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ORIGINAL ARTICLE

p53 and *c-myc* alterations in patients with non-small cell lung carcinoma in Serbia

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Summary

Purpose: *To determine the incidence of c-myc amplification and p53 mutations in patients with stage I, II and III of histologically confirmed non-small cell lung cancer (NSCLC) of different histological subtypes: adenocarcinomas (AC), squamous cell carcinomas (SCC), large cell carcinomas (LCC) and adeno-squamous carcinomas (AC-SCC) and of histological grade (G) 2 and 3.*

Materials and methods: *DNA was isolated from 41 frozen tumor samples by standard phenol-chloroform extraction. Amplification of c-myc gene was determined by the differential polymerase chain reaction (PCR) method, followed by polyacrylamide gel electrophoresis, and mutations in exons 5, 6, 7 and 8 p53 gene were detected by the PCR single-strand conformation polymorphism (SSCP) method.*

Results: *c-myc gene amplification was found in 2 of 41 (4.9%) analyzed tumors and both amplifications were found in stage III tumors. Mutations in the p53 gene were found in 19 of 41 (46.3%) of the analyzed tumors. SCC and LCC were more likely to contain mutations in p53 gene (68.4% and 66.7%, respectively).*

Conclusion: *Our results indicate that p53 mutations are common in NSCLC with higher incidence in SCC compared with other histological subtypes. Since the mutations are more frequent in early-stage NSCLC, it appears that mutations in p53 gene could be an early event in lung carcinogenesis. On the other hand, c-myc amplification is a rare event in NSCLC and occurs in the late phase of development of this type of lung cancer.*

Key words: *c-myc* gene, non-small cell lung carcinoma, *p53* gene, PCR-SSCP analysis

Introduction

Lung cancer is a major cause of cancer deaths in the world, with over 1,000,000 cases diagnosed every year [1]. NSCLC represents 75-80% of all lung cancer cases, and, annually, only in the United States are diagnosed approximately 170,000 new NSCLC patients [2]. Among them, only 30% have local disease at the time of diagnosis, whereas others have disease spread to lymph nodes and/or with distant metastases. Prognosis of the patients is largely dependent on the stage of the disease, and even in patients with stage I NSCLC, the 5-year survival rate is 65-75%, and this rate has not been changed significantly over the past 20 years [3]. Considering all these, it is quite clear how important is identifying a marker for high-risk early-stage patients. Recently, a number of molecular markers have been identified, which are implicated in the causation of cancer. These markers, including overexpression of certain oncogenes or inactivation of tumor suppressor genes, have been found in significant numbers of patients with lung cancer and are therefore logical candidates as prognostic or predictive factors.

One of the oncogenes, which are commonly activated in lung cancer, is the *c-myc* gene. The protein product of this gene forms heterodimers, which act as transcriptional factors of a number of genes significant for the cell proliferation and differentia-

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tion. Activation of *c-myc* gene occurs by the mechanism of gene amplification or by transcriptional dysregulation, both of which lead to protein overexpression. In lung cancer, *c-myc* gene is activated only by gene amplification and it appears that *c-myc* amplification occurs more frequently in small-cell lung cancer (SCLC) (18-31%) than in NSCLC (8-20%) [4].

The *p53* gene is a tumor suppressor gene encoding for a nuclear phosphoprotein, a transcription factor involved in the regulation of the cell cycle and apoptosis. This gene is mutated frequently in human cancers and there is some data indicating that more than 50% of lung cancers carry such a mutation. *p53* mutations also have been found in premalignant lesions in lung tissue indicating an early loss of this tumor suppressor's function in lung carcinogenesis [5].

Therefore, the objectives of our study were to detect amplification of c-*myc* gene and to determine the frequency of *p53* point mutations in samples of NSCLC from patients in Serbia. We correlated the incidence of gene alterations with prognostic features like stage of disease, together with histological subtype and grade of tumors as indices further describing prognosis of NSCLC patients.

Table 1. Patient characteristics

	No.	*%*
Patients	41	
men	34	82.9
women	7	17.1
Age, years		
median	58	
range	43 - 69	
Smoking history		
smokers	29	70.1
ex-smokers	12	29.3
Histology		
SCC	19	46.3
AC	17	41.5
LCC	3	7.3
AC-SCC	2	4.9
TNM stage		
I	20	48.8
II	4	9.8
III	17	41.5
Grade		
G2	19	46.3
G3	22	53.7

For abbreviations see text

Materials and methods

Patients and tissues

Primary lung tumors obtained from 41 patients with NSCLC were analyzed. All patients had undergone curative operations at the Military Medical Academy, Beograd, from October 2000 to December 2002. Among them, there were 34 men and 7 women aged 43-69 years (median 58 years) at the time of diagnosis. All patients were smokers or ex-smokers. Patients were classified in stage I (n=20), stage II (n=4) and stage III (n=17) of disease according to the TNM classification system. Tumors were histologically classified as SCC (19; 46.3%), AC (17; 41.5%), LCC (3; 7.3%) and AC-SCC (2; 4.9%), according to the "Histological Typing of Lung Tumors" by the World Health Organization [6]. Nineteen (46.3%) tumors were G2 and 22 (53.7%) G3 (Table 1).

Tumors were frozen in liquid nitrogen and stored at –80^0 C until DNA extraction. Genomic DNA was extracted by digestion with proteinase K and phenol extraction-ethanol precipitation method [7]. The concentration of DNA was determined by spectrophotometric method and the quality of the DNA was checked using 1% agarose electrophoresis. The DNA solutions were stored at 4^0 C.

Analysis of c-myc amplification

To determine whether *c-myc* gene was amplified in the tumor specimens, we used the differential PCR method. This semi-quantitative method is based on a simultaneous amplification of two different fragments of the DNA (by using two different sets of primers) in the same PCR reaction. The reaction conditions were as follows: 95^0 C, 2 min; (95^0 C, 1 min; 50^0 C, 1 min; 72^0, 1 min) ×25 cycles; 72^0 C, 10 mins. Fragments of the *c-myc* gene were amplified together with fragments of the D_2R gene, which is present in one copy per cell. Sequences of all primers were as follows:

c-myc, sense: 5' - GCT CCA AGA CGT TGT GTG TTC G - 3'

c-myc, antisense: 5' - GGA AGG ACT ATC CTG CTG CCA A - 3'

D_2R, sense: 5' - CCA CTG AAT CTG TCC TGG TAT G - 3'

D_2R, antisense: 5' - GTG TGG CAT AGT AGT TGT AGT GG - 3'

The PCR products were separated by electrophoresis on 8% polyacrylamide gel, which was then stained with silver nitrate. The intensity of bands was determined by densitometer (LKB Ultro Scan XL Laser Densitometer, Sweden).

PCR - SSCP analysis of p53 gene

p53 mutations in exons 5 to 8 were evaluated by SSCP analysis. Genomic DNA (500 ng) was amplified in a 50 µl reaction mixture, containing 0.2 mM deoxynucleotide triphosphate mix (Pharmacia Biotech, Sweden), 0.6 mM of sense and antisense primers and 1.5 units of Taq polymerase (Pharmacia, Upsala, Sweden), with buffer containing 2 mM $MgCl_2$. One negative control reaction with DNA isolated from the blood of a healthy person and one control reaction without a template were included. Four portions of genomic DNA fragments covering the region between exon 5 and exon 8 *p53* gene were amplified by PCR using *p53*-specific set of primers [8], under the following conditions: 95° C, 5 min; (95° C, 60 sec; 60° C, 60 sec) ×35 cycles.

After amplification, the PCR products were analyzed by the SSCP method. Samples were denatured by heating at 95° C for 5 min and electrophoresed (LKB, Pharmacia, Sweden) at 4° C on an 8% and 10% non-denaturing polyacrylamide gel in 0.5×TBE buffer (45 mM Tris-borate, 1 mM EDTA). After electrophoresis was completed, gels were stained with silver nitrate (Serva Heidelberg, Germany).

Statistical analysis

The statistical significance of the association between histological subtype, grade and stage and the gene alterations found was determined by the χ^2-test.

Results

We amplified the genomic DNA and evaluated the c-*myc* and *p53* abnormalities in all 41 tumors. The obtained results are summarized in Table 2.

Status of the c-myc gene

Analysis of the c-*myc* gene amplification was determined by differential PCR, followed by PAGE (Figure 1). Amplification of the *c-myc* gene was found in 2 of 41 (4.9%) analyzed carcinomas. One of them had stage III, G2 SCC and the other one had stage III, G3 AC. *c-myc* amplification was found in 1 of 19 (5.3%) analyzed SCC and in 1 of 17 (5.9%) AC; the difference in the incidence of *c-myc* amplification between these histological subtypes was not significant. Both amplifications were found in stage III carcinomas (2 of 17).

Table 2. Association of *p53* and *c-myc* status with patient's characteristics

	Patients *n*	*p53 mutations* *n (%)*	*c-myc amplification* *n (%)*
Patients	41	19 (46)	2 (4.9)
men	34	18 (53)	2 (5.9)
women	7	1 (14)	–
Age, years, median (range)	58 (43-69)		
Smoking history			
smokers	29	10 (34)	1 (3.5)
ex-smokers	12	9 (75)	1 (8.3)
Histology			
SCC	19	13 (68)	1 (5.3)
AC	17	4 (24)	1 (5.9)
LCC	3	2 (67)	–
AC-SCC	2	–	–
TNM stage			
I	20	12 (60)	–
II	4	2 (50)	–
III	17	5 (29)	2 (4.9)
Grade			
G2	19	7 (37)	1 (5.3)
G3	22	12 (55)	1 (4.6)

For abbreviations see text

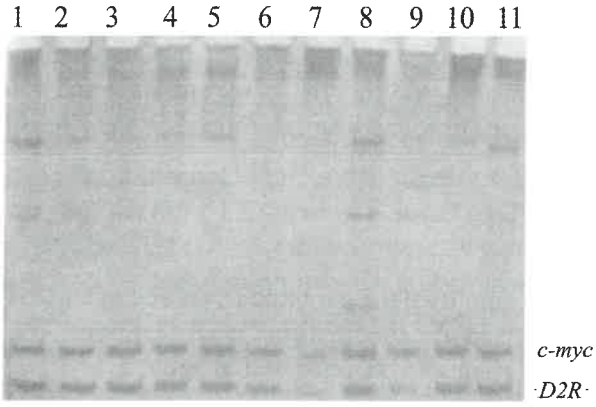


Figure 1. Polyacrylamide gel electrophoresis of *c-myc* gene. Lane 9: amplified *c-myc* gene.

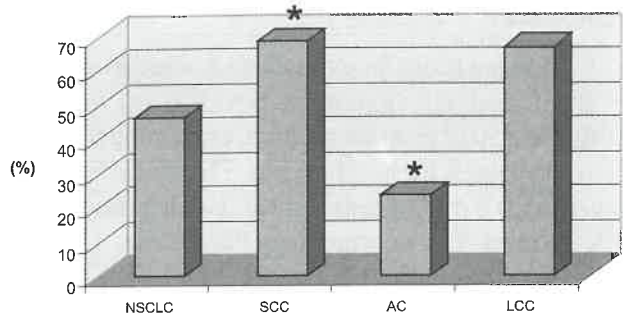


Figure 3. The incidence of *p53* gene mutations in different histological subtypes of NSCLC. *indicates statistically significant difference of mutation incidence found in SCC and AC ($p < 0.01$).

Status of the p53 gene

Forty-one samples of NSCLC were examined for mutations in exons 5-8 of the *p53* gene by the PCR-SSCP method (Figure 2). *p53* mutations were detected in 19 out of 41 samples (46.3%). Two separate mutations were detected in one sample giving a total of 20 mutations in 19 cancers. Mutations were found in 13 of 19 (68.4%) SCC, 4 of 17 (23.6%) AC, and 2 of 3 (66.7%) LCC (Figure 3). No *p53* gene mutation was found in AC-SCC. The difference between mutations in SCC and AC was statistically significant ($\chi^2=7.3$, $p < 0.01$). LCC were not included in the statistical analysis because of the small number of samples. Mutations in *p53* gene were found in 12 of 20 (60%) stage I carcinomas, 2 of 4 (50%) stage II carcinomas and 5 of 17 (29.4%) stage III carcinomas (Figure 4). The difference between mutations in stage I, II and III carcinomas was not statistically significant. The incidence of mutations found in cancers of different histological grade also varied, yet not reaching statistical significance. In G2 tumors the incidence of *p53* mutations was 36.9% (7/19) and in G3 tumors it was 54.6% (12/22) (Figure 5).

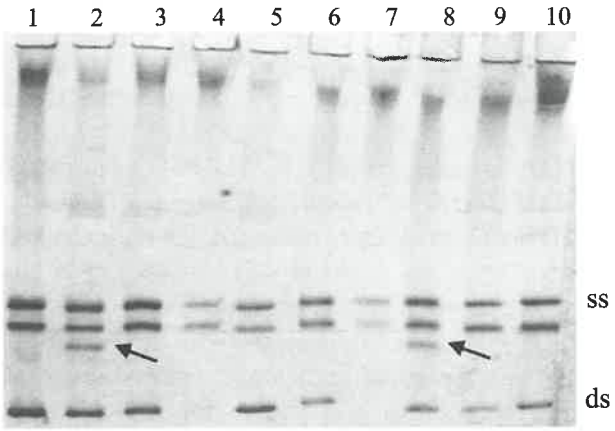


Figure 2. PCR-SSCP analysis of exon 7 of *p53* gene using 10% polyacrylamide gel stained with silver nitrate. Lanes 2 and 8: mutant *p53* genes from NSCLC (arrows); lane 10: wild-type *p53* gene.

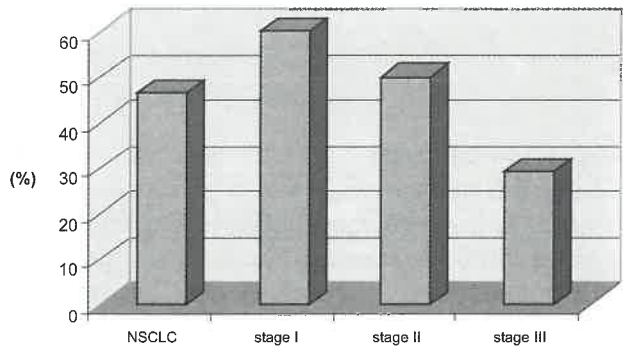


Figure 4. The incidence of *p53* gene mutations in different stages of NSCLC. No statistically significant difference was found.

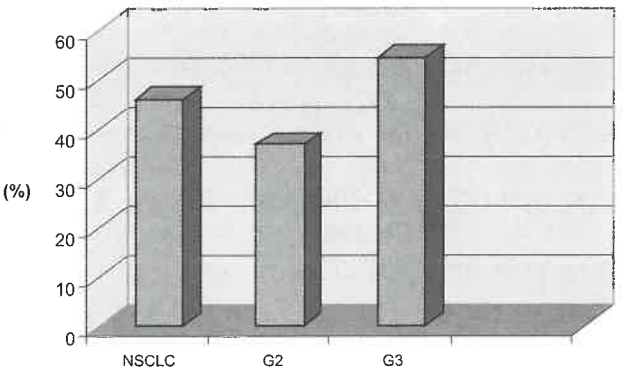


Figure 5. The incidence of *p53* gene mutations in different grades of NSCLC. No statistically significant difference was found.

Discussion

Lung cancer is the end stage of the multistep process of carcinogenesis, which is driven by genetic and epigenetic damages of the DNA. At least 3 classes of cellular genes are involved in this process: proto-oncogenes, tumor suppressor genes and DNA repair genes, and oncogenic activation occurs via point mutation, gene amplification or rearrangement.

It has been published that many cancers are characterized by alterations that ultimately lead to constitutive overexpression of the *myc* oncogene [9]. However, its role in the genesis and development of NSCLC has not yet been established. It has been estimated that the *c-myc* gene is activated via gene amplification mechanism in lung cancer and that it is more frequently activated in SCLC than in NSCLC [10]. Moreover, it seems that the *c-myc* gene is amplified in cell lines derived from NSCLC, which are mostly derived from metastatic lesions rather than from primary carcinomas. Considering all these facts, it could be concluded that amplification of the *c-myc* gene is a rare event in the genesis of NSCLC, which is consistent with our results. We found amplification of this gene in 4.9% (2 of 41) analyzed carcinomas and both amplifications were found in stage III disease, suggesting a rare event in the later phase of the development of NSCLC.

p53 tumor suppressor gene is one of the most mutated genes in human cancers. The first mutations in this gene were found in 1989 in colorectal cancer and cell lines derived from lung cancer. Further investigations showed that mutations in the *p53* gene appear to play a significant role in the genesis and development of different types of cancer. There is growing evidence that over 50% of all human cancers have one *p53* allele deleted, while the remaining one is inactivated by mutation. Besides that, inactivation of the *p53* pathway occurs in most tumors and even in cancer types in which *p53* mutations are rare: *p53* function is indirectly abolished either by nuclear exclusion (neuroblastoma), interaction with a viral protein (cervical cancer) or interaction with overexpressed MDM2 protein (sarcoma) [11].

Mutations in the *p53* gene are detected in 31-55% of NSCLC [12] and over 85% of them are point mutations localized within exons 5-8. That is the main reason why the majority of researchers focused to analyze those 4 exons of the *p53* gene.

We observed a 46.3% incidence of the *p53* mutations by SSCP, which is similar to what has been reported in other studies. Hashimoto et al. and Tomizawa et al. reported an incidence of 45.1% [13] and 47.6% [3], respectively, while Skaug et al. found an even higher incidence of 54% [14]. The lower mutation frequency was reported by Huang et al. (36.8%) [12] and Vega et al. (21%) [15]. Differences between our results and previously published data are a consequence of a different number of analyzed tumor samples, as well as their different histological subtypes, TNM stages and grades. Apart from that, there is a difference in the methods used for mutation detection and spectrum of examined exons of *p53* gene.

The most frequent mutations were found in exon 5 (10 mutations) and in exon 7 (8 mutations), while only 2 mutations were detected in exon 6. In exon 8, mutations in the *p53* gene were not detected. Published data confirm that mutations in exon 5 are the most frequent mutations of *p53* gene found in NSCLC. These data also show a lower incidence of mutations in exons 7 and 8, and the lowest incidence in exon 6 [11]. The existing difference between our results and those of other investigators could be explained by the fact that certain carcinogenic agents induce particular mutations in different genes and different exons [16]. Considering the fact that the mutation spectrum reflects exposure to particular environmental carcinogenic agents, differences among geographic areas and environmental factors induce a different mutation spectrum. Besides that, there is a genetic predisposition of particular populations which, among others, is reflected by the function of metabolic and detoxification mechanisms, as well as mechanisms for DNA repair.

p53 gene mutations are among the most frequent genetic events in lung cancer with different prevalences, depending on the tumor histology. Our results agree with the reported percentages of the *p53* mutations in different histological subtypes of NSCLC. We found a 26.3% (4 of 17) incidence of *p53* mutations in AC, and significantly higher incidence in LCC (2 of 3; 66.7%) and in SCC (13 of 19; 68.4%). This is similar to published data, which vary from 26.2% [12] to 44% [14] in AC, from 57% [14] to 81% [12] in LCC and from 47.9% [12] to 67% [14] in SCC.

Mutations in the *p53* gene were found in carcinomas of all stages and the highest incidence (60%) was found in TNM stage I carcinomas. This could be explained by differences in the number of analyzed cancers of different stages. However, since *p53* gene was not mutated in all NSCLC, other mechanisms of genesis of this type of cancer, independent of mutational inactivation *p53* gene, must be functioning. It is possible that these mechanisms are more frequent in stage III carcinomas, as well as it is possible that this type of carcinoma gives clinical symptoms in later phases of the disease. One of the many ways of inactivation of structurally normal *p53* is by binding to E6 protein of

human papillomavirus (HPV) types 16 and 18 that induce its degradation through the ubiquitin pathway [17]. In the literature, the prevalence of HPV infection in SCC of the lung varies from 0 to 79% [18,19]. The high prevalence of HPV infection in lung carcinomas in countries such as Japan, China, Greece, and Finland reflects the possibility that the geographic variability of HPV infection is associated with these lesions [20].

For the determination of the prognostic significance of *p53* mutations in NSCLC further investigations are necessary. Nevertheless, the status of this gene could be a predictive factor, especially for patients with inoperable carcinomas and candidates for additional cytotoxic therapy, which acts through induction of apoptotic answer including the *p53* gene.

Previously published data indicate that *myc* activation strongly selects for spontaneous inactivation of the *ARF-MDM2-p53* pathway *in vivo*, cancelling its protective checkpoint function and accelerating progression to malignancy [9]. Besides that, *in vitro* studies conducted on human colon carcinoma cells showed that sensitivity to 5-fluoruracil (5FU) was dependent on both, elevated *c-myc* expression and transactivation activity of p53 protein. Moreover, these data predicted that only patients with tumors showing amplified *c-myc* and wild-type *p53* would benefit from 5FU-based adjuvant therapy, which confirmed the results obtained by analyzing the status of these two genes in patients included in a multi-institutional phase III clinical trial of adjuvant therapy for stage II and III colon cancer [21]. It would be interesting to investigate the cooperative effect of *c-myc* and *p53* genes and their influence on the response to adjuvant therapy of patients with NSCLC.

References

1. Toyooka S, Maruyama R, Toyooka K et al. Smoke exposure, histologic type and geography-related differences in the methylation profiles of non-small cell lung cancer. Int J Cancer 2003; 103: 153-160.
2. Grondin SC, Liptay MJ. Current concepts in the staging of non-small cell lung cancer. Surg Oncol 2002; 11: 181-190.
3. Tomizawa Y, Kohno T, Fujita T et al. Correlation between the status of the p53 gene and survival in patients with stage I non-small cell lung carcinoma. Oncogene 1999; 18: 1007-1014.
4. Fong KM, Sekido Y, Minna JD. Molecular pathogenesis of lung cancer. J Thorac Cardiovasc Surg 1999; 118: 1136-1152.
5. Vahakangas KH, Bennett WP, Castren K et al. p53 and K-ras mutations in lung cancers from former and never-smoking women. Cancer Res 2001; 61: 4350-4356.
6. Anonymous. The World Health Organization Histological Typing of Lung Tumours (2nd edn). Am J Clin Pathol 1982; 77: 123-136.
7. Sambrook J, Fritsch EF, Maniatis T. Preparation of organic reagents. In:Nolan C (ed). Molecular Cloning; a Laboratory Manual (2nd edn). Cold Spring Harbor Laboratory Press, New York, 1989: B4-B5.
8. Sakai E, Rikimaru K, Ueda M et al. The p53 tumor-suppressor gene and ras oncogene mutations in oral squamous-cell carcinoma. Int J Cancer 1992; 52: 867-872.
9. Eischen CM, Weber JD, Roussel MF, Sherr CJ, Cleveland JL. Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. Genes Development 1999; 13: 2658-2669.
10. Richardson GE, Johnson BE. The biology of lung cancer. Semin Oncol 1993; 20: 105-127.
11. Soussi T, Beroud C. Assesing TP53 status in human tumours to evaluate clinical outcome. Nature Rev 2001; 1:1-8.
12. Huang C, Taki T, Adachi M, Konishi T, Higashyama M, Miyake M. Mutations in exon 7 and 8 of p53 as poor prognostic factors in patients with non-small cell lung cancer. Oncogene 1998; 16: 2469-2477.
13. Hashimoto T, Tokuchi Y, Hayashi M et al. p53 null mutations undetected by immunohistochemical staining predict a poor outcome with early-stage non-small cell lung carcinomas. Cancer Res 1999; 59: 5572-5577.
14. Skaug V, Ryberg D, Kure EH et al. p53 mutations in defined structural and functional domains are related to poor clinical outcome in non-small cell lung cancer patients. Clin Cancer Res 2000; 6: 1031-1037.
15. Vega FJ, Iniesta P, Caldes T et al. p53 exon 5 mutations as a prognostic indicator of shortened survival in non-small-cell lung cancer. Br J Cancer 1997; 76: 44-51.
16. Bennett WP, Hussain SP, Vahakangas KH, Khan MA, Shields PG, Harris CC. Molecular epidemiology of human cancer risk: gene-environment interactions and *p53* mutation spectrum in human lung cancer. J Pathol 1999; 187: 8-18.
17. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 1990; 63: 1129-1136.
18. Gorgoulis VG, Zacharatos P, Kotsinas A et al. Human papillomavirus (HPV) is possibly involved in laryngeal but not in lung carcenogenesis. Hum Pathol 1999; 30: 274-283.
19. Clavel CE, Nawrocki B, Bosseaux B et al. Detection of human papillomavirus DNA in bronchopulmonary carcinomas by hybrid capture II: a study of 185 tumors. Cancer 2000; 88: 1347-1352.
20. Cheng YW, Chiou HL, Sheu GT et al. The association of human papillomavirus 16/18 infection with lung cancer among nonsmoking Taiwanese women. Cancer Res 2001; 61: 2799-2803.
21. Arango D, Corner GA, Wadler S, Catalano PJ, Augenlicht LH. c-myc/p53 interaction determines sensitivity of human colon carcinoma cells to 5-fluorouracil *in vitro* and *in vivo*. Cancer Res 2001; 61: 4910-4915.