Therapy increases poly-ADP-ribose and p53-Ser392-P levels in recurrent squamous cell lung cancer*

A. Holownia[1], R.M. Mroz[1], M. Kozłowski[2], E. Chyczewska[3], J. Laudanski[2], L. Chyczewski[4], J.J. Braszko[1]

[1]*Department of Clinical Pharmacology, e-mail: holow_sinai@hotmail.com,* [2]*Department of Thoracic Surgery,* [3]*Department of Pneumology, and* [4]*Department of Clinical Molecular Biology, Medical Academy of Bialystok, 15 274 Bialystok, Poland*

Received

p53 protein is a critical regulator of the cell cycle and apoptosis and its levels and functions change in response to many stimuli. To assess whether the cytotoxic drugs induce DNA changes, affect phosphorylation and stability of p53 protein, we determined poly-ADP-ribose levels, the expression of p53 protein and its carboxyl-terminal Ser-392 phosphate levels in fiberoptic bronchoscopy biopsy samples taken from patients suffering from recurrent squamous cell lung cancer before and after radiotherapy and chemotherapy.

All 14 patients included in this study were in IA-IIIA clinical stage prior to surgery. Radiation/chemotherapy decreased G2/M cell numbers but increased S-phase cells by almost 50% compared to ploidy status before therapy, while median p53 expression was doubled (109% increase). p53 phosphorylated on Ser-392 was also increased by approximately 70% in patients treated with radiotherapy and with chemotherapy and correlated with elevated poly-ADP-ribose levels. Our data suggest that apart from changes in p53 quantity, posttranslational phosphorylation/dephosphorylation-mediated alterations may play an important role in neoplastic cell proliferation as well as in antiproliferative activity of drugs inducing DNA damage and apoptosis.

Key words: Cisplatin, DNA ploidy, squamous cell lung cancer, vinorelbine.

p53 protein, a product of tumor-suppressor gene is one of the critical regulators of the cell cycle and apoptosis. This key nuclear protein governs cellular growth through regulation of some genes controlling apoptosis or downregulating cell proliferation [6]. Cellular p53 levels are low, because the protein is rapidly degraded via proteosome-mediated reactions with participation of murine double minute oncoprotein (mdm2), a ligase-like enzyme targeting the protein for degradation [8]. Mutation in p53 gene, which is observed in over 50% of human cancers, increases the half-life of p53 protein but decreases its DNA binding and functions [7]. Modifications of p53 levels and alterations in protein functions are frequent in lung cancer but clinical correlation between its expression (both wild-type and mutated) and prognosis is still poor. On the other hand, chemotherapy supported by exogenous p53 significantly improves lung cancer patient outcome and survival stressing important role of p53 in therapy [9].

Changes in the levels of p53 are usually associated with different post-translational modifications of its N-terminal domain and/or its carboxyl-tail [6]. Recently published data indicate that phosphorylation of p53 near mdm2 binding region may be critical for negative targeting of p53 by mdm2 [4]. Even phosphorylation of C-terminal Ser-392 by casein kinase may enhance cell cycle regulation and p53-DNA binding followed by altered p53 transcription resulting in alterations in cancer cell growth [14, 21]. p53 interact with many other proteins to maintain genome integrity and suppress tumorigenesis. Recently published data indicate that the poly(ADP-ribose) polymerase (PARP, PARP-1, EC 2.4.2.30) plays an important role in DNA repair, cell proliferation and death and cooperate with p53 in suppressing tumorigenesis [25]. DNA-damaging therapy of cancer strongly activate PARP and increase poly-ADP-ribose (PAR) which levels correlate with biochemical markers of apoptosis [22].

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The aim of the present study was to assess the effect of DNA-damaging and proapoptotic therapy typical in lung cancer treatment, consisting of multiple radiation/cisplatin/vinorelbine cycles on tumor cell DNA status, its poly-ADP-ribose levels, p53 expression and p53 Ser-392~P levels.

Material and methods

Clinical samples. Bronchial tissue was taken using fiberoptic bronchoscopy from 14 male smoking patients, aged 48–73 years (mean 63 ± 8) previously subjected to surgical resection of their primary lung tumor histologically confirmed as squamous cell lung cancer (Tab. 1). Patients were admitted to hospital due to clinical signs of recurrent lung cancer. Bronchoscopy was performed before and at day 22nd–24th of the last chemotherapy cycle.

Bronchoscopy procedure. Bronchial biopsy specimens were fixed separately in 10% buffered formaldehyde or 95% ethanol and routinely processed for light microscopy. Sections were independently examined by pathologists who were blind to the clinical and smoking histories of the subjects and interpreted using standard protocols of macro- and microscopic evaluations including: histological grade, tumor depth of invasion (T), lymph node metastasis (TNM), and patterns of tumor infiltrations. All data presented in this paper come from patients suffering from recurrent squamous cell lung cancer, initially (prior to surgery) graded T1-3.N0-3.M0 (Tab. 1). Bronchial tissue for DNA ploidy determination, Poly-ADP-ribose levels quantification and analysis of p53 protein were taken from the same area as for pathology assay.

All patients were treated with standard post-operative chest radiation (30 daily 2-Gy fractions for a total of 60 Gy) and 4 cycles of chemotherapy with cisplatin/vinorelbine starting at 120 mg/m^2 cisplatin every 3 weeks and 30 mg/m^2 vinorelbine on the 1st and the 8th day of each 21-day cycle [29].

Tumor preparation and DNA staining. Unfixed biopsy samples were mechanically minced using tissue dissociation sieves (Tissue Grinder Kit, Sigma) with 200 μm diameter mesh followed by 100 μm diameter mesh. Then, samples were layered on Ficoll (Pharmacia) and damaged cells and aggregates were separated using differential gradient centrifugation.

To determine DNA ploidy the obtained pellets containing mostly cell nuclei were resuspended in Tris buffer (pH 7.5) containing 20 μg/ml RNase A and 0.1% Triton X-100 and stained with propidium iodide (PI) at 50 μg/ml final concentration (16). 12 hours latter, samples were filtered by 50 μm mesh filters (Beckton-Dickinson) to remove clumps and cell aggregates and run on aligned Coulter Epics Elite flow cytometer (Coulter Electronics) equipped with 488 nm argon laser. 10.000 nuclei in each sample was assayed at pulse area >580 nm with 1024 linear scale channel resolution and minimal contribution of doublets. The modal G0/G1 peak channel of PI fluorescence was set to 200th channel by adjustment of the Fl2 detector voltage using healthy bronchial cells surrounding resected tumors of the same type. DNA fluorescence histograms were analysed for DNA ploidy, S-phase fraction (SPF) and G2/M fraction using Multicycle Phoenix software (Phoenix Flow Systems). DNA index (DI) was calculated as the mean channel position of the tumor G0/G1 peak divided by the mean channel position of the diploid G0/G1 peak obtained with healthy bronchial cells surrounding resected tumors. Assayed sample was assigned as diploid, when its DNA fluorescence histogram showed a single, symmetric G0/G1 peak with CV<6. Samples were assigned as aneuploid when G0/G1 peak had 0.8< DI<1.2 and showed at least bivariate distribution, while tetraploid sample had 1.8<DI<2.2.

Poly-ADP-ribose, (PAR) levels were quantified in dot blot using mouse IgG3 monoclonal antibody against poly-ADP-ribose, (Clone 10H, Alexis Biochemicals) and secondary peroxidase-coupled antibody [18]. Briefly, equal amount of tissue homogenate proteins were blotted into nitrocellulose membrane (Bio-Rad), dried, blocked with milk (Bio-Rad) and incubated overnight with the first antibody (1:500) with constant agitation. Controls were prepared using unspecific antibody of the same isotype. Then, the membrane was washed with PBS-T and incubated with peroxidase-conjugated anti-mouse secondary antibody (1:1000) for 1 hour. The blot was washed, revealed using ECL kit (Amersham, U.K.) and quantified using Image-Quant software.

Table 1. Patients with previous surgical resection of the primary lung tumor were admitted to hospital due to clinical signs of recurrent lung cancer. Bronchial tissue samples were taken for assay before and after recurrence treatment with radiation and 4 cycles of chemotherapy with cisplatin/vinorelbine.

Patient number	Age	TNM	Type of resection	Site of bronchial biopsy
1	69	T1N2M0	R.L.B.	Stump
2	67	T1N1M0	R.U.L.	Stump
3	53	T2N2M0	L.P.	Carina
4	48	T3N1M0	L.P.	Stump
5	68	T2N0M0	R.U.L.	Stump
6	66	T2N1M0	R.P.	Stump
7	61	T1N2M0	R.P.	Carina
8	71	T2N0M0	L.L.L.	Left main bronchus
9	64	T2N1M0	L.P.	Stump
10	67	T2N1M0	L.L.L.	Stump
11	66	T2N1M0	R.P.	Stump
12	54	T2N0M0	R.P.	Stump
13	73	T1N0M0	R.U.L.	Right main bronchus
14	52	T2N1M0	R.L.B.	Stump

R.L.B. – right lower bilobectomy, R.U.L. – right upper lobectomy, L.P. – left pulmonectomy, R.P. – right pulmonectomy, L.L.L. – left lower lobectomy.

Western blot analysis of p53 protein [13]. For immunoblot analysis, samples were incubated in a lysis buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylsulfonyl fluoride (PMSF), 10 mg/ml leupeptin and 10 mg/ml aprotinin), resulting lysates were resolved on 10% PAGE, (50 μg protein per lane) and transferred onto nitrocellulose membranes (BioRad). The membranes were blocked with TBST buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% milk and then hybridised with monoclonal mouse IgG Ab recognizing both wild and mutated human p53 (Oncogen Research Products). Chemiluminescence kit (Amersham) was used as a detection system and p53 bands were quantitated using densitometry, numerized and compared.

Determination of p53 levels phosphorylated on Ser-392 was performed using imunoprecipitation followed by SDS-PAGE and immunoblot [10]. Briefly, bronchial tissue was lysed for 1 hour on ice, in a buffer containing 50 mM Tris-acetate (pH 7.2), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, 0.3 M NaCl, 1 mg/ml BSA, 1% NP-40, 0.2% NaN_3, freshly added Na_3VO_4 to 1 mM final concentration, 5 mM NaF and spun at 15.000 rpm for 10 minutes. All samples were equalized for protein (BCA assay kit, Pierce) and then incubated for 1 hour at 4 °C with monoclonal mouse IgG Ab against human p53 (clone BP53.12; Oncogen Research Products) recognizing both wild-type and mutant gene product, following by addition of protein A-sepharose beads (Sigma). Control reaction was performed using the same incubation conditions but antibody was replaced by foetal blood serum (Sigma). The beads were sedimented by centrifugation (11.000 x g), washed 3 times with lysis buffer, resuspended in SDS-PAGE sample buffer (BioRad), equalized for p53 protein and immunoblotted on SDS-PAGE using 10% separation gel and 4% stacking gel with loaded molecular weight markers (BioRad). Then, gels were blotted on nitrocellulose membrane (BioRad) and revealed using rabbit polyclonal antibody recognizing p53 protein phosphorylated on Ser-392 (BioSource) and chemiluminescence detection system (Amersham).

Statistical analysis was performed with the Statistica (Statsoft) package using the Kolmogorof-Smirnof distribution test and the Wilcoxon signed rank test to compare median p53 expression in samples taken before and after treatment. Linear regressions and correlations between p53, p53-Ser392-P and poly-ADP-ribose levels before and after therapy were calculated according to Pearson test.

Results

DNA ploidy and cell cycle. The obtained DNA histograms were considered reproducible in 22 out of 28 lung cancer samples. Among these, 4 samples were assigned as diploid, 7 aneuploid and 1 tertraploid or near tetraploid (Fig. 1A-C) and these patterns did not change after treatment. DNA histograms analyzed using zero and first order models evidenced that mean S-phase (SPF) and G2/M cell fractions before therapy were $8.7 \pm 3.4\%$ and $4.8 \pm 2.9\%$ of total cell counted, respectively. After therapy, corresponding values were $13.2 \pm 4.8\%$, (52% increase, $p<0.05$) and 2.2 ± 2.1 (54% decrease, $p<0.05$) for SPF and G2/M fractions respectively. Apart from described changes in cell cycle profiles, therapy did not change DNA ploidy-status in particular patients. Due to scarce patient numbers in this study and relatively high margin of error we did not attempt to quantify these data separately in euploid and aneuploid subgroups.

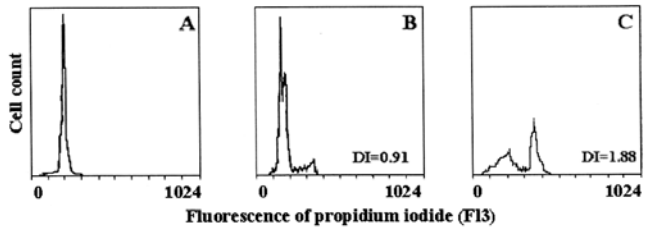

Figure 1. Representative DNA fluorescence histograms from recurrent squamous cell lung cancer patients. A-euploid, B-aneuploid, C-tetraploid histograms. Radiotherapy/cisplatin/ vinorelbine treatment altered particular cell cycle fractions but did not change DNA ploidy patterns.

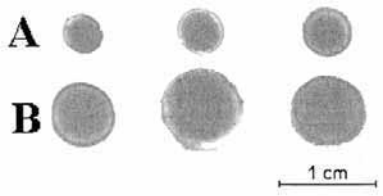

Figure 2. Representative dot-blot spots of poly-ADP-ribose assay in bronchial biopsy specimens from recurrent squamous cell lung cancer patients (A) and from the same patients after radiotherapy/cisplatin/vinorelbine treatment (B). Mean relative poly-ADP-ribose content is 2.7 fold higher after therapy (268 ± 91; n=14, $p<0.05$) than before therapy (100 ± 76; n=14).

Poly-ADP-ribose was detected in all samples tested with significantly increased values after treatment. Representative spots are shown in Figure 2A and B, respectively, for patients before and after therapy. Mean PAR content before therapy was expressed as 100 ± 76 relative units and corresponding value after therapy was 268 ± 91 ($p<0.05$).

p53 expression was found in 23 out of 28 bronchial samples assayed by SDS-PAGE and WB. p53 was detected in 11 out of 14 samples taken before therapy (representative samples are shown in Fig. 3A) and in 12 samples obtained from the same patients after therapy (Fig. 3B). Relative mean p53 band-intensities were increased after therapy by about two

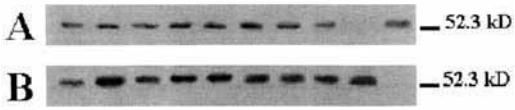

Figure 3. Western blot analysis of the p53 protein levels in lung tissue taken from recurrent squamous cell lung cancer patients before (A) and after (B) radiotherapy/cisplatin/vinorelbine therapy. The p53 protein was analyzed by SDS/ PAGE, hybridized with p53 Ab and revealed using ECL system. Position of molecular weight standard used (ovalbumin) is shown. Mean relative p53 levels were two fold higher (105% increase) after therapy ($p<0.05$).

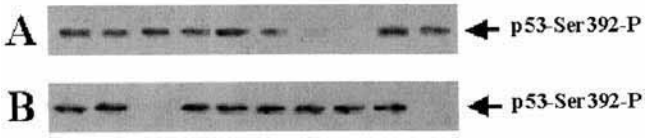

Figure 4. Immunoprecipitation/Western blot analysis of the p53 protein phosphorylated at Ser392 in recurrent squamous cell lung cancer patients before (A) and after (B) radiotherapy/ cisplatin/vinorelbine therapy. The p53 protein was immunoprecipitated by monoclonal Ab and the precipitate was analyzed on an SDS/PAGE. The transferred bands were hybridized with p53-Ser-392-P Ab and revealed using ECL system. Mean relative p53-Ser392-P level was 71% higher after therapy ($p<0.05$).

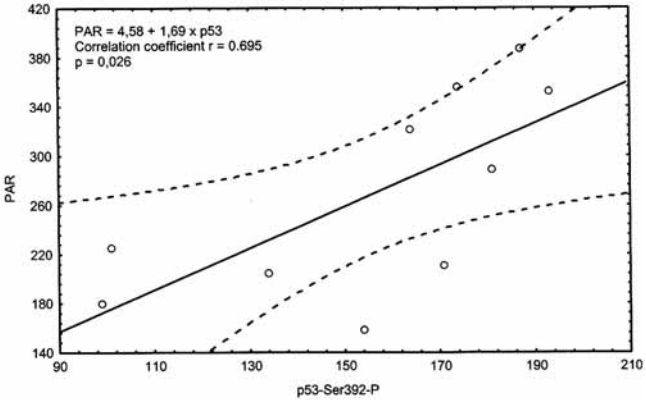

Figure 5. Regression and correlation analysis between poly-ADP-ribose (PAR) levels and p53Ser-393-P levels in recurrent squamous cell lung cancer patients exhibited no correlation (r=0.15) before radiotherapy/cisplatin/vinorelbine treatment (not shown) and good correlation (r=0.70, $p<0.05$) after therapy.

fold ($105 \pm 59\%$) and this difference was statistically significant ($p<0.05$). Alterations in p53 expression did not correlate with changes in poly-ADP-ribose levels or p53-Ser392~P.

p53 phosphorylation on Ser-392. p53 immunoprecipitation followed by immunoblot detection of phosphorylated p53 protein at Ser392 showed positive staining in 11 and 12 bronchial samples taken before and after therapy, respectively (Fig. 4A and B). Relative p53-Ser392~P levels after therapy were higher by 71% ($p<0.05$) comparing protein levels before therapy. Regression and correlation analysis showed no correlation (r=0.15) between poly-ADP-ribose and p53-Ser392-P levels before therapy, but good correlation (r=0.7; $p<0.05$) after therapy (Fig. 5).

Discussion

Non-small cell lung cancer has variable clinical phenotype even in patients with early stages of the disease and for several reasons, there is no reliable prognostic index of cancer progression which may serve to assess patients outcome. Independently on the therapy, long-term survival remains unsatisfactory, even in patients having complete surgery with following radio/chemotherapy [26].

Alteration of p53 suppressor genes is most frequently identified in human neoplastic diseases including lung cancer, although its prognostic value is usually poor due to low correlation between protein expression and function [2, 6, 7]. Most data on p53 functional alterations are based on experimental studies. Our pilot results indicate, that radiotherapy/cisplatin/vinorelbine treatment do not alter DNA ploidy patterns but increase S-phase cell fractions and decrease G2/M cell numbers in bronchial biopsy samples from patients treated for recurrent squamous cell lung cancer. Moreover elevated levels of p53, its Ser392~P and poly-ADP-ribose are observed and increased PAR levels due to the therapy positively correlate with elevated p53-Ser392~P. These results are interesting, although reasons of described alterations are still to be defined.

The mechanisms of DNA damage signalling are important in the therapeutic management of cytotoxicity. Increased poly-ADP-ribosylation was described in apoptotic cells and PAR-producing enzymes, are described as early markers of apoptosis-associated DNA damage [22]. Increased levels of PAR due to therapy may indicate that PAR-producing enzymes are activated. This effect can trigger several secondary reactions and some of them may affect p53 protein. It is known, that poly-ADP-ribose polimerase-1 regulates not only the stability of the wild-type p53 protein [15] but also its transcriptional activation [28]. Immunoprecipitation assay, followed by immunoblot detection of p53-Ser392~P protein evidenced increased phosphoprotein levels after therapy. Ser-392 is located at the carboxyl terminus of p53 protein and this region enable the protein to bind to DNA and to catalyze DNA renaturation and transfer [12, 24]. Phosphorylation of the carboxyl terminus of p53 regulates also transcription transactivator function of p53 including up-regulation of p21 encoding G1 checkpoint protein [11] and this pathway may stimulate antiproliferative properties of p53 and induce apoptosis [1]. *In vitro*, this reaction is controlled by casein kinase II and this enzyme is probably also responsible for Ser-392 phosphorylation *in vivo*. Our results agree with the recently published data indicating that postoperative radio/che-

motherapy may trigger phosphorylation and/or acetylation of the p53 protein resulting in protein activation and changes in apoptotic mechanisms of cell death [3, 17]. Another data indicate, that cisplatin may increase p53 phosphorylation [5]. It is not surprising, that DNA-damaging therapy exerts similar effects. On the other hand, potential p53 activation may have minor role in regulation of cancer response to the therapy, due to majority of mutated, inactive protein. Positive correlation of PAR and p53-Ser392~P levels merit further attention, but our results should still be confirmed in larger series of patients. Several reports indicate, that both DNA-damage recognition proteins functionally cooperate [27]. It is possible, that increased PAR trigger p53-Ser392 phosphorylation resulting in p53 activation and apoptosis.

Radiation/chemotherapy DNA damage frequently results in p53 overexpression, growth arrest and apoptosis or occurrence of mutations, (also including p53 gene) and even in promotion of carcinogenesis [19]. Apart from DNA damage, such radical treatment release cytochrome c, activate caspase-3 and induce apoptosis, via p53-dependent or p53-independent pathways [19]. Several reports describe accumulation of mutated p53 proteins in different neoplastic diseases [23]. These mutated proteins are usually unable to initiate apoptosis and cancer regression. We have shown two-fold increase in the levels of p53 protein after therapy and it seems that this protein may represent, at least in part, mutated p53. Unfortunately, we were not able to separate wild type and mutated p53 due to scarce tissue availability. This assay should be included in future investigations.

Our study indicates, that posttranslational modification of p53 protein, especially multisite wild type and mutated p53 phosphorylation along with poly-ADP-ribosylation assay may be promising in improving our prediction of lung cancer response to therapy.

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