

# Complex analysis of the p53 tumor suppressor in lung carcinoma

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**Abstract.** Lung cancer is the leading cause of cancer-related deaths worldwide. The p53 tumor suppressor is a transcription factor controlling expression of its target genes in response to various stress stimuli. Mutations of the *TP53* gene occur very frequently in lung carcinomas and they play an important role in both oncogenic transformation of lung epithelial cells and lung carcinoma progression. We determined the *TP53* status in 42 samples of squamous cell lung carcinoma (SQCC) and 56 samples of lung adenocarcinoma (AC) by the functional analysis FASAY and its variant called split assay. Altogether, we detected 64 *TP53* mutations in 63 patients and analyzed them by cDNA and gDNA sequencing. The *TP53* mutations were found in 76.2% (32/42) of SQCC cases, and 55.4% (31/56) of ACs. Immunoblotting revealed the p53 protein accumulation in 18 samples (42.9%) among SQCC cases and 19 samples (33.9%) among AC cases. Using fluorescence *in situ* hybridization we detected loss of the *TP53*-specific 17p13.3 locus in 23 from 41 analyzed SQCC samples (56.1%) and in 20 from 54 analyzed AC samples (37.0%). We did not find any statistically significant differences in overall and disease-free survival in relation to *TP53* status.

## Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1,2). Despite improvements in therapy, the 5-year survival rate for all patients diagnosed with lung cancer remains as low as 15-20%. Approximately 75-80% of human lung

cancers are non-small cell lung cancers (NSCLC). NSCLC represents a heterogeneous group of cancers and includes two major forms: lung squamous cell carcinoma (SQCC) and lung adenocarcinoma (AC).

Development of lung cancers involves formation of multiple genetic aberrations, including those affecting the p53 tumor suppressor. p53 mediates an adequate reaction of cells to stress. It functions as a transcription factor responding to various stress signals by controlling expression of its target genes. The p53 protein then participates in control of cell cycle, DNA repair, apoptosis, senescence and maintaining of genome integrity (3,4). Mutations and allelic deletions of the *TP53* gene are the most frequent genetic alterations detected in human tumors, including lung cancers. Inactivation of p53 usually results from missense mutations in the core region of the *TP53* gene coding for the sequence-specific DNA-binding domain. Due to inability to transactivate its negative regulator, the E3-ubiquitin ligase MDM2 (5-7), the mutant p53 protein often accumulates in tumor cells.

Mutations of the *TP53* gene are very frequent in lung carcinomas and they play an important role in both oncogenic transformation of lung epithelial cells and lung carcinoma progression (8). There are multiple lines of evidence documenting that the presence of *TP53* aberrations worsen the prognosis of patients with lung cancer (8-11). Frequency of the *TP53* aberrations in lung cancers has been tested repeatedly and showed higher frequency of the *TP53* mutations in lung SQCC (50-70%) than in lung AC (~40%) (11-13). Integrated and comprehensive genomic characterization was performed both in AC and squamous cell lung cancers (14,15). By detailed analysis of 188 cases of lung AC, 26 genes were identified as mutated at significantly high frequencies and thus they are probably involved in development of the disease. Among them, *TP53* was mutated in 66 cases (34%). Notably, the rate of missense mutations was rather low (65.2%) and some specific mutations were detected repeatedly, namely p.G266\* (4x), p.R273L (3x), p.R282W (3x), and others (14). For squamous cell lung cancers, 178 cases were analyzed and statistically recurrent mutations in 11 genes were found. One of them, the *TP53* gene was mutated in 90% of the samples (15).

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In the present study, we analyzed the *TP53* status in 42 samples of lung SQCC and 56 samples of lung AC by the functional analysis FASAY and its variant called split assay followed by cDNA and gDNA sequencing. Each sample was also tested for the p53 protein accumulation by immunoblotting and loss of the *TP53*-specific 17p13.3 locus by fluorescence *in situ* hybridization. We examined relationship between the *TP53* status and the disease outcome.

## Materials and methods

**Clinical materials.** Ninety-eight patients (37 females and 61 males) were included in the study. They underwent surgical removal of the tumor tissue in University Hospital in Brno from March 2006 to January 2014. All samples were classified according to WHO guidelines. Forty-two patients (9 females and 33 males) were diagnosed with spinocellular lung carcinoma (cases labeled S1 to S42; Table I). The average age of patients at the time of surgical intervention was 65.5 years, ranging from 40 to 79 years. Fifty-six patients (28 females and 28 males) were diagnosed with AC (cases labeled A1 to A56; Table II). The average age of patients at the time of surgical intervention was 66.5 years, ranging from 41 to 86 years. Among SQCC patients 9 (24.3%) were non-smokers, 9 (24.3%) ex-smokers and 19 (51.4%) smokers. Among AC patients the ratio was 42.0% (21 non-smokers), 20.0% (10 ex-smokers) and 38.0% (19 smokers). For all included patients, fresh frozen tissue samples as well as formalin-fixed, paraffin-embedded tumor tissue blocks (FFPE) were available. Patients were included in the present study only after signing the informed consent form approved by the Ethics Committee of the hospital.

**FASAY and split assay.** FASAY was performed as previously described (16,17) with small modifications. Total RNA was extracted using NucleoSpin RNA kit (New England BioLabs Inc., Ipswich, MA, USA) and stored at -80°C until further processing. cDNA was synthesized by ProtoScript® II (New England BioLabs Inc.) using primer oligo(dT)<sub>12</sub>. 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 Phusion DNA Polymerase (New England BioLabs Inc.). Yeast cells were co-transformed with the PCR product, linearized pSS16 plasmid and salmon sperm DNA carrier (Life Technologies Inc., Carlsbad, CA, USA) by the lithium acetate procedure (18). 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 at room temperature for 2-3 days. For split assay, PCR of the *TP53* 5'-part was performed with primers P3 and P17 (5'-GCC-GCC-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 (19).

FASAY deduces the functional status of p53 from the color of colonies of transformed yeast cells. Expression of functional p53 results in formation of large white colonies, inactive p53 leads to smaller red ones. The background frequency of red yeast colonies typically does not exceed 10%. Thus, samples providing <10% of red colonies are considered to contain

only wild-type *TP53* alleles, while samples providing >10% of red colonies are suspicious to possess a clonal *TP53* mutation. The ratio of red colonies scoring between 10 and 20% can result from a presence of clonal *TP53* mutation in rather small fraction of cells or from increased rate of RNA degradation. To distinguish these two possibilities, version of FASAY, called split assay was established. In the split assay, the 5'- and 3'-parts of the *TP53* cDNA were tested separately (19).

**Purification of the plasmids from transformed yeast cells and sequencing of the *TP53* cDNA.** Yeast cells from individual yeast colonies were harvested, resuspended in TSN (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA), and grinded by vortexing with glass beads; plasmid DNA was extracted by phenol/chloroform procedure. The *TP53* cDNA was amplified using the P3 and P4 primers and *Taq* polymerase (Life Technologies Inc.) and subjected to agarose gel electrophoresis. The PCR product was purified by MinElute PCR purification kit (Qiagen, Hilden, Germany) and sequenced by BigDye terminator v3.1 cycle sequencing kit using ABI PRISM 3130 Genetic Analyzer (both from Applied Biosystems, Darmstadt, Germany).

**Isolation and sequencing of the *TP53* gDNA.** Genomic DNA was isolated from FFPE blocks or from the frozen tumor samples using Purogene DNA Isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The *TP53* exons were amplified by PCR and automated fluorescent sequencing was performed using BigDye terminator kit and ABI 3100 sequencer (Applied Biosystems, Carlsbad, CA, USA). Either a picked exon of interest or all exons 2-11 were analyzed. Primers and conditions were adopted from IARC TP53 database (20).

**Immunoblotting.** Tissue samples were lysed in 150 mM NaCl, 50 mM NaF, 50 mM Tris (pH 8.0), 5 mM EDTA, 1% NP40 and 1 mM phenylmethylsulfonyl fluoride in ice for 30 min, and the cell extract was centrifuged at 17,000 x g for 30 min to remove cell debris. Protein concentration was measured by the Bradford assay. Solubilized proteins were resolved by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were blocked in 0.1% Tween-20 and 5% low-fat milk in PBS for 1 h and probed with anti-p53 mouse monoclonal antibody DO-1 (Dako, Glostrup, Denmark) at 4°C. Blots were developed with Dako peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako) using the ECL chemiluminescence detection kit (GE Healthcare UK Ltd., Little Chalfont, UK).

**Fluorescence *in situ* hybridization (FISH).** FISH was performed in tissue sections prepared from FFPE blocks. For the *TP53*-specific locus analysis, the ZytoLight® SPEC TP53/CEN17 Dual Color Probe was used (ZytoVision GmbH, Bremerhaven, Germany). Hybridization was performed according to the manufacturer's instructions. Images were scanned by DM 5500 B microscope equipped with Leica DFC 290 HD camera. Fluorescence signals were analyzed using Leica LAS AF software (Leica Microsystems GmbH, Wetzlar, Germany). Cells (50-100) per case were analyzed. The cut-off level was defined by the mean value plus three

Table I. Summary of the *TP53* status analyses in samples of squamous cell lung carcinoma.

Case	Gender/age	FASAY <sup>a</sup>	DNA sequencing		<i>TP53</i> status	FISH <sup>b</sup>	IB
			cDNA	gDNA			
1	M/59	70.3	r.1024c>u <sup>c,d</sup>	c.1024C>T <sup>f</sup>	c.1024C>T p.R342*	27	-
4	M/79	WT	ND	WT <sup>f</sup>	WT	78	-
5	M/76	95.6	r.464c>a <sup>c</sup>	ND	c.464C>A p.T155N	46	++
6	F/65	20.1	r.921-939del <sup>d</sup>	c.920-1G>A <sup>e</sup>	c.920-1G>A p.L308Pfs*31	34	-
7	M/77	32.8	r.207delu <sup>d</sup>	c.207delT <sup>f</sup>	c.207delT p.A70Lfs*53	27	-
8	M/67	82.4	r.461g>u <sup>c</sup>	ND	c.461G>T p.G154C	ND	+++
9	F/68	WT	ND	ND	WT	7	-
10	F/55	WT	ND	WT <sup>f</sup>	WT	33	-
11	M/53	28.1	r.488a>g <sup>c</sup>	ND	c.488A>G p.Y163C	9	-
12	M/50	71.2	r.764u>g <sup>c</sup>	c.764T>G <sup>f</sup>	c.764T>G p.I255S	7	+++
13	M/63	34.9	r.422dupg <sup>d</sup>	c.422dupG <sup>e</sup>	c.422dupG p.C141Wfs*7	6	-
14	M/69	WT	ND	ND	WT	37	-
15	M/69	74.7	r.[782+2u>g; 782_783ins78 2+1_783-1] <sup>c,d</sup>	c.782+2T>G <sup>f</sup>	c.782+2T>G p.S261Rfs*63	13	-
17	M/48	18.4	r.734g>u <sup>d</sup>	ND	c.734G>T p.G245V	68	-
18	M/62	85.2	r.313_375del <sup>c,d</sup>	c.314G>T <sup>f</sup>	c.314G>T p.G105_T125del	6	++
19	M/68	41.7	r.764u>a <sup>c</sup>	c.764T>A <sup>f</sup>	c.764T>A p.I255N	5	+++
20	M/64	29.6	r.746g>c <sup>c</sup>	ND	c.746G>C p.R249T	76	+++
21	F/40	WT	ND	ND	WT	2	-
22	F/63	WT	ND	WT <sup>f</sup>	WT	11	+
23	M/61	73.5	r.848g>c <sup>c</sup>	c.848G>C <sup>f</sup>	c.848G>C p.R283P	11	-
24	M/57	99.2	r.706u>g <sup>c</sup>	c.706T>G <sup>e</sup>	c.706T>G p.Y236D	9	+++
25	M/68	WT	ND	ND	WT	81	-
26	M/67	15.4	r.403delu <sup>d</sup>	c.403delT <sup>f</sup>	c.403delT p.C137Afs*35	8	-
27	M/66	95.9	r.380c>u <sup>c</sup>	ND	c.380C>T p.S127F	12	+++
87	M/65	86.2	r.848g>c <sup>c</sup>	ND	c.848G>C p.R283P	4	+++
88	M/60	94.1	r.404g>u <sup>c</sup>	c.404G>T <sup>e</sup>	c.404G>T p.C135F	41	+++
89	F/65	92.6	r.359a>u <sup>c</sup>	c.359A>T <sup>e</sup>	c.359A>T p.K120M	36	+++
90	M/60	88.0	r.422g>a <sup>c</sup>	c.422G>A <sup>e</sup>	c.422G>A p.C141Y	29	++
91	F/58	93.9	r.820g>u <sup>c</sup>	c.820G>T <sup>e</sup>	c.820G>T p.V274F	46	+++
92	M/63	WT	ND	ND	WT	28	-
93	M/66	92.7	r.752u>a <sup>c</sup>	c.752T>A <sup>f</sup>	c.752T>A p.I251N	14	+++
94	M/66	21.3	r.637c>u <sup>d</sup>	c.637C>T <sup>f</sup>	c.637C>T p.R213*	60	-
95	M/64	WT	ND	ND	WT	41	-
96	M/71	26.8	r.461g>u <sup>d</sup>	c.461G>T <sup>f</sup>	c.461G>T p.G154V	68	-
97	M/69	24.7	r.176_375del (3/15) <sup>c,g</sup> r.[375+5g>a; 375_376ins37 5+1_375+109] (3/15) <sup>c,g</sup> r.[375+5g>a; 375_376ins37 5+1_376-1] (3/15) <sup>c,g</sup>	c.375+5G>A <sup>f</sup>	c.375+5G>A p.G59Vfs*23 (3/15) <sup>d</sup> p.Y126Vfs*59 (3/15) <sup>d</sup> p.Y126Vfs*49 (3/15) <sup>d</sup>	10	-
98	M/75	98.6	r.832c>u <sup>c</sup>	c.832C>T <sup>f</sup>	c.832C>T p.P278S	6	+++
99	F/43	WT	ND	ND	WT	30	-
100	M/68	20.4	r.418_422deli nsgcccuu <sup>d</sup>	c.418_422delins6G CCCTT <sup>f</sup>	c.418_422delins GCCCTT p.T140Afs*9	26	-
101	F/76	81.0	r.473g>u <sup>c</sup>	c.473G>T <sup>f</sup>	c.473G>T p.R158L	79	+++

Table I. Continued.

Case S	Gender/age	FASAY <sup>a</sup>	DNA sequencing		<i>TP53</i> status	FISH <sup>b</sup>	IB
			cDNA	gDNA			
102	M/70	93.1	r.461g>u <sup>c</sup>	c.461G>T <sup>f</sup>	c.461G>T p.G154V	42	+++
103	M/76	11.0	WT <sup>d</sup>	c.511G>T <sup>f</sup>	c.511G>T p.E171*	44	-
104	M/77	96.3	r.739_752deli nsgg <sup>c,d</sup>	c.739_752delinsGG <sup>f</sup>	c.739_752delinsGG p.N247_I251delinsG	10	+++

M, male; F, female; IB, immunoblotting; ND, not done; WT, wild-type; <sup>a</sup>% of red colonies; <sup>b</sup>% of positive cells; <sup>c</sup>cDNA prepared from tumor tissue; <sup>d</sup>cDNA isolated from yeast colonies; <sup>e</sup>gDNA isolated from FFPE; <sup>f</sup>gDNA isolated from frozen tumor tissue; <sup>g</sup>fraction of positive colonies.

times the SD of the frequency of control cells exhibiting one red and two green signals (9.6%).

**Statistical analyses.** Standard descriptive statistics was used; median supplemented with min-max range for continuous variables and absolute and relative frequencies for categorical variables. The influence of patient characteristics on their survival was analyzed using the Cox proportional hazards model and described using hazard ratios, their 95% confidence interval and statistical significance. Analysis was computed using SPSS 22.0.0.1 (IBM Corporation, 2014).

## Results

**Assessment of the *p53* status by FASAY and split assay.** We used FASAY to determine the *TP53* status of all 98 enrolled samples and assessed the ratio of red colonies. Among 42 samples of SQCCs, 7 samples scored under the 10% background level and were classified as bearing the standard *TP53* variant. Split assay was performed in all samples exceeding the cut-off 10% level. Three samples only slightly exceeding the background level in FASAY (10.8-11.4%) scored under the cut-off 10% level in both parts of split assay. Therefore, these samples were also classified as having the standard *TP53* variant. Remaining 32 samples were suspected to contain a clonal *TP53* mutation and they underwent further analyses (Table I). The same procedure was performed for 56 enrolled lung AC samples. Twenty-five samples scored under the background level, while the remaining 31 samples were assigned for further analyses (Table II).

**Sequencing of the *TP53* cDNA.** All 63 cases positive for *TP53* mutation as determined by FASAY/split assay were analyzed by cDNA sequencing. Two strategies were used. In 45 cases, usually with higher ratio of red colonies in FASAY, cDNA to be sequenced was prepared directly from tumor tissues. In 29 cases, the *TP53* expression vector was recovered from 3 to 10 red yeast colonies per case, and this cDNA was used as template for DNA sequencing. In 11 cases, both approaches were combined with affirmative results (Tables I and II). With exception of three cases, unambiguous clonal *TP53* mutations were identified in each sample (60/63): 29 cases of AC and 31 cases of SQCC. No clonal mutation was found in the AC

case A53 with score 16.8% of red colonies in FASAY. In AC case A35 the results of FASAY and split assay were only slightly above the cut-off level (score 14.7% in FASAY and 6.1/13.4 in split assay) and no potential clonal mutation was suggested by the *TP53* cDNA sequencing of randomly picked eight yeast colonies. Each colony contained different *TP53* mutation, one of them was nonsense p.K321\* mutation. We repeated the whole procedure and analyzed 10 more yeast colonies. The mutation p.K321\* was found in two other colonies and was subsequently confirmed by gDNA analyses. In the SQCC case S41 (score 11.0% in FASAY) the presence of missense mutation p.G154V was proposed as it was detected in 2 from 8 red colonies tested. Although the mutation was not confirmed by subsequent gDNA analysis, the nonsense mutation p.E171\* was clearly disclosed instead.

**Sequencing of the *TP53* gDNA.** Two main strategies were used for *TP53* gDNA sequencing. First, the gDNA was isolated from FFPE blocks, the *TP53* exons 2-11 were amplified by PCR and sequenced. Fifteen cases with mutated *TP53* and five randomly chosen cases with standard *TP53* as determined by cDNA analyses were subjected to this procedure. In all 15 positive cases, the same mutation identified by cDNA sequencing was confirmed. Four cases lacking *TP53* mutation scored standard also by gDNA sequencing. In AC sample A23 which was classified as standard by FASAY (scoring 9.2%), the presence of two independent *TP53* mutations was revealed (A and B). One mutation A23-A: p.G154V was confirmed by sequencing of cDNA isolated from background red yeast colonies obtained by FASAY (in 3/9 colonies tested), the other mutation (A23-B: c.557\_558ins17N) was not traced in this way (0/9).

Second, the gDNA was isolated from frozen tumor tissues and then subjected to the sequencing analysis. For 34 *TP53* mutation positive samples revealed by cDNA sequencing (including the cases A35 and S41) or gDNA sequencing from FFPE samples (case A23 A and B) only the relevant *TP53* exons were PCR amplified and sequenced. In all cases, the proposed mutations were confirmed. The same technical procedure comprising exons 2-11 was employed in seven other cases with the wild-type *TP53* output determined by FASAY. They included six cases carrying deletion of the *TP53* locus assessed by FISH analysis (see below). No *TP53* mutation was disclosed in any of these samples.

Table II. Summary of the *TP53* status analyses in samples of lung adenocarcinoma.

Case A	Gender/age	FASAY <sup>a</sup>	DNA sequencing		<i>TP53</i> status	FISH <sup>b</sup>	IB
			cDNA	gDNA			
28	M/62	WT	ND	ND	WT 8	-	
30	F/78	WT	ND	ND	WT 12	-	
31	F/71	WT	ND	ND	WT ND	-	
32	M/73	WT	ND	WT <sup>f</sup>	WT 38	-	
33	M/65	WT	ND	WT <sup>f</sup>	WT 39	-	
34	M/57	92.3	r.596g>u <sup>c</sup>	c.596G>T <sup>f</sup>	c.596G>T p.G199V	10	+++
35	M/67	97.8	r.313_375del (11/13) <sup>c,d,g</sup> r.314g>u (1/13) <sup>c,d,g</sup>	c.314G>T <sup>f</sup>	c.314G>T 7 p.G105_T125del (11/13) <sup>d</sup> p.G105V (1/13) <sup>d</sup>	-	
36	F/76	WT	ND	WT <sup>f</sup>	WT 41	-	
37	F/68	58.3	r.358a>u <sup>c,d</sup>	c.358A>T <sup>f</sup>	c.358A>T p.K120*	8	-
38	M/54	18.9	r.461delg <sup>d</sup>	c.461delG <sup>f</sup>	c.461delG p.G154Afs*15	18	-
39	F/67	WT	ND	ND	WT 7	-	
40	F/68	83.4	r.743g>a <sup>c</sup>	c.743G>A <sup>f</sup>	c.743G>A p.R248Q	14	+++
41	M/68	82.9	r.820g>u <sup>c</sup>	c.820G>T <sup>f</sup>	c.820G>T p.V274F	6	+++
42	F/57	89.2	r.733g>a <sup>c</sup>	c.733G>A <sup>f</sup>	c.733G>A p.G245S	25	+++
43	M/61	88.3	r.743_744gg>uu <sup>d</sup>	c.743_744GG>TT <sup>f</sup>	c.743_744GG>TT p.R248L	13	+++
44	M/60	27.9	r.1013delu <sup>d</sup>	c.1013delT <sup>f</sup>	c.1013delT p.F338Sfs*7	20	-
45	F/63	19.4	r.839g>c <sup>d</sup>	ND	c.839G>C p.R280T	18	+++
46	F/67	27.3	r.406c>g <sup>d</sup>	ND	c.406C>G p.Q136E	33	-
47	F/53	WT	ND	ND	WT 40	-	
48	F/63	WT	ND	ND	WT 7	-	
49	F/72	WT	ND	ND	WT 8	-	
50	F/76	WT	ND	ND	WT 7	-	
51	M/76	9.2	a) r.461g>u (3/9) <sup>d,g</sup> B) - (0/9) <sup>g</sup>	A) c.461G>T <sup>e</sup> B) c.557_558insA GCTGCTCAG ATAGCGA <sup>e</sup>	A) c.461G>T4 B) c.557_558insAGCTG CTCAGATAGCGA A) p.G154V B) p.G187Afs*67	-	
52	M/58	23.1	r.533a>u <sup>d</sup>	ND	c.533A>T p.H178L	36	-
53	F/60	98.3	r.711g>u <sup>c</sup>	ND	c.711G>T p.M237I	3	++
54	M/69	90.2	r.512a>g <sup>c</sup>	ND	c.512A>G p.E171G-ts	4	-
55	M/61	WT	ND	WT <sup>e</sup>	WT 7	-	
56	M/65	WT	ND	WT <sup>e</sup>	WT 3	-	
57	F/71	95.7	r.470u>g <sup>c,d</sup>	c.470T>G <sup>f</sup>	c.470T>G p.V157G	7	+++
58	F/65	65.9	r.844c>u <sup>c</sup>	c.844C>T <sup>e</sup>	c.844C>T p.R282W	10	+++
59	F/70	86.6	r.839g>c <sup>c</sup>	c.839G>C <sup>e</sup>	c.839G>C p.R280T	6	+++
60	M/76	WT	ND	WT <sup>e</sup>	WT 30	-	
61	F/86	WT	ND	ND	WT ND	-	
62	F/75	WT	ND	ND	WT 63	-	
63	F/50	14.7	r.961a>u <sup>d</sup>	c.961A>T <sup>f</sup>	c.961A>T p.K321*	8	-
64	M/63	WT	ND	ND	WT 6	-	
65	F/74	WT	WT	WT	WT 59	-	
66	M/78	51.6	r.176_375del <sup>c,d</sup>	c.375+2T>A <sup>f</sup>	c.375+2T>A p.G59Vfs*23	62	-
67	M/62	WT	ND	ND	WT 65	-	
68	F/67	WT	ND	ND	WT 7	-	
69	F/41	99.9	r.722c>u <sup>c</sup>	c.722C>T <sup>e</sup>	c.722C>T p.S241F	6	+++
71	M/62	62.1	r.824g>u <sup>c</sup>	c.824G>T <sup>e</sup>	c.824G>T p.C275F	4	+++
73	F/64	83.4	r.644g>c <sup>c,d</sup>	c.644G>C <sup>e</sup>	c.644G>C p.S215T-ts	10	++

Table II. Continued.

Case A	Gender/age	FASAY <sup>a</sup>	DNA sequencing		<i>TP53</i> status	FISH <sup>b</sup>	IB
			cDNA	gDNA			
74	M/60	86.5	r.488a>g <sup>c</sup>	c.488A>G <sup>e</sup>	c.488A>G p.Y163C	14	+++
75	M/76	WT	ND	ND	WT 20	-	
76	M/66	WT	ND	ND	WT 12	-	
77	F/74	85.4	r.848g>c <sup>c</sup>	ND	c.848G>C p.R283P	10	+++
78	F/65	WT	ND	ND	WT 84	-	
79	M/69	46.7	r.814g>u <sup>d</sup>	c.814G>T <sup>e,f</sup>	c.814G>T p.V272L-ts	10	-
80	M/60	WT	ND	ND	WT 8	-	
81	M/69	91.2	r.659a>g <sup>c</sup>	c.659A>G <sup>f</sup>	c.659A>G p.Y220C	73	+++
82	M/71	72.9	r.997_1003del <sup>c,d</sup>	c.997_1003del <sup>f</sup>	c.997_1003del p.R333fs*10	7	++
83	F/59	16.8	WT	WT	WT 52	-	
84	M/69	64.5	r.176_375del (1/8) <sup>c,d,g</sup> r.[375+1g>u; 375_376ins375 +1_375+109] (2/8) <sup>c,d,g</sup> , r.[375+1g>u; 375_376ins375 +1_376-1] (5/8) <sup>c,d,g</sup>	c.375+1G>T <sup>f</sup>	c.375+1G>T 3 p.G59Vfs*23 (3/15) <sup>d</sup> p.Y126Vfs*59(3/15) <sup>d</sup> p.Y126Vfs*49 (3/15) <sup>d</sup>	-	
85	F/55	93.5	r.473g>u <sup>c</sup>	c.473G>T <sup>e</sup>	c.473G>T p.R158L	36	+++
86	M/63	98.2	r.843c>g <sup>c</sup>	c.843C>G <sup>e</sup>	c.843C>G p.D281E	10	+++

M, male; F, female; IB, immunoblotting; ND, not done; WT, wild-type; fraction of positive colonies; <sup>a</sup>% of red colonies; <sup>b</sup>% of positive cells; <sup>c</sup>cDNA prepared from tumor tissue; <sup>d</sup>cDNA isolated from yeast colonies; <sup>e</sup>gDNA isolated from FFPE; <sup>f</sup>gDNA isolated from frozen tumor tissue; <sup>g</sup>fraction of positive colonies.

**Collection of *TP53* mutations.** Altogether we detected 64 *TP53* mutations in 63 lung cancer cases (Tables I and II). Thirty-two mutations were detected in 32 cases of squamous cell carcinoma. Frequency of lung SQCC cases with *TP53* mutation (32/42) thus reached 76.2%. Among 56 lung AC cases, 31 were *TP53* mutation positive representing 55.4%. One AC case contained two independent *TP53* mutations. Majority of the mutations (49/64) were confirmed by both cDNA and gDNA analyses. Thirteen mutations were detected by cDNA-based analyses and were not further analyzed by gDNA sequencing. Two mutations (A23-B and S41) were found by gDNA analysis but were not recognized by cDNA based techniques. The majority of detected *TP53* mutations were missense. They represented 59.4% (19/32) of mutations detected in lung SQCC and 71.9% (23/32) of mutations detected in AC. Three mutations (p.E171G, p.S215T and p.V272L), all among AC cases, were recognized as temperature sensitive according to the typical phenotype of positive yeast colonies. We further detected 7 mutations affecting splicing (4 in SQCC, 3 in AC), 5 nonsense mutations (3 in SQCC, 2 in AC), and 10 deletions/insertions (6 in SQCC, 4 in AC), all of them causing the reading frame shift and formation of premature termination codon.

In collection of the 64 *TP53* mutations, some were repeatedly detected. The p.G154V mutation appeared in four cases

(1 AC, 3 SQCCs), p.R283P in three cases (1 AC, 2 SQCC), and mutations p.Y163C, p.R158L, p.V274F and p.R280T were detected in two cases. Notably, less frequent mutation c.314G>T was also detected twice (A7 and S15), once in SQCC and once in AC case. This mutation potentially creates a new donor splice site (GT). Altogether, we sequenced the cDNA isolated from 28 yeast colonies (13 from case A7 and 15 from S15). Only single colony (case A7) revealed the presence of the transcript carrying missense mutation r.314g>u (p.G105V), the rest of 27 colonies contained the alternatively spliced variant r.313\_375del (p.G105\_T125).

Furthermore, two splice site mutations with the same effect on splicing of intron 4 were detected: mutation c.375+2T>A in AC sample A38, and mutation c.375+1G>T in AC sample A54. Sequencing performed on cDNA from the colonies obtained by FASAY proved that these mutations indeed affected splicing. We detected even three different aberrant transcripts: i) partial deletion of exon 4, ii) insertion of 109 nucleotides of intron 4, and iii) insertion of whole intron 4. Moreover, intronic variant c.375+5G>A detected in SQCC sample S35 was shown to have the same effect on splicing although the nucleotide change did not directly affect in the consensus splice site.

**Analysis of the *p53* protein accumulation.** We examined the *p53* protein accumulation in tumor tissues of all enrolled cases

by immunoblotting using the p53-specific DO-1 monoclonal antibody (IB). Among 42 cases of SQCC, 18 (42.9%) were strongly positive (+++) or positive (++) providing intermediate level of p53. Twenty-four samples (57.1%) exhibited low (+) or none level of the p53 protein (-). Concordance between presence of the *TP53* mutation and the p53 protein accumulation occurred in 29 cases (69.0%). All 18 samples positive for the p53 protein (+++ or ++) exhibited clonal *TP53* mutation. From 24 negative samples (+ or -), 13 cases featured truncating *TP53* mutation, including 9 frameshifts and 1 splicing mutation. Among 56 AC cases, 19 (33.9%) were positive (+++ or ++). All of them exhibited clonal *TP53* mutation. Thirty-seven samples (66.1%) were negative (+ or -). Among them, 12 samples featured truncating *TP53* mutation including 5 frameshift and 2 splicing mutations. Concordance between *TP53* mutational status and p53 protein accumulation occurred in 44 AC cases (78.6%).

**FISH.** We performed FISH analysis of the *TP53* alleles using the locus-specific and centromeric probes. We analyzed 61 cases. Three cases (1 SQCC and 2 AC) were excluded from analysis due to suboptimal tissue fixation. Among SQCC cases, 18 (43.9%) were negative as the portion of positive nuclei did not reach the 15% cut-off level. Fifteen of them featured presence and three absence of the *TP53* mutation. In 23 cases (56.1%), the *TP53*-specific locus was clearly lost. The portion of positive nuclei ranged from 26 to 81%. The FISH-positivity was detected in 16 cases having the *TP53* mutation in the other allele and in 7 cases with standard *TP53* gene. Among adenocarcinomas, 20 cases (37.0%) were positive and 34 (63.0%) negative. From positive cases, 9 cases contained the *TP53* mutation and 11 cases did not. Among negative cases, 21 featured the *TP53* mutation while 13 standard *TP53* gene. In total, cDNA/gDNA analyses did not detect any *TP53* mutation in 18 cases positive in FISH: 7 SQCCs and 11 ACs.

**Correlation between the p53 status and the disease outcome.** In this part of the analysis, only patients with complete clinical records were included. For cohort of 36 patients with SQCC the median follow-up was 22.8 months (ranging from 0.3 to 77.2 months), the median follow-up to progression (PFS) was 21.8 months (0.3-76.9 months). For cohort of 46 AC patients the median follow-up was 24.4 months (2.5-103.5 months), the median follow-up to progression was 21.4 months (2.0-103.5 months). We examined relation between the disease outcome (progression-free survival and overall survival) and the *TP53* status: namely presence of the *TP53* mutation, type of the *TP53* mutation (missense vs. non-missense), loss of the *TP53* locus, the p53 protein accumulation or any p53 aberration. We did not find any statistically significant difference in survival rate (both OS and PFS) in relation to any examined factor but some trends for their relationship were demonstrated. The most pronounced association was found between the presence of any p53 aberration in tumor tissue and decreased PFS ( $p=0.074$ ) and OS ( $p=0.121$ ) of patients with lung AC.

## Discussion

In the present study, we performed detailed analyses of the *TP53* status in 98 cases of lung carcinoma. Our cohort comprised

of 42 cases of squamous cell carcinoma and 56 cases of lung adenocarcinoma (AC). We analyzed cases which were sequentially collected in tissue bank of the University Hospital Brno from March 2006 to January 2014. Parameters of our cohort (e.g. age, male/female, SQCC/AC and non-smokers/smokers ratios) are similar to corresponding cohorts worldwide. Results of our analysis are in good agreement with previous findings. Proportion of the *TP53* mutation-positive cases is very high among our lung AC and particularly among SQCC cases reaching 55.4 and 76.2%, respectively. Frequency of cases with any somatic *TP53* aberrations, mutation and/or deletion, is even higher reaching 75% for AC and 92.9% for SQCC cases. To some extent, it corresponds to the results of the comprehensive studies describing 34% of mutated cases for lung AC (15) and 90% for lung SQCC (14). The complex and detailed analysis employing several methodically diverse approaches and particularly powerful and highly sensitive FASAY are the likely reasons for more efficient detection of the *TP53* aberrations in our study. FASAY has been repeatedly shown to be potent and robust tool for detection and further analyses of *TP53* mutations (21). In the present study, we carefully searched for a *TP53* mutation in 12 cases classified as wild-type by FASAY. Using cDNA and gDNA sequencing we found the *TP53* mutation in only one of them (AC case A23). In fact, two different *TP53* mutations were traced in the sample. One of them (A23-B: c.557\_558insAGCTGCTCAGATAGCGA) caused frame shift and formation of premature termination codon and mRNA degradation which, in combination with its low prevalence in sample (~15% based on gDNA sequencing estimation), explains why it was missed by FASAY. The other mutation found in the same sample by gDNA sequencing (A23-A: p.G154V)-which should be detectable by FASAY-had comparably low prevalence in the sample. This one initially unrevealed and then revised positive case represents 8% from 12 comprehensively reassessed *TP53* mutation-negative cases providing an estimation of false negativity of FASAY. Similarly, the nonsense mutations could also be missed in the examination. In case of the mutation p.E171\* (case S41) only our examination of another suspected (and finally not confirmed) mutation resulted in its disclosure. Detection of the mutation p.K321\* in AC sample A35 was also difficult. It was detected in only 3 from 18 red yeast colonies and subsequently confirmed by gDNA sequencing. The mutation p.E171\* has already been reported 22 times in tumors, notably 10 times just in lung carcinomas. The mutation p.K321\* is rather rare as it has been previously reported only twice and never in lung carcinoma (<http://www-p53.iarc.fr>; R17; 20). Nevertheless, FASAY again proved to be considerably successful even in detection of the *TP53* mutations associated with nonsense-mediated mRNA decay as demonstrated by other cases, for example SQCC cases S4, S23, S38, AC cases A10, A16 and others. Altogether, we detected 16 mutations leading to formation of premature termination codon. Five mutations were nonsense and 11 mutations were deletions and/or insertions causing reading frame shift. These mutations represent as much as 25% of all detected mutations in our whole cohort and even 31.25% (10/32) among SQCC cases (while only 18.75%-6/32 in AC cases).

Inactivation of p53 usually results from a missense mutation in the core region of the *TP53* gene coding for the sequence-

specific DNA-binding domain. According to the IARC database, proportion of the missense mutations represents 73.2% on average and 72.8% in lung tumors (<http://www-p53.iarc.fr>; R17; 20). In our samples, missense mutations represented 71.9% of mutations detected in AC but occurred less frequently in SQCC representing only 59.4%. An interesting, though missense, mutation was detected in AC sample A15: p.R248L. Its determination was unequivocal but quite surprising as the mutation represents exchange of dinucleotide (c.743\_744GG>TT). Nevertheless, the mutation has already been reported four times, three of them in lung carcinoma. This mutation, similarly to p.E171\* seems to occur rarely but predominantly in lung carcinoma (<http://www-p53.iarc.fr>; R17; 20).

We repeatedly detected several mutations, namely p.G154V (4x), p.R283P (3x), p.Y163C, p.R158L, p.V274F, p.R280T and mutation p.[G105V, G105\_T125del]. Among them, only mutations in codons 158 and 163 were found repeatedly in other cohorts (14,22). On the contrary, mutations suggested as lung carcinoma hot-spots by other authors were also found by us, namely mutations in codons 155, 245, 248, 275, 282 and also in codon 157 (AC case A29) which is considered to be associated with smoking (11,14,23,24). From mutations detected repeatedly in our cohort, the mutation c.314G>T was the most unusual. The mutation, though not typical for the *TP53* gene and occurring with low frequency, appeared twice: in SQCC case S15 and in AC case A7. Though the analyses of these cases were not performed at the same time, we were aware of risk of sample swapping. To rule out this possibility, both cases were analyzed repeatedly, both providing consistently the same results. We observed that while the case S15 was heterozygous in polymorphism p.P72R/p.P72P, the case A7 was homozygous p.P72R. This convinced us that indeed the two samples correspond to two different persons. In addition, this conclusion was unequivocally verified by the chimerism test. According the IARC *TP53* database (<http://www-p53.iarc.fr>; R17; 20), the mutation c.314G>T has predicted its effect on splicing. The FASAY, as its full name (functional analysis of separated alleles in yeast) indicates it provides the way to assess frequency of particular *TP53* alleles. Indeed, using FASAY we were able to detect the alternatively spliced transcript p.G105\_T125del, which was prevalent in both cases (12/13 and 15/15 colonies, respectively).

Notably, we found three different splicing mutations in intron 4 (c.375+5G>A, c.375+2T>A, c.375+1G>T) resulting in creation of multiple aberrant transcripts in all cases. All the three splicing mutations are rather rare, but each has been already reported: once, five times and once, respectively; none of them in lung carcinoma (<http://www-p53.iarc.fr>; R17; 20).

We detected accumulation of the p53 protein in 57.1% of SQCC cases and 33.9% of AC cases. This result fits well with other studies (11,25). Similarly, the concordance we found between the *TP53* mutations and the p53 protein accumulation for SQCC cases (69.0%) and AC cases (78.6%) is comparable to published data (60 and 70%) (11). Notably, the level of concordance would be even higher if the type of the *TP53* mutation was taken into account. We clearly showed that several non-concordant p53 proteins, negative/*TP53* mutation-positive cases, contained mutation causing formation of premature termination codon and mRNA degradation. In contrast, the concordance between *TP53* mutation and loss of

the *TP53* specific locus 17p13 was considerable lower reaching 46.3% in SQCC and 40.7% in AC. However, it has been also previously shown that in NSCLC the *TP53* mutations appear both with and without the allele loss (11). Our results confirm this observation and show that the *TP53* allele loss appears both with and without *TP53* mutation. It indicates the overall high loss of the p53 function in lung carcinomas confirming its importance in process of the lung carcinoma development and progression.

Several studies, including a large meta-analysis showed that abnormal p53 expression was associated with decreased survival across all stages in NSCLC in both adenocarcinoma and squamous cell carcinoma (11,26,27). However, results of our study did not support this conclusion. Although we examined relation between the disease outcome and several p53 parameters as presence and type of the *TP53* mutation, loss of the *TP53* locus, the p53 protein accumulation or p53 aberration, we did not find any statistically significant difference. The insufficient size of the cohort of patients can provide one possible explanation for this discrepancy. Due to high proportion of cases positive for a p53 aberration, the subgroup without a p53 hit was not sufficient. It may also explain why the only association coming close to statistical significance concerned the AC cases among which the proportion of p53 aberrations was lower. In addition, similar results demonstrating p53 as significant prognostic marker in AC but not squamous cell carcinoma has been reported earlier (10,27).

In conclusion, although our results failed to boost the role of the p53 inactivation as a negative prognostic marker in lung carcinoma, they clearly support its critical role in lung carcinoma development. Last but not least, we demonstrated the usefulness and convenience of FASAY as a fast, simple and reliable instrument for detection of *TP53* mutations in human tissues.

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