Immunohistochemical evaluation of P21^{ras} and P53 proteins expression in human non-small-cell lung cancers*

B. Przybojewska[1], K. Rydzynski[1], M. Stepnik[1], M. Jakubiak[2], J. Kozak[3], W. Szymczak[1]

[1]Department of Toxicology and Carcinogenesis, The Nofer Institute of Occupational Medicine, 90-950 Lodz, Poland, e-mail: mstep@imp.lodz.pl; [2]Department of Pathological Anatomy, and [3]Department of Thorax Surgery, M. Copernicus Provincial Specialistic Hospital, 93-513 Lodz, Poland

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Human non-small-cell lung cancers (NSCLCs) of 48 patients were analyzed immunohistochemically to detect P21^{ras} and P53 proteins expression. The relationship between P21^{ras} and P53 proteins expression and clinicopathologic findings was also assessed. DAKO EnVision^{TM} detection system was employed in the study. The P21^{ras} and P53 proteins expression was shown in 75% (36/48) and 33.3% (16/48) studied NSCLCs, respectively. In both cases the difference was significant when compared with adequate negative control. Simultaneous expression of both studied proteins was observed in all cases in which P53 expression was noticed. No significant association of P21^{ras} and P53 expression was found with age, histologic type, histologic grade, tumor size or lymph node metastasis of the studied NSCLCs. Therefore, our study suggests that P21^{ras} and P53 protein play a role in the pathogenesis of NSCLCs but they have no value as a prognostic markers in the case of lung cancers.

Key words: Human lung cancer, P21^{ras}, P53, histologic type, tumor size, histologic grade, lymph node metastasis.

Lung cancer is one of the leading causes of cancer death in most developed countries [30]. Less than a quarter of new cases of lung cancer diagnosed every year are amenable to curative surgery, and with the best therapeutic approach, less than 10% survive 5 years [27]. The events leading to the development and progression of the malignant phenotype are complex and interactive. The knowledge of the events can direct the action to prevent and inhibit the development of cancer. It was detected that mutations of different proto-onogenes and inactivation of tumor suppressor genes are involved in the development and progression of human cancers arising in a variety of tissues [10, 18, 35]. Available data [1, 10, 20, 36] indicate that genetic changes in proto-oncogenes: *Ras, Raf1, Jun, ErbB-2(Neu), Fur, Myb, Myc, Src, Sis, Fes* and tumor suppressor genes: *P53, P16, Rb* are implicated in the pathogenesis of lung cancers. There are evidences that some of the activated protooncogenes and tumor supressor genes are more selectively expressed or absent in small-cell lung cancers (SCLCs) (*Myc, Myb, Src, Rb* gene) or non-small-cell lung cancers (NSCLCs) (*ErbB-2, Sis, Fes*) [1]. For example, alterations in the *P53, Rb* and *P16* suppressor genes, and *Myc* amplification appear in both SCLCs and NSCLCs with the frequencies higher in SCLCs [1, 20, 36], while the *Ras* family genes mutations have been detected only in NSCLCs [36]. Among the NSCLCs subtypes, adenocarcinomas generally show higher frequency of *Ras* genes mutation than squamous-cell carcinomas, ranging from 15 to 60% (2, 5, 13, 22, 28). Approximately in one third of resected lung adenocarcinomas mutations of K-*Ras* gene have been observed and the most of these mutations have been detected in codon 12, less frequently in codon 13 and 61 [2]. N-*Ras* and H-*Ras* mutations have seldom been stated in lung cancers [32].

The *Ras* gene family genes (H-, K- and N-*Ras*), each located on a different chromosome, code similar highly conserved 21 kiloDalton (kD) proteins (P21^{ras}) that bind and

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hydrolyze GTP [21]. The P21^{ras} proteins play a role in the transduction of mitogenic signals from activated growth-factor receptors, leading to cell-cycle entry [21]. In consequence of the single base substitution the *Ras* genes code mutated P21^{ras} proteins which are defective in hydrolysing GTP and constitutively "switched on".

P53 gene mutations belong to the most frequent genetic alterrations in human tumors [3, 4, 9, 12, 23]. They appear in 70–100% of SCLCs and 40–60% of NSCLCs [16, 23]. The normal allele of the *P53* tumor suppressor gene codes a nuclear phosphoprotein called P53, of 53 kD molecular weight, involved in the cell cycle regulation, DNA repair, senescence and apoptosis. Loss of its function leads to defective DNA-repair, genomic instability, abnormal cell proliferation [7, 19]. As a consequence of the mutation in the *P53* gene, the half-life of the P53 protein increases. This event leads to an overexpression of the P53 protein. Activation P21^{ras} and P53 proteins have not been found in normal tissues and therefore their detection in neoplastic and preneoplastic lesions may have clinical utility.

The aim of this study was to investigate the expression of the P21^{ras} and P53 proteins in human lung cancers and to find out the relationship between positive staining for both proteins with clinicopathologic features.

Material and methods

Human NSCLCs specimens from 48 patients (39 men and 9 women) were obtained during surgical procedures at M. Copernicus Provincial Specialistic Hospital in Lodz, Poland. The median age of the patients was 57 years, with a range of 38–75 years. No chemo-, or radiotherapy was performed prior to surgery. Tumors were diagnosed histopathologically according to the WHO International Histological Classification [34] and included 33 squamous cell carcinomas, 10 adenocarcinomas and 5 other cell type NSCLCs (adenosquamous and large-cell carcinomas). The above mentioned classification was also used to assessment of histologic grade of the studied NSCLCs. The clinical stage was determined according to TNM classification given by FRANKLIN [8].

The tissue sections were fixed in 10% formalin overnight and embedded in paraffin. The 4-μm paraffin sections were adhered to slides pretreated with poly-L-lysine. Immunohistochemical analysis was performed using DAKO EnVision^{TM} method. Briefly, sections were heated to 65 °C and deparaffinized in three changes of xylene. This was followed by three rinses in gradient alcohols. Sections were than treated with blocking serum to block nonspecific reactions. For P21^{ras} immunostaining the monoclonal mouse anti-human p21^{ras} antibody (clone NCC-RAS-001; DAKO, Denmark) was applied for 30 min. The concentration of antibody used in the study was 1:200. For P53 immunostaining the mono-

Table 1. Expression of the P21^{ras} and P53 proteins in correlation with clinicopathologic findings in human lung cancers

Case	Age/ sex	Histologic type[a]	Histologic grade[a]	TNM clinical classification[b]	Immunohistochemical expression	
					P21^{ras}	P53
1	66/F	SQ	G3	$pT_2N_1M_x$	+	+++
2	66/M	SQ	G1	$pT_1N_oM_x$	–	–
3	68/M	SQ	G2	$pT_2N_oM_x$	+++	+++
4	54/M	SQ	G2	$pT_2N_1M_o$	+++	–
5	46/M	SQ	G3	$pT_2N_1M_o$	+	–
6	68 M	SQ	G2	$pT_2N_1M_o$	+++	–
7	52/M	SQ	G2	$pT_2N_oM_o$	+++	–
8	59/M	SQ	G2	$pT_2N_oM_o$	–	–
9	54/M	SQ	G2	$pT_1N_oM_o$	+++	+++
10	71/M	SQ	G3	$pT_2N_1M_x$	–	–
11	49/M	SQ	G2	$pT_1N_1M_x$	–	–
12	69/F	SQ	G1	$pT_1N_oM_x$	+++	+
13	62/M	SQ	G3	$pT_3N_oM_o$	+++	++
14	75/M	SQ	G3	$pT_3N_oM_o$	+	+
15	68/M	SQ	G2	$pT_1N_xM_x$	+++	–
16	60/F	SQ	G2	$pT_3N_1M_x$	+++	–
17	61/M	SQ	G3	$pT_3N_oM_o$	–	–
18	72/F	SQ	G2	$pT_2N_oM_x$	++	–
19	46/M	SQ	G1	$pT_2N_2M_o$	-	–
20	54/M	SQ	G2	$pT_2N_oM_o$	+	–
21	47/M	SQ	G3	$pT_2N_oM_o$	+++	–
22	54/M	SQ	G2	$pT_1N_oM_o$	+++	+++
23	66/M	SQ	G3	$pT_2N_oM_o$	++	+++
24	57/M	SQ	G2	$pT_3N_2M_o$	+++	–
25	67/M	SQ (atque tbc productive)	G3	$pT_3N_oM_o$	++	++
26	38/F	SQ	G3	$pT_3N_xM_x$	+++	–
27	56/M	SQ	G3	$pT_3N_oM_x$	+++	–
28	55/M	SQ	G2	$pT_4N_oM_o$	+++	+++
29	61/M	SQ	G3	$pT_2N_oM_x$	–	–
30	51/F	SQ	G2	$pT_1N_xM_x$	+++	+++
31	46/M	SQ	G1	$pT_2N_oM_o$	+++	–
32	57/M	SQ	G3	$pT_1N_oM_o$	+++	–
33	39/F	SQ	G3	$pT_3N_oM_x$	–	–
34	51/F	AD	G2	$pT_2N_oM_x$	+++	++
35	71/M	AD	G3	$pT_3N_2M_o$	+++	–
36	54/M	AD	G3	$pT_2N_oM_x$	+++	–
37	69/M	AD	G2	$pT_2N_oM_o$	–	–
38	56/M	AD	G3	$pT_1N_2M_x$	–	–
39	68/M	AD	G4	$pT_3N_oM_o$	+	–
40	68/M	AD	G2	$pT_2N_oM_o$	+	+
41	59/M	AD	G2	$pT_2N_2M_o$	–	–
42	61/M	AD	G1	$pT_2N_1M_x$	+++	+++
43	65/M	AD	G3	$pT_2N_oM_x$	–	–
44	66/M	AS	G3	$pT_1N_oM_o$	+++	–
45	58/M	AS	nd	$pT_1N_1M_x$	++	–
46	66/M	AS	nd	$pT_2N_2M_o$	++	+
47	65/F	L	G1	$pT_2N_oM_o$	+++	+++
48	66/M	L	G4	$pT_2N_2M_o$	+++	–

[a] – according to the WHO International Histological Classification [34], [b] – according to TNM classification given by Franklin [8], M – male, F – female, SQ – squamous cell carcinoma, AD – adenocarcinoma, AS – adenosquamous cell carcinoma, L – large cell carcinoma, nd – no data, – – no positive cells, + – <10% positive cells, ++ – 11–40% positive cells, +++ – >40% positive cells.

Figure 1. Expression of P21^{ras} protein in non-small-cell lung cancer (DAKO EnVision^{TM} system; P21^{ras} antibody).

Figure 2. Expression of P53 in non-small-cell lung cancer (Dako EnVision^{TM} system; P53 antibody).

Figure 3. Negative control for P21^{ras} (section of the P21^{ras} and P53 positive squamous cell carcinoma; DAKO EnVision^{TM} system; P21^{ras} antibody).

Figure 4. Positive control for P53 (section of the P21^{ras} and P53 positive squamous cell carcinoma; DAKO EnVision^{TM} system; P53 antibody).

clonal mouse anti-human P53 antibody (clone DO-7) was used for 30 min. The staining of P53 was optimal when the concentration of antibody was 1:50.

In the next step, the slides were incubated in Dextran (DAKO) and than Fast Red (DAKO) as a chromogen was used. The sections were lightly counterstained with hematoxilin. Each step was followed by two washing cycles and bathing in a 0.05M TRIS- buffered saline (pH 7.6) for 3 min each. Negative control sections were incubated with mouse immunoglobulin IG1 instead of the primary antibody to assess nonspecific staining. As a positive control, sections of human squamous-cell carcinoma showing strong immunostaining of P21^{ras} and P53 treated with mouse anti-human p21^{ras} antibody or 53 antibody, were used. Staining of sections was evaluated under light microscope at objective of x20, x40. All positive cells were counted, regardless of the intensity of staining. The expression of P21^{ras} was assessed by the percentage of positively labeled cytoplasm of the tumor cells in the case of P21^{ras} and positively labeled tumor cell nuclei in the case of P53 of the three fields (500–700 cells in each field). The intensity of staining was graded subjectively using four categories as follows: –, indicates negative; +, less than 10% of tumor cells are positive (weakly positive); ++, 11% to 40% of tumor cells are positive (moderately positive) and +++, over 40% of tumor cells are positive (strongly positive).

Evaluation of the frequency of P21^{ras} and P53 expression in total studied lung cancers was analyzed on the base of 95% confidence interval in binomial distribution to compare with adequate frequency at negative control. The association of P21^{ras} and P53 expression with tumor age, tumor grade, tumor stage and nodal metastasis was made by Fisher's exact test. All p values were calculated as two sides and if a p value was $0<0.05$, comparing frequencies were considered significant.

Table 2. Relationship between P21^{ras} and P53 proteins expression and clinicopathological findings

	No. of cases	No. of cases with P21^{ras} expression (%)	p	No. of cases with P53 expression (%)	p
Age					
<60 yr	21	16 (76.1)	0.88*	10 (47.6)	0.19*
60–69 yr	23	17 (73.9)		5 (21.7)	
>70 yr	4	3 (75.0)		1 (25.0)	
Histologic type[a]					
Squamous-cell carcinoma	33	25 (75.77)	0.29*	11 (33.3)	0.84*
Adenocarcinoma	10	6 (60.0)		3 (30.0)	
Other cell types[b]	5	5 (100.0)		2 (40.0)	
Total	48	36 (75.0)	(60.4 ÷ 86.4)**	16 (33.3)	(20.4 ÷ 48.4)
Histologic grade[a,c]					
G1	6	4 (66.6)	0.73*	3 (50.0)	0.56*
G2	19	15 (78.9)		7 (36.8)	
G3	19	13 (68.4)		5 (26.3)	
G4	2	2 (100.0)		0 (0.0)	
Tumor size[d]					
T1	11	8 (72.7)	0.82*	4 (36.4)	0.54*
T2	25	18 (72.0)		8 (32.0)	
T3	11	9 (81.8)		3 (27.3)	
T4	1	1 (100.0)		1 (100.0)	
Lymph node metastasis[d]					
N_o	22	22 (75.9)	0.54*	12 (41.4)	0.34*
N_1	9	7 (77.8)		2 (22.2)	
N_2	7	4 (57.1)		1 (14.3)	
N_x	3	3 (100.0)		1 (33.3)	

[a] – according to the WHO International Histological Classification [34], [b] – large cell carcinoma and adenosquamous cell carcinoma, [c] – we have data for 46 non-small-cell cancers, [d] – according to TNM Staining System for Lung Carcinoma [8], * – value for Fischer's exact test, ** – the frequency is significantly greater than 0 on the base of 95% confidence interval, of frequency in binomial distribution.

Results

The clinicopathologic characteristics determined at the time of surgery, including age of the patients, histologic type, histologic grade, TNM stage classification of the studied NSCLCs and also the results of P21^{ras} and P53 proteins expression are presented in Table 1. The relationship between P21^{ras} and P53 proteins expression, and clinicopathological findings is summarized in Table 2. An example of P21^{ras} protein and P53 expression in examined NSCLCs is given in Figure 1.

The frequency of P21^{ras} expression was found in 75% (36/48) while P53 expression in 33.3% (16/48) NSCLCs (Tab. 1). The strong positive P21^{ras} immunostaining (+++) was observed in 52.08% (25/48), moderate (++) in 10.41% (5/48) and weak (+) in 12.5% (6/48) NSCLCs. The strong positive P53 immunostaining (+++) was revealed in 18.75% (9/48), moderate (++) in 6.25% (3/48) and weak (+) in 8.33 (4.48) studied NSCLCs. Interestingly, the study showed that all of P53 positive NSCLCs exhibited P21^{ras} expression.

As presented in Table 2 the frequency of P21^{ras} and P53 proteins expression in the studied NSCLCs was significant (p=60.4 ÷ 86.4; p=20.4 ÷ 48.4, respectively), when compared with adequate frequency at negative control on the base of 95% confidence interval in binomial distribution. However, no significant differences in P21^{ras} protein (p=0.29) and P53 protein (p=0.84) expression has been noticed between squamous-cell carcinomas, adenocarcinomas and other cell type NSCLCs. Besides, the study detected no association between the P21^{ras} protein (p=0.73) or P53 protein (p=0.56) expression in a well differentiated (G1), moderately differentiated (G2), poorly differentiated (G3) and undifferentiated (G4) NSCLCs. No significant association of P21^{ras} and P53 protein expression was either found with tumor size (p=0.82 and p=0.54) and nodal lymph metastasis (p=0.54 and p=0.34, respectively). There was also no relationship between P21^{ras} (p=0.88) and P53 (p=0.19) proteins expression and age of the patients.

Epidemiological studies leave no doubt that most human lung cancers are caused by exposure to tobacco smoke [14].

We have data concerning tobacco smoke for 26 of 50 studied patients. It is worth mentioning that all of them without one were smokers or former smokers. The mean number of cigarettes per day was equal to 30 and the median smoking time 39 years (range 20–57).

Twelve of twenty seven (44.44%) patients for whom we have survival data passed away within one year after surgical procedure.

Discussion

The results of the numerous reports show that the P21^{ras} and P53 proteins are involved in the development of lung cancers. Miyamoto et al [26] obtained positive immunoreaction with P21^{ras} protein in 56% (24/43) squamous cell carcinomas and 68% (27/40) adenocarcinomas. Volm et al [37] detected that 79% of the studied adenocarcinomas were positive for all three *Ras* proteins. According to Harada et al [11] 63% of the NSCLCs were positive for *Ras* proteins. Kim et al [17] detected P21^{ras} protein expression in 54.68% studied NSCLCs. Our results concerning P21^{ras} expression in NSCLCs are similar to these obtained by the others. We found P21^{ras} protein expression in 75% (36/48) analyzed NSCLCs.

Jassem et al[15] stated that positive staining for P53 protein was present in 46% (44/94) patients with NSCLC. Kim et al [17] detected P53 protein expression in 18.1% examined NSCLCs. In our study we observed the P53 protein expression in 33.3% (16/48) studied NSCLCs. The results of our study confirmed the results obtained by the others suggesting that the both studied proteins play a role in the pathogenesis of NSCLCs.

The Miyamoto et al [26] study demonstrated that P21^{ras} expression was associated with the stage of the disease and that higher level of P21^{ras} expression is significantly correlated with a shorter survival time for patients with NSCLCs. According to Miyamoto et al [26] P21^{ras} expression and the stage of the disease considered together may improve prognostic evaluation of lung cancer. However, prognostic significance both P21^{ras} and P53 proteins is still controversial. In our study no association between P21^{ras} protein and P53 protein expression and tumor size, tumor grade or lymph node metastasis was detected.

Some studies have demonstrated that the P53 mutation in NSCLCs is associated with poor prognosis [24, 25, 31, 33] but the others have reported no significant effect [15, 29]. According to Jassem et al [15] there is no significant difference between survival in the group with and without P53 protein expression. The works of Dworakowska et al [6] and Passlick et al [29] support these findings suggesting the lack of prognostic relevance of P53 expression in surgically treated NSCLCs patients.

In conclusion, this study confirmed suggestion of the others that P21^{ras} and P53 proteins are involved in the pathogenesis of NSCLCs. However, no significant association between P21^{ras}ras or P53 proteins expression and clinicopathological findings was observed. Promising tumor markers still need to be evaluated.

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