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Alterations of p53 in tumorigenic human bronchial epithelial cells correlate with metastatic potential

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The cellular and molecular mechanisms of radiation-induced lung cancer are not known. In the present study, alterations of p53 in tumorigenic human papillomavirus-immortalized human bronchial epithelial (BEP2D) cells induced by a single low dose of either α-particles or 1 GeV/nucleon ^{56}Fe were analyzed by PCR–single-stranded conformation polymorphism (SSCP) coupled with sequencing analysis and immunoprecipitation assay. A total of nine primary and four secondary tumor cell lines, three of which were metastatic, together with the parental BEP2D and primary human bronchial epithelial (NHBE) cells were studied. The immunoprecipitation assay showed overexpression of mutant p53 proteins in all the tumor lines but not in NHBE and BEP2D cells. PCR–SSCP and sequencing analysis found band shifts and gene mutations in all four of the secondary tumors. A G→T transversion in codon 139 in exon 5 that replaced Lys with Asn was detected in two tumor lines. One mutation each, involving a G→T transversion in codon 215 in exon 6 (Ser→Ile) and a G→A transition in codon 373 in exon 8 (Arg→His), was identified in the remaining two secondary tumors. These results suggest that p53 alterations correlate with tumorigenesis in the BEP2D cell model and that mutations in the *p53* gene may be indicative of metastatic potential.

Introduction

The *p53* tumor suppressor gene has been shown to be inactivated either by gene mutations or loss of function of the gene product in a wide variety of human tumors and in as many as 50% of human lung cancers (1). p53 function can also be lost through interaction with several viral oncoproteins, including HPV-16, HPV-18, SV40 large T and adenovirus E1B. Binding of p53 to the HPV-E6 oncoprotein results in increased degradation of the p53 protein through a ubiquitin-mediated pathway (2), whereas the opposite is true for SV40 large T and E1B (3). An understanding of the functional role of p53 mutations in neoplastic transformation of human bronchial epithelial cells by various environmental carcinogens such as radon and cigarette smoke may provide new diagnostic and therapeutic approaches.

The mechanisms of human bronchial carcinogenesis induced by high LET radiation such as radon α-particles and heavy ions are not clear. The use of lung tumor tissues from exposed underground miners to identify consistent cellular and molecular alterations, as in the case of human colorectal cancers, is complicated by the fact that the majority of miners are also cigarette smokers. It would be ideal to use a human bronchial epithelial cell line that has been malignantly transformed by α-particles to assess the various changes leading to malignancies. *In vitro* oncogenic transformation studies using rodent cell systems have shown that high LET radiation is more efficient in transforming cells than X- or γ-rays at equivalent doses, with a relative biological effectiveness ranging from 2.2 to 10 (4,5). In contrast, human cells in culture have proven to be refractory to malignant transformation *in vitro*, due partly to their extremely low spontaneous immortalization incidence.

We have recently demonstrated that HPV-immortalized human bronchial epithelial BEP2D cells can be malignantly transformed by a single 30 cGy dose of high LET α-particles at a frequency estimated to be ~4×10^{-7} after successive cultivation for 3–4 months post-irradiation (6,7). Transformed cells progress through sequential stages characterized by altered growth kinetics and anchorage-independent growth before becoming tumorigenic and producing progressively growing subcutaneous tumors upon inoculation into athymic nude mice. While BEP2D cells express normal E6 oncoprotein which binds and increases the degradation of wild-type p53 protein (2), this does not preclude expression of mutated p53 proteins in tumorigenic BEP2D cells, as shown previously (7). There is evidence to indicate that cervical carcinoma cells that harbor HPV have a more aggressive phenotype if they also acquired a missense mutation in a p53 allele (8). In addition, clinical pathology studies have indicated that overexpression of p53 proteins and *p53* gene mutations frequently correlate with advanced tumor stage (reviewed in ref. 9). While there is a large database available on the correlation of p53 alterations with the clinicopathological progression of human tumors using immunohistochemical or PCR–single-stranded conformation polymorphism (SSCP) analysis, the prognostic value of altered p53 expression in lung cancer is still being debated. It is reasonable to assume that the use of a single technique may not be sufficiently accurate to estimate p53 abnormalities among samples. In the present studies, we used several independent techniques to determine the correlation between p53 mutations with tumor progression and metastatic potential in tumorigenic BEP2D cell lines induced by either α-particles, such as those emitted by radon progeny, or by heavy ions.

Materials and methods

Generation of tumor cell lines

A human papillomavirus-immortalized human bronchial epithelial cell line (BEP2D) was irradiated with either a single 30 or 60 cGy dose of high LET α-particles accelerated at the Radiological Research Accelerator Facility of Columbia University as described previously (6,10) or with a single 60 cGy dose of 1 GeV/nucleon ^{56}Fe particles accelerated at the Alternating Gradient Synchrotron of the Brookhaven National Laboratory (Upton, NY). Irradiated cells were subcultured for 12–15 passages before being inoculated s.c. into

Abbreviations: Rb, retinoblastoma; SSCP, single-stranded conformation polymorphism.

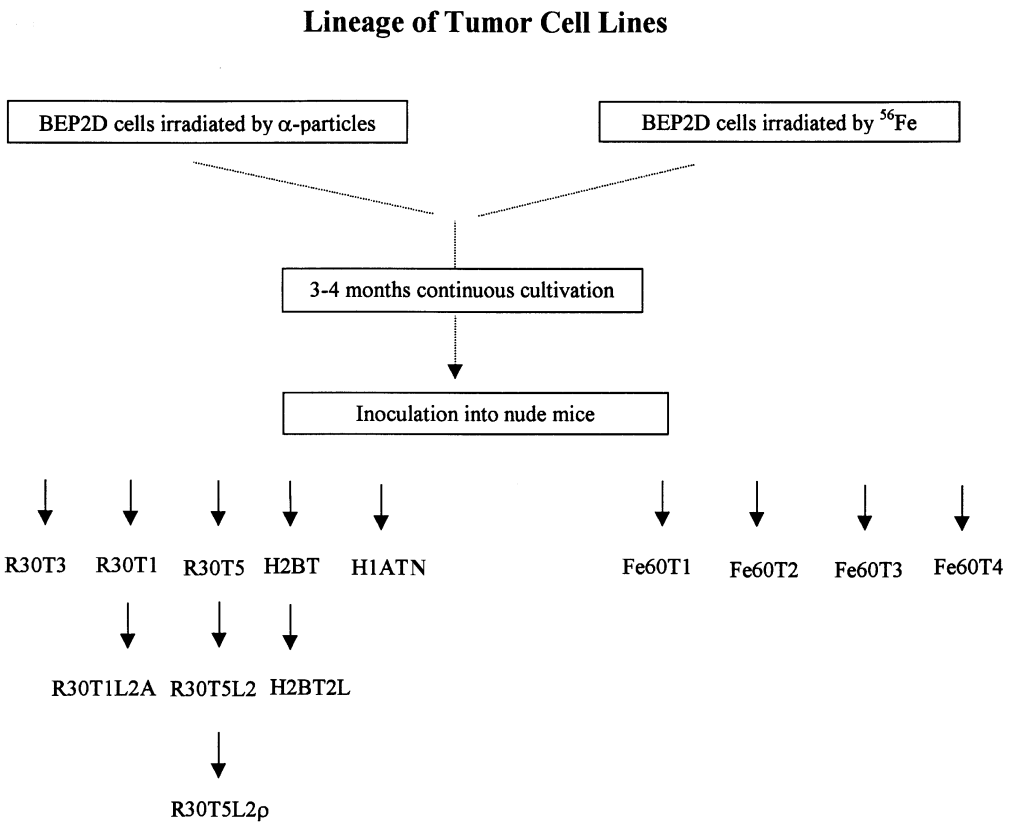

Fig. 1. Schematic diagram showing the lineage of the various tumor cells.

Table I. Tumorigenic BEP2D cell lines analyzed

Cell line	Irradiation (cGy)	Tumor in nude mice			
		P/S/T[a]	Latency period (weeks)	Size (cm)	Metastasis
	(α-particles)				
H1ATN	30	P	17	1.0	–
H2BT	60	P	13	1.5	–
R30T3	30	P	17	1.0	–
R30T1	30	P	12	2.0	–
R30T5	30	P	12	1.5	–
R30T5L2	30	S	5	2.0	–
H2BT2L	60	S	5	2.0	+
R30T1L2A	30	S	4	2.5	+
R30T5L2P	30	T	2	3.0	+
	(^{56}Fe ions)				
Fe60T1	60	P	14	1.2	–
Fe60T2	60	P	14	1.0	–
Fe60T3	60	P	14	1.0	–
Fe60T4	60	P	14	1.0	–
NHBE	–		>20	0	
BEP2D	–		>20	0	

[a]P/S/T, primary/secondary/tertiary tumor.

nude mice for tumorigenic assay as described (6,7,10). Tumors >1 cm in diameter were resected from nude mice and primary tumor cell lines were established using enzymatic treatment (7; Figure 1). Secondary tumor cell lines were established by injecting the primary tumor cells into nude mice. These tumors had a shorter latency period and a higher growth fraction as compared with the primary tumors. Metastasis was determined by injecting the secondary tumor cells into spleens of nude mice (11). A total of 13 tumor cell lines in addition to the control normal human bronchial epithelial (NHBE) cells (Clonetics, San Diego, CA) and the immortalized, non-tumorigenic BEP2D cells were analyzed (Table I).

PCR amplification

Genomic DNA was isolated from individual tumor cell lines by a simple salt-out procedure as described previously (12,13). Briefly, tumorigenic cells were lysed in 0.2% SDS containing 300 μg/ml proteinase K for 20–25 min at 65°C. The lysates were then treated with a 5 N salt solution and centrifuged at 1500 r.p.m. for 15 min to precipitate the protein. DNA was precipitated with absolute ethanol, rinsed with 70% ethanol, air dried and dissolved in 1× TE buffer. Exons 5–9 of the *p53* gene were amplified using specific intronic oligonucleotide primers as described previously (14). Exon 5: sense, 5′-TGACTTTCAACTCTGTCTCC; antisense, 5′-TCAGTGAGGAATCAG-

AGGCC. Exon 6: sense, 5′-CTGGAGAGACGACAGGGCTG; antisense, 5′-CCAGAGACCCCAGTTGCAAAC. Exon 7: sense, 5′-CTCGCGCACTG-GCCTCATCTT; antisense, 5′-TCAGCGGCAAGCAGAGGCTG. Exon 8: sense, 5′-GGACAGGTAGGACCTGATTTCCTTAC; antisense, 5′-TGCAC-CCTTGGTCTCCTCCAC. Exon 9: sense, 5′-GGTGGAGGAGACCAAGGG-TGCACTT; antisense, 5′-CTGGAAACTTTCCACTTGAT.

PCR reactions were performed in a 20 μl volume containing 200 ng of DNA template, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM dNTPs, 0.5 mM each primer. Amplifications were performed for 30 cycles using a DNA Thermal Cycler model 480 (Perkin Elmer, Foster City, CA). After a hot start at 95°C for 2 min with the addition of *Taq* polymerase (1.25 U; Perkin Elmer), each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 63°C for 1 min and extension at 72°C for 1 min. After the last cycle, an extension period of 2 min at 72°C was added. All PCR reactions were repeated two to three times to rule out potential contamination.

SSCP and sequence analysis

For non-radioactive SSCP analysis, the GenePhor System (Pharmacia Biotech, Piscataway, NJ) was used. Samples consisting of 2 ml of PCR products and 2 ml of denaturing solution were heated at 95°C for 5 min and thereafter placed immediately on ice to prevent re-annealing of the single-stranded product. Two milliliters of loading buffer were added to each sample before electrophoresis using the GeneGel Excel gel (Pharmacia Biotech) run at 10°C, 600 V for 90 min. The gel was stained using the DNA Sliver Staining Kit (Pharmacia Biotech). Alterations of bands relative to controls were analyzed. Sequencing of PCR fragments was completed by the DNA Sequencing Facility of Columbia University.

Immunoprecipitation analyses of p53 proteins

Expression of mutated p53 proteins was detected by immunoprecipitation using mouse monoclonal antibody PAb 240 (Oncogene Science, Uniondale, NY) as described (7). Exponentially growing control and tumor cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), centrifuged and protein concentration was determined (15). A total of 300 μg of protein for each sample was pre-absorbed with protein A/G+–agarose and rabbit polyclonal anti-p53 antibody FL-393, which reacts with both wild-type and mutant p53 proteins (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h. The immunocomplexes formed were then dissolved and separated by 10% SDS–PAGE and transferred to PVDP membrane (Millipore Corp., Bedford, MA) as described (7). Mutant p53 proteins were detected by primary antibody PAb 240 and alkaline phosphatase-conjugated secondary antibody using the ProtoBlot II AP system with stabilized nitroblue tetrazolium/bromochloroindolyl phosphate (Promega, Madison, WI) as substrate.

Results

Non-radioactive PCR–SSCP analysis was used to screen DNA samples from tumorigenic BEP2D cells for changes in the *p53* gene. All of these tumor cells, with the exception of R30T5L2 and R30T5L2P, which originated from the same parental culture, were derived from different populations of irradiated BEP2D cells. Figure 2 shows the migration pattern of the amplified fragments for exons 5, 6 and 8 in normal human bronchial epithelial cells, parental BEP2D cells and their tumorigenic variants. Alterations of bands were found in four of four secondary and one of nine primary tumor cell lines examined (Table II). No band shift was found in exons 7 and 9.

Tumor samples that were positive for *p53* mutations based on PCR–SSCP analyses were further examined by direct sequencing. DNA sequence analysis showed that the secondary tumor line R30T5L2 contained a G→T transversion in codon 139 in exon 5 resulting in the substitution of Lys by Asn (Table II). The tertiary tumor cells, R30T5L2P, derived from injecting R30T5L2 into nude mice, showed an additional mutation in exon 6 with a G→A transition. However, this was a sequence polymorphism and resulted in no amino acid change. In contrast, R30T1L2A, a secondary tumor cell line, showed a G→T transversion in codon 215 in exon 6 resulting in the substitution of Ser by Ile. Mutation in the *p53* gene was also confirmed for the secondary tumor line H2BT2L, which

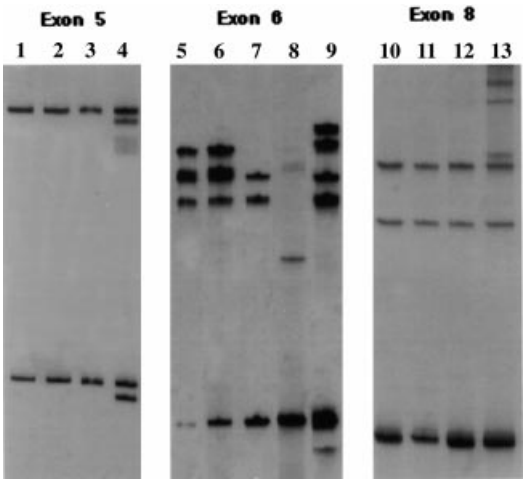

Fig. 2. PCR–SSCP analysis of exons 5, 6 and 8 of the *p53* gene. Individual exons were amplified using intronic primers to yield fragments with nucleotide lengths of 290, 184 and 237, respectively. Lanes 1, 5 and 10 are amplified fragments from primary NHBE cells; lanes 2, 6 and 11 represent immortalized BEP2D cells; lanes 3 and 12 are R30T3; lanes 4 and 7 are R30T5L2P; lane 8 is R30T1L2A; lane 9 is Fe60T1; lane 13 is H2BT2L.

Table II. Sequencing analysis of *p53* mutations

Cell line	Tumor type[a]	Exon	Codon	Change in amino acid
R30T5L2	S	5	139	Lys→Asn
R30T5L2P	M	5	139	Lys→Asn
		6	192	No change
R30T1L2A	M	6	215	Ser→Ile
H2BT2L	M	8	273	Arg→His
Fe60T1	P	No mutation found		

[a]P/S/M, primary/secondary/metastatic tumor.

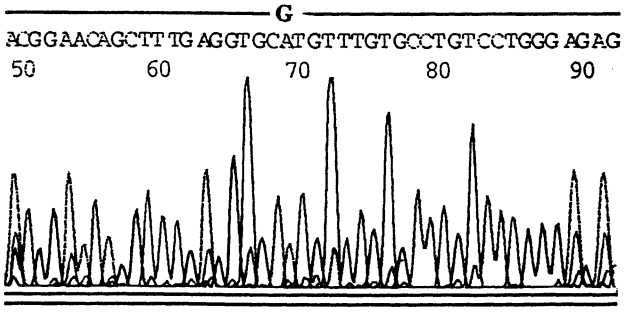

Fig. 3. Sequence analysis of exon 8 (fragment size 237 nt) of the *p53* gene in the metastatic tumor line H2BT2L resulting in the substitution of Arg by His as a result of a G→A transition.

showed a G→A transition in codon 273 in exon 8 resulting in the substitution of Arg by His (Figure 3). However, DNA sequence analysis showed no mutational change in the *p53* gene of the primary tumor cell line F60T1, which had a detectable band shift in the SSCP analysis (Table II). This anomaly was, therefore, regarded as a false positive. All four cell lines with *p53* mutations by sequence analysis were secondary tumors induced by α-particles. All except R30T5L2 were metastatic as well. These tumors were bigger (>2 cm) and had a shorter latency period compared with the primary tumors. The tertiary tumor cell line, R30T5L2P, had a latency period of only 2 weeks (Table I).

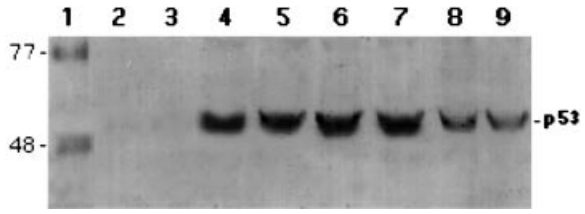

Fig. 4. Immunoprecipitation followed by western blot analysis of mutant p53 protein expression in primary NHBE cells (lane 2), BEP2D cells (lane 3), metastatic tumor R30T1L2A (lane 4), secondary tumor R30T5L2 (lane 5), metastatic tumor R30T5L2P (lane 6), metastatic tumor H2BT2L (lane 7) and primary tumors R30T1 (lane 8) and H2BT (lane 9). Lane 1 is a size marker.

Expression of mutated p53 proteins in normal human bronchial epithelial cells and parental and tumorigenic BEP2D cells was assessed by immunoprecipitation assay. The antibody PAb 240 detects only mutant p53 under conditions for immunoprecipitation. As shown in Figure 4, neither NHBE nor parental BEP2D cells showed any significant expression of mutant p53. In contrast, all tumor cell lines, including those primary tumor cells which failed to show any mutations in the *p53* gene detected by PCR–SSCP (R30T1 and H2BT, lanes 8 and 9), showed overexpression of mutant p53 protein. No wild-type p53 protein was detected in either NHBE, parental BEP2D cells or any of the tumorigenic cell lines examined (data not shown).

Discussion

In the present study, immunoprecipitation, PCR–SSCP and direct sequencing were used to examine the status of the *p53* gene in tumorigenic BEP2D cells malignantly transformed by either α-particles, such as those emitted by radon progeny, or by heavy ions. The BEP2D cells were initiated by lipofectin transfection of cloned full-length HPV-18 into normal bronchial epithelial cells obtained as an outgrowth of a bronchial explant (16). BEP2D cells are near diploid and are relatively stable genotypically over many passages (16,17). Furthermore, they are anchorage dependent and non-tumorigenic in nude mice (6,7,18). BEP2D cells express normal E6 oncoprotein which binds and increases the degradation of wild-type p53 protein (2). As a result, wild-type p53 protein is usually present at a non-detectable level when assayed using an immunoprecipitation assay (7). However, this does not preclude expression of mutated p53 proteins in some human cancers harboring HPV (8,19,20) and in tumorigenic BEP2D cells, as shown in this report. Our finding that mutations in the *p53* gene occurred only in secondary tumors, of which three out of four were also metastatic, is consistent with previous reports that HPV-positive cervical carcinomas are likely to be more aggressive if they contain *p53* mutations (8,21). More importantly, our findings suggest that the presence of the HPV gene in BEP2D cells may not decrease the likelihood of *p53* mutations contributing to the carcinogenic mechanisms essential in radon-induced malignant conversion of the cells. It should be noted that BEP2D cells also express E7 oncoprotein, which binds preferentially to under-phosphorylated retinoblastoma (Rb) protein and inactivates the growth regulatory function of normal Rb (22). The finding that HPV-immortalized BEP2D cells are non-tumorigenic even in late passages (6,7,18) suggests that abnormal p53 and Rb functions are not sufficient criteria for tumorigenic development and that additional genetic changes are needed. The observation that most HPV-negative human cervical cancers do not contain *p53* mutations suggests that these tumors must have achieved their malignant phenotype by a different pathway, including genetic alterations other than p53 inactivation. It is possible that the inactivation of p53 by E6 protein only leads to the loss of functional p53, whereas mutations in the *p53* gene result in positive transforming activity. Our results also support the notion that mutant *p53* may act as an oncogene in HPV-immortalized human cells.

The human *p53* gene is 20 kb in size and contains 11 exons. The majority of *p53* mutations in human cancers occur in the highly conserved regions, primarily in exons 5–8 (codon 126–306), which cover >90% of the reported *p53* mutations (9). Most of the cancer-related mutations of the *p53* gene are clustered in four hotspot areas in codons 175, 248, 273 and 281, of which mutations in codon 273 are the second most frequent among human cancers. Analysis of the *p53* mutation spectrum has indicated an etiological correlation with certain tumor types, as in the case of a G→T transversion in codon 248 in aflatoxin B1-associated hepatocellular carcinoma (1). Likewise, a missense mutation in codon 249 of the *p53* gene has been found to be a hotspot in lung cancers from radon-exposed uranium miners (23). However, the results have not been confirmed by others (24,25) and may reflect the significantly higher radon dose experienced by the miners in the former case. None of the four tumor samples found to be positive for *p53* mutations based on direct DNA sequencing in the present study contained mutations in codon 249. Although two out of four tumor cell lines examined contained a mutation in codon 139 in exon 5, our data indicate no clear correlation between sequence-specific mutations of the *p53* gene and metastatic phenotype.

It is of interest to note that while our immunoprecipitation data indicate that all tumor cell lines generated overexpressed mutant p53 oncoproteins, PCR–SSCP and direct DNA sequencing analyses only detected mutations of the gene among the secondary and tertiary tumor cells examined. The discrepancy in results could, perhaps, be explained by the fact that the later assays involved only exons 5–9 of the *p53* gene. It is conceivable that some of the primary tumors could have acquired mutations in other exons of the *p53* gene. However, the possibility that all nine primary tumors contain mutations in such regions is highly unlikely. It has been shown that radiation, particularly high LET radiation such as α-particles, is an efficient inducer of multilocus deletions and complex mutations in both human and rodent cells and at various gene loci examined (13,26,27). It is possible that mutations occurring outside the coding regions of the *p53* gene in some of these tumor samples might have occurred and not been detected.

The immunoprecipitation assay clearly depends on the specificity and quality of the antibody used. PAb240 is specific for mutant p53 under non-denaturing conditions, whereas it binds to both mutant and wild-type p53 under denaturing conditions. However, experimental conditions are unlikely to be a factor of concern in our study since the BEP2D cells express little wild-type p53 due to the presence of E6 oncoprotein. This is consistent with the negative immunohistochemical staining for wild-type p53 protein among the parental BEP2D and all the tumorigenic cells examined.

There is evidence that the oncogenic activities of p53 depend on mutations in its coding sequence and that different mutations of the gene exert different effects on tumor development (28,29). Mutations that are recessive can result in the expression of an inactive protein whereas dominant mutations often lead

to loss of function in which mutant p53 overcomes the suppressor activity of the wild-type protein by modifying its conformation and stability. The actual number of p53 aberrations can be underestimated by immunohistochemistry, since tumors with nonsense or frameshift mutations that result in the production of unstable, truncated proteins are often negative by immunostaining (30). The PCR–SSCP technique is a quick and simple way to detect structural DNA aberrations, including point mutations. However, as demonstrated in our study with the primary tumor Fe60T1, SSCP analysis does not achieve a 100% detection level. Consequently, direct sequencing of the *p53* gene is the only sure way to assess the mutational event that inactivates this gene. In this regard, our data suggest that mutation in the *p53* gene is likely to be a late event in the neoplastic transformation process of human bronchial epithelial cells by radon.

Acknowledgements

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