Increased Δ133p53 mRNA in lung carcinoma corresponds with reduction of *p21* expression

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Abstract. Modification of *p53* expression levels and its principle apoptosis and cell cycle regulatory partners, mouse double minute 2 homolog (*MDM-2)* and *p21*, has been previously reported in various types of cancer. In the current study, the expression of Δ133p53 isoforms was investigated in lung carcinomas with respect to the expression of the aforementioned genes. The expression of *p53* full-length transcript and Δ133p53 isoforms α, β and γ transcripts, *MDM-2* and *p21* transcripts were determined by reverse transcription-quantitative polymerase chain reaction, in total RNA isolated from 17 lung carcinoma specimens and 17 corresponding adjacent non-cancerous tissues. RNA expression analysis was performed according to the Pfaffl equation and Rest tool using *β-actin* as a reference gene. Detection of the above proteins was additionally performed by western blotting. Significant overexpression of the Δ133p53 mRNAs was observed in cancerous as compared with adjacent non-cancerous tissues (3.94-fold), whereas full-length *p53* and *MDM-2* expression exhibited a smaller, however significant, increase. The expression of the *p21* transcript was significantly reduced in cancerous specimens. Δ133p53 and *p21* expression levels varied in parallel, however were not significantly correlated. p53 full-length protein expression observed by western blot analysis strongly varied from the Δ133p53 isoforms, however MDM-2 protein isoforms were not detectable and p21 protein was more abundant in non-cancerous tissues. In conclusion, Δ133p53 mRNA levels is suggested as a potentially useful marker of malignancy in lung cancer. The absence of Δ133p53 protein in lung carcinomas, which overexpress Δ133p53 transcripts, may indicate the role of the latter in post-transcriptional regulation through RNA interference in the cell cycle and apoptosis.

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Introduction

p53 is a key regulator of growth arrest, senescence and apoptosis in response to a wide array of cell damage events (1). Rapid induction of high p53 protein levels under different stress conditions prevents inappropriate propagation of cells carrying potentially mutagenic, damaged DNA (2). The central role of p53 in the cell stress response lends significance to the existence of at least nine different p53 isoforms arising from differential splicing and promoter usage (3). These isoforms are expressed in normal tissue in a tissue-dependent manner and their differentiated expression in human cancer suggests that they may be involved in tumor development or progression (4).

The Δ133p53 isoform(s) of tumor suppressor *p53* are transactivated by p53 in response to stress (5). The Δ133p53 isoforms are lacking the first 133 amino acids and the expression of their mRNA is derived from an alternative internal promoter located in intron 4 of *p53*. Lack of the p53 N-terminus is responsible for a dominant negative inhibition of normal apoptotic function of the p53 protein. Therefore, the excessive expression of these isoforms may serve a critical role and interfere with normal *p53* function. The Δ133p53 isoforms are implicated in controlling cellular senescence and elevated levels of these isoforms have been observed in a variety of tumors. Elevated expression of Δ133p53 isoform(s) has been observed in breast cancer and in renal cell carcinoma, whereas in colon cancer, progression from colon adenoma to carcinoma is accompanied by an increase of Δ133p53 mRNA (6). In addition to its multiple functions, p53 has been additionally identified to suppress metastasis and inhibit angiogenesis, a process strongly contributing to tumor development (7). Bernard *et al* (8) established that angiogenesis and growth of glioblastoma U87 tumors are inhibited upon depletion of the Δ133p53α isoform and that this isoform induces pro-angiogenic gene expression and represses anti-angiogenic gene expression. Furthermore, previous analysis of germline *p53* mutations in breast cancer by Kouidou *et al* (9) demonstrated that the Li-Fraumeni and Li-Fraumeni-like syndromes are

closely associated with the loss of the initiation codon 133 of the Δ133p53 isoforms, thus indicating that these isoforms serve a regulatory role and are associated with carcinogenesis.

In the current study, the expression levels of transcripts leading to the expression of Δ133p53 isoforms were identified in lung cancer. In addition, due to the fact that p53 serves a pivotal role within the cell and is subjected to a tight and orchestrated control, creating a network of positive and negative regulations (10), the association of Δ133p53 mRNA expression with respect to that of mouse double minute 2 homolog (*MDM-2)* and *p21,* two of its principle regulatory partners, was investigated. MDM-2 has been identified as the principal cellular antagonist of p53 by limiting p53 tumor suppressor activities (11). Through binding to the N-terminal transactivation domain, MDM-2 is involved in p53 degradation in the cytoplasm (12), thus suppressing its activity. MDM-2, an E3 ubiquitin ligase of the RING-finger family, is involved in p53 regulation. The RING domain permits the direct binding of ubiquitin enzymes resulting in mono- or poly-ubiquitination of p53 (13). p21 is a notable effector of p53 and is a general inhibitor of cyclin-dependent kinases, functioning to negatively regulate the cell cycle. The expression of *p21* is upregulated by the *p53* response to DNA damage, leading to cell-cycle arrest at the G_1 checkpoint (14). Similar to other types of human cancer, approximately 50% of lung cancer types exhibit mutations in *p53* (15), however the relative expression of the Δ133p53 isoforms remains to be fully elucidated in association with this disease.

Table I. Epidemiological characteristics of samples used in the experiments of the current study, documentation of the histopathology analysis of each patient, the age, gender and the smoking habits (packs per years) are presented.

Sample no.	Histopathology	Age	Gender	Smoking (packs/year)
1	Squamous	44	M	60
6	NSCLC	N/A	F	70
9	NSCLC	N/A	M	Non-smoker
11	NSCLC	73	M	55
12	Adenocarcinoma	42	M	63
13	Squamous	N/A	M	40
20	Adenocarcinoma	47	M	60
27	NSCLC	N/A	N/A	N/A
38	Adenocarcinoma	75	M	Non-smoker
39	NSCLC	58	M	30
40	NSCLC	66	F	Non-smoker
41	Adenocarcinoma	67	M	150
42	NSCLC	65	M	140
43	NSCLC	66	M	200
44	NSCLC	60	M	60
45	NSCLC	77	M	60
46	Adenocarcinoma	48	F	70

N/A, not available; NSCLC, non-small cell lung cancer.

Materials and methods

Tissue specimens. Surgical specimens, primary tumor samples and corresponding non-malignant tissues, were obtained from 17 patients admitted to Theageneion Anticancer Hospital (Thessaloniki, Greece) immediately after the excision of non-small cell lung carcinoma during resection surgery (Table I). Thirteen of the patients were male (mean age, 61.27 years) and three female (mean age, 58 years). Macroscopically non-cancerous lung tissue was obtained from a distal site at the excision limit from the same individual. Samples were immediately snap-frozen and stored in liquid nitrogen prior to long-term storage at -80˚C. This study was approved by the ethics committee of Theageneion Anticancer Hospital. The human lung adenocarcinoma epithelial cell line (A549) and the human lung fibroblast cell line (MRC-5) were also analysed. The cell lines were kindly provided by Dr George Geromichalos (Theageneion Anticancer Hospital).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays for detection of p53, full-length and Δ133p53, MDM-2 and p21 mRNA and statistical analysis of the results. Total RNA was extracted with the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA) treated with DNase (Promega Corporation), both integrity and purity were confirmed via spectrophotometry and agarose gel electrophoresis. cDNA was synthesized using oligodT and Superscript II Reverse Transcriptase (Promega Corporation). cDNA (2 μg) were then used for the PCR reaction. For quantification of *p53*, full-length and Δ133p53 isoforms, *MDM-2* and *p21* transcripts, RT-qPCR was performed using 125 ng RNA with 2X Power SYBR-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions and performed on Applied Biosystems StepOnePlus Real Time PCR System (Thermo Fisher Scientific, Inc.). The cycling conditions were as follows: 95˚C for 10 min, then 40 cycles of 95˚C for 30 sec, 56˚C for 30 sec, 72˚C for 30 sec, followed by melting curve analysis up to 95˚C. The primer sequences used were as follows: Full-length *p53*, P53EX3S 5'-TCCATG GGACTGACTTTCTGC-3' and P53EX4AS 5'-CTGGCA TTCTGGGAGCTTCA-3'; Δ133p53 isoforms, P534b 5'-TAG ACGCCAACTCTCTCTAG-3' and P53RE5 5'-TTGGCA AAACATCTTGTTGAGGGC-3'; *MDM-2,* MDM2S 5'-CTG AAATTTCCTTAGCTGAC-3' and MDM2AS 5'-TTCAGG AAGCCAATTCTCAC-3'; *p21,* P21S 5'-TGGACCTGT CACTGTCTTGT-3' and P21AS 5'-TCCTGTGGGCGG ATTAG-3' and *β-actin*: B-ACTINS 5'-CGTCTTCCCCTC CATCGTG-3' and B-ACTINAS 5'-CTTCTGACCCATGCC CACCA-3'. The primers were designed in our laboratory with the exception of primers P21S and P21AS, which were designed as described previously (16). All primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) The molecular weight of PCR products was verified by agarose gel electrophoresis.

In order to quantify and compare the amplification products, Cq data corresponding to the target genes were normalised relative to those of an internal housekeeping gene, *β-actin*. Each data point was obtained twice. Quantitative values were estimated from the quantification PCR cycle

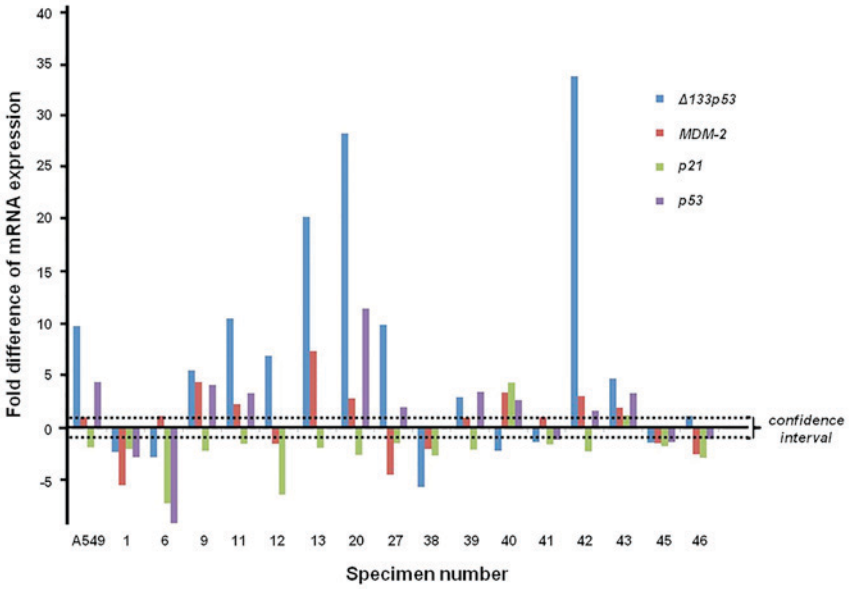

Figure 1. mRNA expression of *p53*, full-length and Δ133p53, *MDM-2* and *p21* relative to *β-actin* (reference gene) for each individual lung carcinoma and corresponding adjacent non-cancerous specimens. The C_q values were analyzed by the $2^{-\Delta\Delta Cq}$ method using Pfaffl analysis. ▪▪▪, 95% confidence interval limit. MDM-2, mouse double minute 2 homolog.

number (Cq) at which the increase in signal associated with an exponential growth for PCR product starts to be detected. The relative mRNA levels in each sample were normalised to its *β-actin* mRNA content. The relative expression level of the target gene was analysed by the $2^{-\Delta\Delta Cq}$ method (17). For each sample, the difference in C_q values for the gene of interest and the endogenous control was calculated (ΔC_q) (17). In addition, the REST© tool (relative expression software, version 2009; Qiagen GmbH, Hilden, Germany) which compares two groups (cancerous vs. non-cancerous tissue) with up to 16 data points in each group for reference, and up to four target genes (18).

Protein extraction. The surgical specimens were homogenized into a fine powder using a mortar and pestle in liquid nitrogen and maintained at -80˚C. For the total protein extraction 100 mg of homogenized tissue was resuspended in 300 μl lysis buffer (50 mM Tris/HCl pH 8, 150 mM NaCl, 0.02% (w/v) sodium azide, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 0.4 mM EDTA, 10 mM NaF, 0.75 mM PMSF, 1% v/v protease inhibitor cocktail EDTA-free). The cells were then lysed by performing 5 freeze-thaw cycles in liquid nitrogen and 37˚C water bath. Subsequent to incubation for 4.5 h at 4˚C under rotation, the suspension was centrifuged at 15,000 x g for 25 min at 4˚C. The supernatant was maintained at -80˚C until later use.

Semi-dry western blotting analysis. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in the presence of blotting buffer (12.5 mM H_3BO_3, 12.5 mM Tris/HCl pH 8.5, 0.02% w/v SDS) by applying 64 mA for 15 min. The membranes were blocked for 1 h in 5% non-fat dry milk in phosphate-buffered saline with 0.1% Tween-20 at room temperature. Incubation with the primary antibodies was performed overnight at 4˚C, followed by incubation with the secondary antibody conjugated to alkaline phosphatase for 1 h at room temperature. The signals in the immunoblots were detected indirectly by staining with NBT/BCIP (Biotium, Fremont, CA, USA). The mouse anti-p53 antibody (Pab 240; sc-99) raised against amino acids 156-214 of p53 of human origin and the rabbit anti-p21 (C-19, sc-397) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and used at a working dilution of 1:500. The rabbit anti-MDM-2 antibody (S166; AP1253e) was purchased from Abgent (San Diego, CA, USA) and used at a 1:1,000 working dilution. The secondary anti-rabbit (7054F) and anti-mouse (7056) IgG-alkaline phosphatase antibodies (1:4,000 working dilution) were purchased from Cell Signalling Technology, Inc. (Danvers, MA, USA). The image capturing was performed with the Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Due to the fact that staining of the β-actin protein was inadequate, Coomassie Blue Staining was used as a loading control for the western blot analysis (19). Variation of β-actin steady-state expression levels in the lung may be responsible for the limited use of this protein as a control (19).

Results

Expression of p53, full-length and Δ133p53, MDM-2 and p21 transcripts, using RT-qPCR. Using specific primers and RT-qPCR, the full-length *p53* transcript, Δ133p53 (for Δ133p53α, Δ133p53β and Δ133p53γ a common set of primers was used), in addition to *MDM-2* and *p21* transcripts were amplified. In each case, the data were verified by replicate tests. The normalized average for each sample relative to the actin control was calculated using the Pfaffl equation (Fig. 1). Fig. 1 illustrates the wide variation of the relative expression of *p53*, full length and Δ133p53, *MDM-2* and *p21* transcripts in the majority of samples. However, the most considerable variation was observed in Δ133p53 transcript expression (3.94-fold increase), which according to the Pfaffl criteria (>1.5) was statistically significant.

Table II. Expression of full-length *p53*, Δ133p53, *MDM-2* and *p21* transcripts in the different tissues and cell lines.

A, Cancerous tissues relative to non-cancerous tissues for the carcinoma specimens

Gene	Type	Reaction efficiency	Expression	P(H1)	Result
β-actin	REF	1.0	0.872	0.845	
p53	TRG	1.0	1.379	0.787	
Δ133p53	TRG	1.0	3.436	0.012	Up
MDM-2	TRG	1.0	1.232	0.756	
p21	TRG	1.0	0.509	0.470	

B, The adenocarcinoma epithelial cell line A549 and the human lung fibroblast cell line MRC-5

Gene	Type	Reaction efficiency	Expression	P(H1)	Result
β-actin	REF	1.0	0.521	0.509	
p53	TRG	1.0	2.346	0.000	Up
Δ133p53	TRG	1.0	5.169	0.000	Up
MDM-2	TRG	1.0	0.570	0.509	
p21	TRG	1.0	0.287	0.000	Down

Values were normalized relative to the *β-actin* using the Rest statistical analytical tool. REF, reference gene; TRG, target gene; MDM-2, mouse double minute 2 homolog.

Table III. Comparison of Δ133p53 expression vs. *MDM-2* and *p21* in cancerous vs. non-cancerous tissue using the Rest statistical analytical tool.

Gene	Type	Reaction Efficiency	Expression	P(H1)
Δ133p53	REF	1.0	1.000	
MDM-2	TRG	1.0	0.384	0.208
p21	TRG	1.0	0.157	0.052

MDM-2, mouse double minute 2 homolog; REF, reference gene; TRG, target gene.

Analysis of the relative expression of p53, full-length and Δ133p53, MDM-2 and p21 transcripts in cancerous vs. non-cancerous tissue. In order to quantitfy the relative alterations in expression of the genes studied, REST© analysis was used. The results presented in Table IIA demonstrate significant overexpression of the Δ133p53 transcripts in cancerous vs. non-cancerous tissue, in addition to a smaller overexpression of *p53* and *MDM-2* in the above tissues. On the contrary, *p21* was observed to undergo a small reduction of expression in the cancerous tissue relative to the non-cancerous tissues. The same conclusions were drawn for the relative expression of *p53*, full-length, Δ133p53 and the *MDM-2* transcripts in the cancerous A549 cell line compared with the non-cancerous MRC-5 cell line (Table IIB). *p21* was significantly underexpressed in the cancerous A549 cell line. Thus, Δ133p53 appears to be the most overexpressed of the above transcripts in the cancerous tissues.

Comparison of MDM-2 and p21 expression relative to p53 full-length and to Δ133p53. In order to acquire a more sensitive marker indicative of the alterations associated with cancer, the relative expression differences of *MDM-2* and *p21* vs. Δ133p53 were investigated in cancerous tissues compared with non-cancerous tissues. The results presented in Table III indicate that the relative expression of *p21* vs. Δ133p53 exhibited a marginally stronger, however not significant, variation (P=0.052). Furthermore, investigation of the *MDM-2*, *p21* and Δ133p53 expression vs. full-length *p53* expression in cancerous and non-cancerous tissue (results not shown) identified smaller differences of the above transcripts with respect to both *MDM-2* and *p21* expression.

Comparison of p53, full-length and Δ133p53, MDM-2 and p21 expression relative to smoking. Due to the fact that cigarette consumption (packs/year) may be another parameter affecting the expression of these genes, the transcript differences with respect to smoking were additionally measured. Of the patients, three were heavy smokers (140-200 packs/year), while, three other patients had never smoked and the remaining 10 patients were intermediate smokers who consumed a smaller number of packs/year (40-70 packs/year). The analysis conducted for cancerous vs. non-cancerous tissues relative to smoking (Fig. 2) indicated that there were several consistent differences in gene expression. One difference was the absence of Δ133p53 overexpression in cancerous vs. non-cancerous tissue among non-smokers (Fig. 2A), detectable overexpression among intermediate smokers (Fig. 2B) and strong overexpression in heavy smokers (Fig. 2C). On the contrary, *p53* and *MDM*-2 differences were similar in all groups in cancerous and non-cancerous tissues and thus not differentiated with respect to smoking. *p21* expression did not vary considerably between cancerous and non-cancerous tissue with respect to smoking. In the small number of these cases, the above results were consistent although not statistically significant.

Western blotting analysis of p53, full-length and Δ133p53 isoforms, MDM-2 and p21. Comparison to mRNA expression. p53 protein expression and the presence of its different isoforms, in addition to MDM-2 and p21 proteins were then analyzed in representative samples with varying levels of Δ133p53 transcript expression: High (samples 13 and 42); intermediate level (sample 9); and low (sample 38). For this purpose the Pab-240 anti-p53 antibody was used, specific for the common exons 5-6 in the DNA binding region of p53, amino acids 156-214, which does not differentiate between α, β and γ variants. Using this antibody, almost all p53 isoforms were detected at differing in addition to the Δ40p53 β and γ isoforms (42 kDa) formed major intensity products (Fig. 3). Detectable truncated isoform Δ133p53α (35 kDa) was observed in sample 13C and Δ133p53β and γ (29 kDa) were weakly detected in sample 9NC. The C-terminus truncated

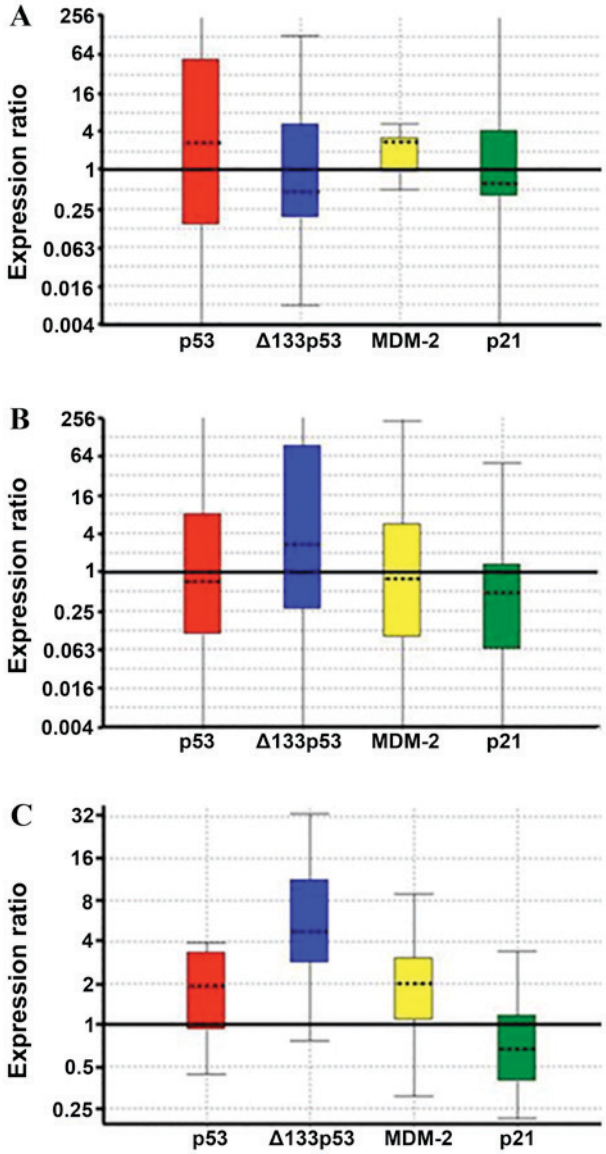

Figure 2. Expression of *p53*, full-length and Δ133p53, *MDM-2* and *p21* transcripts in cancerous vs. non-cancerous tissue in (A) non-smokers (B) intermediate smokers (40-70 packs/year) and (C) heavy smokers (140-200 packs/year) using the Rest statistical analytical tool. ▬, difference limit for the relative expression levels. MDM-2, mouse double minute 2 homolog.

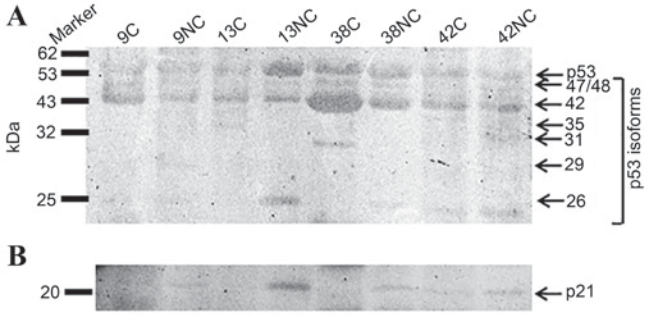

Figure 3. Western blot analysis of (A) p53 and (B) p21 proteins in C and NC specimens. The samples represent varying levels of Δ133p53 transcript expression: High (samples 13 and 42); low (sample 38) and intermediate (sample 9). Molecular weight markers are displayed on the left, whereas the predicted isoforms and corresponding molecular weights (kDa) are displayed on the right. Positive samples are indicated with arrows corresponding to their molecular weights in A. p53 was present at 53 kDa and its isoforms with variable molecular weights, and p21 is presented in B at 21 kDa. C, carcinoma; NC, adjacent normal lung specimens.

p53β and p53γ were detectable in almost all samples. The relative abundance of the smaller isoforms could not be correlated with the corresponding transcript abundance (Fig. 1). Thus, in samples 9 and 42, overexpressing the Δ133 transcripts, corresponding isoforms were not observed.

In the majority of cases, p21 protein was more abundant in adjacent normal lung tissue (Fig. 3B) compared with cancerous samples, and in accordance with the corresponding RT-qPCR results. Western blotting analysis did not provide conclusive evidence for MDM-2 protein isoforms (results not shown).

Discussion

Expression of full-length p53 mRNA, its isoforms and their principle co-regulators, apoptosis effector *MDM-2* and cell cycle regulator *p21*, serve a critical role in the process of lung carcinogenesis. In the current study, it was reported, to the best of our knowledge, for the first time, that in addition to the parallel *MDM-2* and *p53* expression variation in cancerous compared with adjacent non-cancerous lung tissue, Δ133p53 isoforms are significantly overexpressed in cancerous lung tissue. On the contrary, *p21* is significantly under-expressed in cancerous lung tissue samples. The observed inverse association of Δ133p53 isoforms and *p21* expression was not statistically significant.

Although the Δ133p53/p53 mRNA was most abundant in the cancerous samples, the Δ133p53 protein isoforms were not detectable in samples overexpressing the mRNA of these isoforms. By contrast, p21 protein expression was correlated with p21 mRNA expression, in the cancerous and non-cancerous lung tissues used in the present study. The differences observed by western blotting analysis demonstrated inconsistencies between mRNA and protein production, which are observed primarily in cancerous tissues and also verified in previous studies in other types of cancer (20,21). It is suggested that the overexpression of Δ133p53 isoforms may exert its effect through *p53* transcript interference. Transcript interference appears to be a common mechanism by which mRNAs lacking their 5' sequences act (22,23). It has been previously reported that Δ133p53 isoforms differentially modulate *p53* target gene expression to antagonize p53 apoptotic activity at the physiological level in zebra fish and that the knockdown of Δ133p53 enhances p53-mediated apoptosis under stress conditions (24). A recent study did however demonstrate that the Δ133p53 isoforms are also negatively involved in DNA damage repair and inhibition of cell senescence (25). The observations of the current study demonstrate that the Δ133p53 transcript overexpression may additionally have a negative effect on DNA damage repair in lung cancer where mutations are very frequent. This condition paralleled by the *p21* transcript reduction is suggested to strongly affect carcinogenesis in the lung.

Numerous studies have identified the impact of different isoforms on cancer biology (26-28). Alternatively spliced tissue factor (TF) isoform expression is differentially modulated on a post-transcriptional level via regulatory factors including serine/arginine-rich (SR) proteins, SR protein kinases and microRNAs. These isoforms participate in a variety of

physiological and pathophysiological functions, including thrombogenicity, angiogenesis, cell signalling, tumor cell proliferation and metastasis (26). Specifically, Goldin-Lang *et al* (27) observed that upregulation of human full length tissue factor and, particularly, an alternatively spliced human tissue factor in pulmonary adenomatosis, suggested raised risk of thrombosis and tumor progression, thereby indicating poor prognosis in these patients. In addition, in A549 human lung cancer cells, modulation of TF isoform expression, which is controlled by alternative splicing, modulates the pro-angiogenic potential of these cells under hypoxic conditions (28).

In conclusion, the present study verifies the potential value of Δ133p53 isoform(s) overexpression as a marker for lung malignancy, and contributes to our understanding of its role in cell cycle regulation. This information is vital for elucidating lung carcinogenesis and investigating its drug sensitivity.

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