferating cells originating from glial cells. Although new therapy is available, prognosis remains very poor due to the tumor-resistance to standard therapy.

Majority of glioblastomas, primary or *de novo* glioblastomas, arise from less malignant precursor lesions without clinical or histological evidence; they are usually manifested in people over 55 years of age. Secondary glioblastomas develop more slowly by malignant progression from diffuse (WHO grade II) or anaplastic astrocytoma (WHO grade III), and they are manifested in younger patients (mean age 40 year) [1]. Thus, glioblastomas are divided into two subtypes according to patients' age, specificity of developmental genetic pathway and RNA- and protein profiles. Integrated and comprehensive genomic characterization of

human glioblastoma helped to recognize aberrations of three key signaling pathways as a core requirement for pathogenesis of glioblastoma: the RTK/RAS/PI3K signaling pathway and the p53- and RB pathways [2, 3].

The p53 tumor suppressor mediates an adequate reaction of cells to stress. It functions as a transcription factor responding to various stress signals by transactivating expression of its target genes. Proteins coded by these genes then participate in control of cell cycle, DNA repair, apoptosis, senescence, and maintaining of genome integrity [4, 5]. Mutations and allelic deletions of the *p53* gene are the most frequent genetic alterations detected in human tumors, including glioblastomas. Inactivation of p53 usually results from missense mutations in the core region of the *p53* gene coding for the sequence-specific DNA-binding domain. Due to inability to transactivate its negative regulator, the E3-ubiquitin ligase MDM2 [6–8], the mutant p53 protein often accumulates in tumor cells.

In glioblastoma, the mutational status of the p53 tumor suppressor has been analyzed several times. The most common methodical approach was based on PCR-SSCP screening of exons 4 (or 5) to 8 followed by DNA sequencing. Frequency of detected *p53* mutations ranged between approximately 20 and 60 %, and extensive collection of *p53* mutations was compiled. It allowed to establish the *p53* mutational spectrum of glioblastoma and to suggest the hot-spot codons [9–17]. Similarly, the p53 protein overexpression in glioblastoma was assessed immunohistochemically several times reaching positivity from 25 to 60 % [9, 10, 13, 17–19].

In this study, we analyzed the p53 status in 36 samples of glioblastoma multiforme by functional analysis FASAY and its variant called split assay. We detected 17 *p53* mutations in 17 patients (47.2 %) and analyzed them by cDNA and gDNA sequencing. Fifteen (88.2 %) of the mutations were missense mutations causing amino acid substitutions, seven of them exhibited temperature-sensitivity. Two mutations were determined as short deletions. Using FISH, we determined the loss of the *p53*-specific locus in four (12.1 %) of 33 samples. We tested 30 samples for p53 protein accumulation by immunoblotting and found 12 positive samples (40.0 %).

## Material and Methods

### Clinical Material

All samples were classified according to WHO guidelines. During the study, 36 tumors and 1 normal tissue were collected at the time of surgery. Tissues were immediately placed into the RNA later solution (Ambion, USA) and stored at −20 °C. Patients were included in this study only after signing the informed consent form that was approved by the Ethical committee of the hospital. Patients' characteristics are summarized in Table 1.

### Cell Lines

As a reference, human glioblastoma cell lines A172 (p53wt), U87MG (p53wt) and T98G (p53mt) were used. Cells were cultivated in DMEM media supplemented with 2 mML-glutamine, nonessential amino acids, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10 % fetal calf serum in 10 % $CO_2$.

### FASAY and Split Assay

FASAY was performed as described earlier [20, 21] with minor modifications. Prior to RNA extraction, 50 mg of each of the frozen tumor sample was homogenized using MagNA Lyser Green Beads (Roche, Basel, Switzerland). Total RNA was extracted using High Pure RNA Tissue Kit (Roche) and stored at −80 °C until further processing. cDNA was synthesized by SuperScript II (Life Technologies Inc., Carlsbad, CA, USA) using primer oligo$(dT)_{12}$. PCR was performed using primers P3 (5′-CCT-TGC-CGT-CCC-AAG-CAA-TGG-ATG-AT-3′), P4 (5′-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCT-GGA-GT-3′), and *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). Yeast cells were co-transformed with the PCR product, linearized pSS16 plasmid and salmon sperm DNA carrier (Life Technologies) by the lithium acetate procedure [22]. Transformed yeast cells were plated on minimal medium lacking leucine and with 5 μg/ml of adenine, followed by incubation at 35 °C for 2–3 days, and then for 2–3 days at room temperature. For split assay, PCR of the *p53* 5′- part was performed with primers P3 and P17 (5′-GCC-GCC-

**Table 1** Summary of clinicopathological data of analyzed samples

| | Total ($n$=36) | % |
|---|---|---|
| Age (years) | | |
| ≤50 | 5 | 14 |
| >50 | 31 | 86 |
| Median (range) | 54–69 | |
| Gender | | |
| Male | 22 | 61 |
| Female | 14 | 39 |
| Performance status | | |
| 1 | 27 | 75 |
| 2 and 3 | 9 | 25 |
| Extent of resection | | |
| Total | 4 | 11 |
| Subtotal | 32 | 89 |

CAT-GCA-GGA-ACT-GTT-ACA-CAT-3′), the 3′- part with primers P4 and P16 (5′-GCG-ATG-GTC-TGG-CCC-CTC-CTC-AGC-ATC-TTA-3′). Yeast cells were transformed with linearized vectors pFW35 and pFW34 [23].

### Purification of the Plasmids from Transformed Yeast Cells and Sequencing of the p53 cDNA

Yeast cells from individual yeast colonies were harvested, resuspended in TSN (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris pH8.0, 1 mM EDTA), and grinded by vortexing with glass beads; plasmid DNA was extracted by phenol/chloroform procedure. The *p53* cDNA was amplified using the P3 and P4 primers and *Taq* polymerase (Life Technologies) and subjected to agarose gel electrophoresis. The PCR product was purified by MinElute PCR purification kit (Qiagen) and sequenced by BigDyeTerminator v3.1 cycle sequencing kit (Applied Biosystems, Darmstadt, Germany) using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

### Isolation of gDNA and Amplification of Exons 4, 7, and 8 of p53

Genomic DNA was isolated from formalin-fixed, paraffin-embedded tissue blocks using the Purogene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Exons 4, 7, and 8 of the *p53* gene were amplified by PCR using primers p53-Pg4F (5′-CCTGGTCCTCTGACTGCTCT-3′), p53-Pg4R (5′-GCCAGGCATTGAAGTCTCAT-3′), p53-Pg7F (5′-CTTGGGCCTGTGTTATCTCC-3′), p53-Pg7R (5′-GGGTCAGAGGCAAGCAGA-3′), p53-Pg8Fb (5′-GCC-TCT-TGC-TTC-TCT-TTT-TCC-3′), p53-Pg8R (5′-TAA-CTG-CAC-CCT-TGG-TCT-CC-3′) and *Taq* DNA polymerase (Life Technologies). PCR products were purified and sequenced as described above.

### Fluorescent in Situ Hybridization (FISH)

FISH was performed in tissue sections prepared from FFPE blocks. For the *p53*-specific locus analysis, the Vysis LSI TP53 (17p13.1) Spectrum Orange probe and the centromeric CEP 17 Spectrum Green DNA probe were used (Abbott Molecular Inc., Abbott Park, IL, USA). Hybridization was performed according to the manufacturer's instructions. Images were scanned by Leica DMRXA2 microscope equipped with CCD camera (COHU). Fluorescence signals were analyzed using Leica Q-FISH software (Leica Microsystems GmbH, Wetzlar, Germany). 50–100 cells per case were analyzed. The cut-off level was defined by the mean value plus three times the SD of the frequency of control cells exhibiting one red and two green signals (9.6 %).

### Immunoblotting

Tissue samples (50 mg) were homogenized by MagnaBeads (Roche, Switzerland) on MagnaLyser according to manufacturer's instructions and then lysed in 2 M Tris, 5 M NaCl, 0.5 M EDTA, 0.5 % NP-40, 2 % NaF, 10 % PhosSTOP and 10 % Complete (Roche, Switzerland). Equal amounts of proteins were resolved by 10 % SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were probed with anti-p53 mouse monoclonal antibody DO-1 (kindly provided by B. Vojtesek) and anti-GAPDH rabbit monoclonal antibody (Cell Signaling Technology, USA) at 4 °C. Blots were developed with Sigma peroxidase-conjugated rabbit anti-mouse immunoglobulin and Sigma peroxidase-conjugated goat anti-rabbit immunoglobulin (Sigma, USA) using the ECL chemiluminescence detection kit (Amersham Biosciences, Austria).

## Results

### Assessment of the p53 Status by FASAY

First, we verified the p53 status of three human glioblastoma cell lines A172 (p53wt), U87MG (p53wt) and T98G (p53mt) using FASAY by obtaining 6.0, 10.0 and 100 % of red colonies, respectively. Next, we used FASAY to determine the p53 status of all 36 enrolled samples and control and assessed the ratio of red colonies. Ten samples scored under the 10 % background level and were classified as having the standard p53 variant [20]. Split assay was performed in all samples exceeding 10 % level. Nine samples (usually scoring only slightly above the background level in FASAY) including control scored in both parts of split assay under the background 10 % level, and they were also classified as having the standard p53 variant. All other 18 samples were suspected to bear clonal *p53* mutation and were further analyzed.

### Sequencing of the p53 cDNA and gDNA

All eighteen cases positive in FASAY/split assay were further analyzed by cDNA sequencing. In ten cases, the cDNA prepared from tumor tissue was subjected to DNA sequencing. In six cases, the *p53* expression vector was recovered from 3 to 6 pink or red colonies per case, and isolated cDNAs were used as templates for DNA sequencing. In two cases, both approaches were combined with affirmative result.

Based on results of the cDNA sequencing, two samples (4 and 21) were identified as potentially having two independent *p53* mutations. To confirm their p53 status, gDNA was isolated from the formalin-fixed, paraffin-embedded tissue blocks. The relevant exons were amplified and sequenced. In both cases, only single *p53* mutations were

detected. The same approach was used to get unambiguous conclusion on the p53 status of case 30. The presence of the suspect *p53* mutation was excluded in this case.

Altogether, we detected 17 clonal p53 mutations in 17 cases (17/36–47.2 %; Table 2). Fifteen of them (88.2 %) were missense mutations. In addition, we detected two short deletions: the 3 bp deletion in position c.528 and the 1 bp deletion in position c.698 causing reading frame shift and formation of premature termination codon in position 247. These *p53* mutations were distributed non-randomly. Six mutations were located in exon 5, four mutations in exon 6, four mutations in exon 8 and three mutations in exon 7. None of these mutations was detected repeatedly. All detected mutations mapped in the DNA-binding domain-coding region. All 17 detected mutations were described previously in various tumors (http://www-p53.iarc.fr, version R15) [24], eleven of them in brain tumors. The presence of *p53* mutations T211N, R249W, I255S, and R267Q in brain tumor is described here for the first time (Table 2). Seven mutations (P151A, P152L, T211N, V216M, Y220C, Y234H, and E285K) were recognized as temperature-sensitive ones according to the typical phenotype of positive yeast colonies (Fig. 1) [20, 25]. Temperature-sensitivity of all these mutations has been reported previously (Table 2).

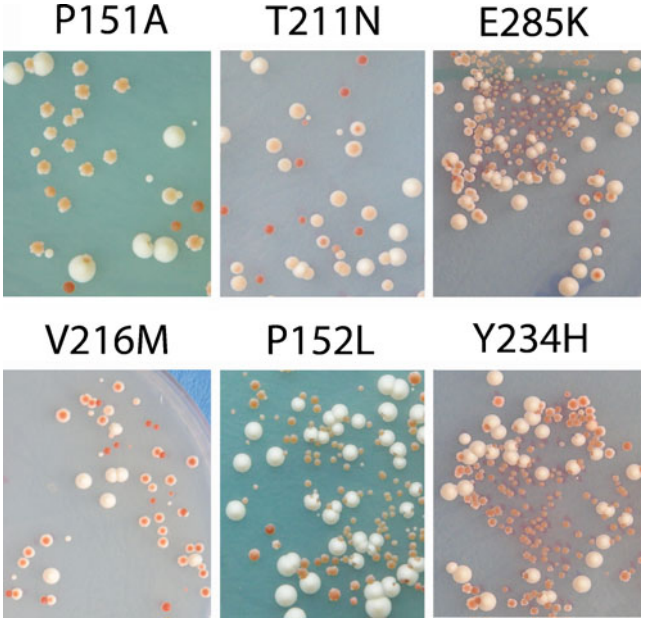


**Fig. 1** Examples of phenotypes of yeast colonies expressing different temperature-sensitive p53 variants

## FISH

We performed the FISH analysis of the *p53* alleles using the locus-specific (red - R) and centromeric (green -G) probes. We analyzed all enrolled cases, but in three of them, the result could not be assessed probably due to suboptimal tissue fixation. Twenty-nine cases were negative as the portion of positive nuclei ranged between 0 and 9 % and did not reach the cut-off level of 9.6 %. In four cases (12.1 %), the *p53*-specific locus was clearly lost. The portion of positive nuclei ranged from 34.6 to 58.1 %. The nuclei were considered positive having either two centromeric signals plus one locus-specific signal (2 G+1R) or

**Table 2** List of detected p53 mutations. [a] according to IARC database R15 (http://www-p53.iarc.fr) [24]; [b] temperature-sensitive mutation

| Case | Mutation | DNA sequence | Exon | Reported as somatic mutation/in brain [a] | Reported as temperature-sensitive | IB |
|---|---|---|---|---|---|---|
| 33 | | 528_530delCAA | 5 | | | +++ |
| 13 | K132R | AAG-AGG | 5 | 57/5 | | ++ |
| 5 | P151A[b] | CCC-GCC | 5 | 18/2 | [31] | ++ |
| 12 | P152L[b] | CCG-CTG | 5 | 83/15 | [43] | ++ |
| 1 | H178Q | CAC-CAA | 5 | 6/1 | | ND |
| 10 | S183P | TCA-CCA | 5 | 3/1 | | +++ |
| 24 | L188W fsX59 | 698delC | 6 | | | – |
| 2 | T211N[b] | ACT-AAT | 6 | 4/0 | [31] | ND |
| 26 | V216M[b] | GTG-ATG | 6 | 76/7 | [25] | +++ |
| 29 | Y220C[b] | TAT-TGT | 6 | 360/19 | [44, 45] | ND |
| 4 | I255S | ATC-AGC | 7 | 9/0 | | ND |
| 18 | Y234H[b] | TAC-CAC | 7 | 27/2 | [43] | ++ |
| 17 | R249W | CGG-TGG | 7 | 40/0 | | + |
| 21 | R267Q | CGG-CAG | 8 | 12/0 | | +/– |
| 3 | G279E | GGG-GAG | 8 | 47/4 | | ND |
| 28 | R280G | AGA-GGA | 8 | 41/5 | | +++ |
| 16 | E285K[b] | GAG-AAG | 8 | 169/6 | [25, 31, 45–47] | +++ |

having one centromeric and one locus-specific signal (1 G+ 1R). We proved earlier that in the later case the lost part of the chromosome comprises also the centromeric region [26]. In all four p53-positive cases determined by FISH, both kinds of positive nuclei were observed. In only one of these cases (26), the *p53* mutation was detected by FASAY. In the other three cases, no other p53 aberration was found.

### Analysis of the p53 Protein Accumulation

We examined the level of the p53 protein in tumor tissue of 30 cases by immunoblotting (IB). Seven glioblastoma samples were strongly p53 positive (+++) when analyzed by immunoblotting. Five samples were positive (++), providing intermediate level of p53. Fourteen samples exhibited low level of the p53 protein (+), four other samples were negative (−) (Fig. 2, Table 2). Overall p53 positivity (+++ or ++) was 40.0 % (12 cases); concordance between p53 mutations and p53 protein accumulation was found in 24 cases (80.0 %). Of the 12 samples positive for the p53 protein (+++ or ++), 9 exhibited clonal p53 mutation. Samples 8, 22 and 32 contained high level of the p53 protein but they did not exhibit any clonal p53 mutation. Of the 18 negative samples (+ or -), only 3 cases (17, 21, 24) featured the p53 mutation including 1 frameshift mutation in case 24. Control sample and the cell lines A172, U87MG, and T98G were also included in the analysis. Only T98G cells having p53 mutation M237I [26] provided high level of the p53 protein, the other samples scored negative (Fig. 2).

## Discussion

In this study, we analyzed the p53 status of 36 samples of primary glioblastoma. We detected *p53* mutations in 17 patients (45.9 %). The p53 tumor suppressor in glioblastoma has been analyzed several times, and the frequency of detected mutations ranged from as low as 20 % to as high as 60 % [2, 9]. Our results thus fit within this range. Previous studies preferentially used PCR-SSCP analyses of exons 4 (or 5) to 8 followed by DNA sequencing to identify the p53 mutations. We used functional analyses called FASAY and its split assay variant followed by DNA sequencing instead. The same approach was already used for analysis of the p53 status in collection of 54 malignant astrocytomas revealing the *p53* mutations in 26 samples (48 %) [27]. Similarly 18 *p53* mutations were detected in 42 patients with glioblastoma (43 %) [28]. This rather high frequency of the *p53* mutations was explained by specificity of the analyzed Northern Japanese population and greater sensitivity of the method. Increased frequency of the *p53* mutations in our study also results from high sensitivity of FASAY. In addition, it corresponds well with two recent comprehensive genomic analyses documenting the *p53* mutations in 41.8 % and 59.1 % of glioblastoma cases, respectively [2, 3].

Inactivation of p53 usually results from a missense mutation in the core region of the *p53* gene coding for the sequence-specific DNA-binding domain. According to the IARC database proportion of the missense mutations represents 73.6 % on average (http://www-p53.iarc.fr) [24, 29]. In our samples, missense mutations occur more frequently representing 82.2 %. This result is well comparable with the data published for glioblastomas previously ranging from about 75 % [2] through about 85 % [9, 12, 15, 30] up to more than 95 % [13]. We also have to consider the fact that there are specific types of non-missense *p53* mutations, such as splite-site mutations, promoter mutations, mutations inducing RNA decay that might be omitted by FASAY, thus causing relative increase of the ratio of detected missense mutations detected. In addition, the results can be also affected by a relatively small size of our data set of the *p53* mutations, at least to some extent.

In one of the fundamental studies in the field [9], codons 175, 273, and 248 of the *p53* gene were identified as hotspots in glioblastoma representing together 43 % of all detected mutations in primary glioblastoma and even more in secondary glioblastomas. In subsequent works, similarly high frequency was rather exceptional (43 %) [13], but some increase was detected also by others (17 %) [15], including

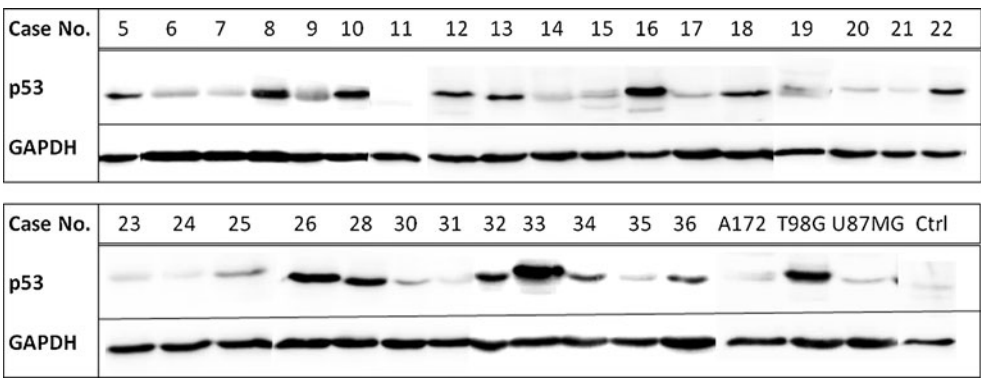


**Fig. 2** Assessment of the p53 protein level in tumor tissue and control samples by immunoblotting using DO-1 antibody; the GAPDH protein was used as a loading control

the team employing functional analysis FASAY. These authors detected 8 mutations (28.6 %) in codons 175, 273, and 248 [27]. Surprisingly, none of 17 *p53* mutations of our collection was detected in these three codons. On the other hand, rather lower percentage of these mutants was also described by others. For example, Schmidt et al. [12] detected mutations in codons 273 and 248 but each in single case while mutation R213R in three cases. Similarly, Schiebe et al. [11] revealed new hot-spot mutation P156R as they detected this mutation in 17 % of their cases. In our study, no mutation was found in more than a single sample. This result presumably reflects limited size of our collection.

Seven of our 17 *p53* mutations exhibited temperature-dependent character. For all of these seven *p53* mutations the temperature-dependency has been already recognized previously (Table 2). This documents reliability of FASAY in exact determination of these mutations. According to the comprehensive study by Siraishi et al. [31], temperature-sensitive mutants comprise about 10 % of all cancer-related p53 mutants exhibiting single amino acid substitutions. In our collection, they represent 41 % of all mutations and even 47 % of missense *p53* mutations, thus extremely exceeding the average estimation. Nevertheless, we have already detected comparably frequent temperature-dependent p53 mutations in AML (38.5 %), HNSCC (head and neck squamous cell carcinomas; 16.7 %) and DLBCL (diffuse large B-cell lymphoma; 21.4 %) [32–34]. In contrast, in other tumors, such as breast and ovarian carcinomas, the proportion of temperature-sensitive mutants was much lower [35, 36]. This raises interesting possibility of cell type- or tissue-specific frequency of temperature-dependent *p53* mutations.

Multiple *p53* mutations per case are not exceptional in various tumors, and they were also reported several times in glioblastomas. Especially, Oghaki et al. [15] detected as many as 26 cases bearing two different *p53* mutations (representing 21 % of their collection) and even 7 cases bearing three different *p53* mutations. The cases featuring multiple *p53* mutations were reported also by others but frequency of these cases has never exceeded 10 % [9, 12, 13, 27, 30]. Based on the sequencing analysis of cDNA isolated from yeast colonies, we captured two suspected samples bearing two independent *p53* mutations in separated alleles: cases 21 and 4. In both cases, only one mutation was confirmed by sequencing of gDNA. The other mutations might be only artificial products of RT-PCR. On the other hand, they might be real, and the negative result might reflect lower sensitivity of gDNA sequencing.

There is an interesting report concerning multiple *p53* mutations as well as partially inactivating (temperature-sensitive) mutations by Fulci et al. [37]. The authors successfully traced progressive loss of the p53 function during development of human astrocytoma. In their study, the p53 abrogation started in germ-line as temperature-sensitive mutation R238H providing pink colonies in FASAY. During development of astrocytoma, the first somatic *p53* mutation R267W inactivated the allele hit by germ-line mutation; the second allele was inactivated by another *p53* mutation, the E258D. This work offers a possibility that the very early phase of astrocytoma development is compatible with only partial abrogation of the p53 function, represented e.g. by temperature-dependent mutation, followed by stronger p53 dysfunction later caused e.g. by fully inactivating mutation. This model would explain higher occurrence of temperature-dependent mutations, as well as higher occurrence of multiple *p53* mutations.

The idea that loss of the p53 function may occur progressively during development of glioblastoma, has been suggested repeatedly and multiple possible scenarios were proposed. Besides missense and other types of mutations of the *p53* gene, also deletions of the *p53*-specific locus 17p13.3 have been suggested [26, 38]. Loss of the locus has been studied in astrocytomas and glioblastomas by several techniques reaching frequency from 12 to 50 % [9, 39–42]. Similarly, both recent comprehensive genomic studies identified 14 and 18 % of positive cases [2, 3]. The 12.1 % frequency of positive cases obtained in our study is rather low but it fits within the range of the previous findings and is in a good agreement with the both comprehensive studies. Interestingly, we found rather low overlap of the *p53* gene mutation as inactivation of one *p53* allele and loss of the *p53*-specific locus as the second hit. But the number of FISH positive cases in this study is too low to make reliable conclusions.

The p53 protein positivity has been repeatedly tested in glioblastoma. It usually scored between 25 and 50 % and concordance ranged between 72 up to 89 % [9, 10, 17–19, 30]. Therefore, our result of 40.0 % positivity and the 80.0 % concordance with mutation analysis perfectly correspond to result described by others. Some discordant results are easily explicable. This is the case of sample 24 scoring negativity by immunohistochemical analyses although the clonal mutation was clearly detected. This mutation, one nucleotide deletion in codon 188, causes frame shift and premature termination codon formation in position 247. We presume that it induces RNA decay mechanism of mRNA degradation, thus interfering with proteosynthesis. Although the majority of discrepancies between the p53 protein level and the detection of *p53* mutation detection are hard to trace, they are rather common and usually connected with impairment of other parts of the p53 functional pathway.

It has been reported several times that age of patients with glioblastoma bearing mutated *p53* gene or high level of the p53 protein is significantly lower than age of patients without *p53* aberration [11, 12, 14, 18]. In contrast, the mean age of our patients having the *p53* mutations was 65.1 years, while the mean age of patients without *p53*

mutations was 57.0 years. Also, from the five patients younger than 50 years in our collection, only one (20 %) was shown to have a *p53* mutation. This confirms that there are many conflicting results and ambiguities in question of contribution of the p53 tumor suppressor aberrations to development of glioblastoma.

In conclusion, we performed complex analysis of the p53 tumor suppressor in glioblastoma by employing combination of several alternative methods to provide insight into the ways of the p53 impairment.

**Acknowledgement** We thank all the patients and their caregivers for participating in this study.

This work was supported by grants NT/13784-4 of the Internal Grant Agency of the Ministry of Health of the Czech Republic, MSM0021622434 of the Ministry of Education, Youth and Sports of the Czech Republic and by Regional Centre for Applied Molecular Oncology (RECAMO; CZ.1.05/2.1.00/03.0101) via the human resources project "IntegRECAMO: Intelectual Anchor" (CZ.1.07/2.3.00/20.0097).

## References

1. Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, Cavenee WK (2002) The WHO classification of tumors of the nervous system. J Neuropathol Exp Neurol 61:215–225
2. Parsons DW, Jones S, Zhang X et al (2008) An integrated genomic analysis of human glioblastoma multiforme. Science 321:1807–1812
3. Cancer Genome Atlas Research Network (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 455:1061–1068
4. Harris SL, Levine AJ (2005) The p53 pathway: positive and negative feedback loops. Oncogene 24:2899–2908
5. Sherr CJ (2004) Principles of tumor suppression. Cell 116:235–246
6. Haupt Y, Maya R, Kazaz A, Oren M (1997) Mdm2 promotes the rapid degradation of p53. Nature 387:296–299
7. Honda R, Tanaka H, Yasuda H (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 420:25–27
8. Wu XW, Bayle JH, Olson D, Levine AJ (1993) The p53 mdm-2 autoregulatory feedback loop. Genes Dev 7:1126–1132
9. Watanabe K, Sato K, Biernat W, Tachibana O, von Ammon K, Ogata N, Yonekawa Y, Kleihues P, Ohgaki H (1997) Incidence and timing of p53 mutations during astrocytoma pregression in patients with multiple biopsies. Clin Cancer Res 3:523–530
10. Kraus JA, Glesmann N, Beck M, Krex D, Klockgether T, Schackert G, Schlegel U (2000) Molecular analysis of the PTEN, TP53 and CDKN2A tumor suppressor genes in long-term survivors of glioblastoma multiforme. J Neuorooncol 48:89–94
11. Schiebe M, Ohneseit P, Hoffmann W, Meyermann R, Rodemann HP, Bamberg M (2000) Analysis of mdm2 and p53 gene alterations in glioblastoma and its correlation with clinical factors. J Neurooncol 49:197–203
12. Schmidt MC, Antweiler S, Urban N, Mueller W, Kuklik A, Meyer-Puttlitz B, Wiestler OD, Louis DN, Fimmers R, von Deimling A (2002) Impact of genotype and morphology on the prognosis of glioblastoma. J Neuropathol Exp Neurol 61:321–328
13. Peraud A, Kreth FW, Wiestler OD, Kleihues P, Reulen HJ (2002) Prognostic impact of TP53 mutations and p53 protein overexpression in supratentorial WHO grade II astrocytomas and oligoastrocytomas. Clin Cancer Res 8:1117–1124
14. Batchelor TT, Betensky RA, Esposito JM, Pham LDD, Dorfman MV, Piscatelli N, Jhung S, Rhee D, Louis DN (2004) Age-dependent prognostic effects of genetic alterations in glioblastoma. Clin Cancer Res 10:228–233
15. Ohgaki H, Dessen P, Jourde B, Horstmann S, Nishikawa T, Di Patre PL, Burkhard C, Schuler D, Probst-Hensch M, Maiorka PC, Baeza N, Pisani P, Yonekawa Y, Yasargil MG, Lutolf UM, Kleihues P (2004) Genetic pathways to glioblastoma: a population-based study. Cancer Res 64:6892–6899
16. Rich JN, Hans C, Jones B, Iversen ES, McLendon RE, Rasheed BKA, Dobra A, Dressman HK, Bigner DD, Nevins JR, West M (2005) Gene expression profiling and genetic markers in glioblastoma survival. Cancer Res 65:4051–4058
17. Momota H, Narita Y, Matsushita Y, Miyakita Y, Shibui S (2010) p53 abnormality and tumor invasion in patients with malignant astrocytoma. Brain Tumor Pathol 27:95–101
18. Birner P, Piribauer M, Fischer I, Gatterbauer B, Marosi C, Ungersbock K, Rossler K, Budka H, Hainfellner JA (2002) Prognostic relevance of p53 protein expression in glioblastoma. Oncol Rep 9:703–707
19. Srividya MR, Thota B, Arivazhagan A, Thennarasu K, Balasubramaniam A, Chandramouli BA, Hegde AS, Santosh V (2010) Age-dependent prognostic effects of EGFR/p53 alterations in glioblastoma: study on a prospective cohort of 140 uniformly treated adult patients. J Clin Pathol 63:687–691
20. Flaman JM, Frebourg T, Moreau V, Charbonnier F, Martin C, Chappuis P, Sappino AP, Limacher JM, Bron L, Benhattar J, Tada M, Van Meir EG, Estreicher A, Iggo RD (1995) A simple p53 functional assay for screening cell lines, blood, and tumors. Proc Natl Acad Sci USA 92:3963–3967
21. Smardova J, Nemajerova A, Trbusek M, Vagunda V, Kovarik J (2001) Rare somatic p53 mutation identified in breast cancer: a case report. Tumor Biol 22:59–66
22. Ishioka C, Frebourg T, Yan YX, Vidal M, Friend SH, Schmidt S, Iggo R (1993) Screening patients for heterozygous p53 mutations using a functional assay in yeast. Nat Genet 5:124–129
23. Waridel F, Estreicher A, Bron L, Flaman JM, Fontolliet C, Monnier P, Frebourg T, Iggo R (1997) Field cancerisation and polyclonal p53 mutation in the upper aerodigestive tract. Oncogene 14:163–169
24. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, Olivier M (2007) Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mut 28:6292–6299
25. Grochova D, Vankova J, Damborsky J, Ravcukova B, Smarda J, Vojtesek B, Smardova J (2008) Analysis of transactivation capability and conformation of p53 temperature-dependent mutants and their reactivation by amifostine in yeast. Oncogene 27:1243–1252
26. Van Meir EG, Kikuchi T, Tada M, Li H, Diserens AC, Wojcik BE, Huang HJS, Friedmann T, de Tribolet N, Cavenee WK (1994) Analysis of the p53 gene and its expression in human glioblastoma cells. Cancer Res 54:649–652
27. Tada M, Iggo RD, Waridel F, Nozaki M, Matsumoto R, Sawamura Y, Shinohe Y, Ikeda J, Abe H (1997) Reappraisal of p53 mutations in human malignant astrocytic neoplasms by p53 functional assay: comparison with conventional structural analyses. Mol Carcinogen 18:171–176
28. Tada M, Matsumoto R, Iggo RD, Onimaru R, Shirato H, Sawamura Y, Shinohe Y (1998) Selective sensitivity to radiation of cerebral glioblastomas harbouring p53 muttations. Cancer Res 58:1793–1797

29. Olivier M, Taniere P (2010) Somatic mutations in cancer prognosis and prediction: lessons from TP53 and EGFR genes. Curr Opin Oncol 23:88–92
30. Kraus JA, Wenghoefer M, Glesmann N, Mohr S, Beck M, Schmidt MC, Schroder R, Berweiler U, Roggendorf W, Diete S, Dietzmann K, Hauser K, Muller B, Fimmers R, von Deimling A, Schlegel U (2001) TP53 gene mutations, nuclear p53 accumulation, expression of Waf/p21, Bcl-2, and CD95 (APO-1/Fas) proteins are not prognostic factors in de novo glioblastoma multiforme. J Neurooncol 52:263–272
31. Shiraishi K, Kato S, Han SY, Liu W, Otsuka K, Sakayori M, Ishida T, Takeda M, Kanamaru R, Ochuchi N, Ishioka C (2004) Isolation of temperature-sensitive p53 mutations from a comprehensive missense mutation library. J Biol Chem 279:348–355
32. Stefancikova L, Moulis M, Fabian P, Vasova I, Zedek F, Ravcukova B, Muzik J, Kuglik P, Vranova V, Falkova I, Hrabalova R, Smardova J (2011) Prognostic impact of p53 aberrations for R-CHOP-treated patiens with difuse large B-cell Lymphoma. Int J Oncol 39:1413–1420
33. Pavlova S, Mayer J, Koukalova H, Smardova J (2003) High frequency of temperature-sensitive mutations of p53 tumor suppressor in acute myeloid leukemia revealed by functional assay in yeast. Int J Oncol 23:121–131
34. Smardova J, Ksicova K, Binkova H, Krpensky A, Pavlova S, Rottenberg J, Koukalova H (2004) Analysis of tumor suppressor p53 status in head and neck squamous cell carcinoma. Oncol Rep 11:923–929
35. Smardova J, Vagunda V, Jandakova E, Vagundova M, Koukalova H, Kovarik J, Zaloudik J (1999) p53 status in breast carcinomas revealed by FASAY correlates well with p53 protein accumulation determined by immunohistochemistry. Neoplasma 46:384–389
36. Nenutil R, Smardova J, Pavlova S, Hanzelkova Z, Muller P, Fabian P, Hrstka R, Janotova P, Radina M, Lane DP, Coates PJ, Vojtesek B (2005) Discriminating functional and non-functional p53 in human tumours by p53 and MDM2 immunohistochemistry. J Pathol 207:251–259
37. Fulci G, Ishii N, Maurici D, Gernert KM, Hainaut P, Kaur B, Van Meir EG (2002) Initiation of human astrocytoma by clonal evolution of cells with progressive loss of p53 functions in a patient with a 283H TP53 germ-line mutation: evidence for a precursor lesion. Cancer Res 62:2897–2905
38. Sidranski D, Mikkelsen T, Schwechheimer K, Rosenblum ML, Cavanee W, Vogelstein B (1992) Clonal expansion of p53 mutant cells is associated with brain tumor progression. Nature 355:846–847
39. James CD, Carlbom E, Nordenskjold M, Collins VP, Cavenee WK (1988) Mitotic recombination of chromosome 17 in astrocytomas. Proc Natl Acad Sci USA 86:2858–2862
40. Bigner SH, Mark J, Burger PC, Mahaley MS, Bullard DE, Muhlbaier LH, Bigner DD (1988) Specific chromosomal abnormalities in malignant human gliomas. Cancer Res 88:405–411
41. James CD, Carlbom E, Dumanski JP, Hansen M, Nordenskold M, Collins VP, Cavenee WK (1989) Clonal genomic alterations in glioma malignancy stages. Cancer Res 48:5546–5551
42. von Deimling A, Eibl RH, Ohgaki H, Louis DN, von Ammon K, Petersen I, Kliehues P, Chung RY, Wiestler OD, Seizinger BR (1992) p53 mutations are associated with 17p allelic loss in grade II and III atsrocytomas. Cancer Res 52:2987–2990
43. Campomenosi P, Monti P, Aprile A, Abbondandolo A, Frebourg T, Gold B, Crook T, Inga A, Resnick MA, Iggo R, Fronza G (2001) p53 mutants can often transactivate promoters containing p21 but not Bax or PIG3 responsive elements. Oncogene 20:3573–3579
44. Di Como C, Prives C (1998) Human tumor-derived p53 proteins exhibit binding site selectivity and temperature sensitivity for transactivation in yeast-based assay. Oncogene 19:2527–2539
45. Dearth LR, Qian H, Wang T, Baroni TE, Zeng J, Chen SW, Yi SY, Brachmann RK (2007) Inactive full-length p53 mutants lacking dominant wild-type p53 inhibition highlight loss of heterozygosity as an important aspect of p53 status in human cancers. Carcinogenesis 28:289–298
46. Yamamoto S, Tada M, Lee CC, Masuda C, Wanibuchi H, Yoshimura R, Wada S, Yamamoto K, Kishimoto T, Fukushima S (2000) p53 status in multiple human urothelial cancers: assessment for clonality by yeast p53 functional assay in combination with p53 immunohistochemsitry. Jpn J Canc Res 91:181–189
47. Scharer E, Iggo R (1992) Mammalian p53 can function as a transcription factor in yeast. Nucl Acid Res 20:1539–1545