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Aberrant Expression of pRb, p16, p14ARF, MDM2, p21 and p53 in Squamous Cell Carcinomas of Lung

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Expression of cell cycle regulatory proteins in both the RB and p53 pathways was investigated in 50 cases of squamous cell carcinoma (SCC) of the lung using immunohistochemical techniques. Abnormality of pRb and p16 expression was seen at the frequencies of 16% and 78%, respectively, and appeared to be in a reciprocal relationship. On the other hand, strong and diffuse p53 immunoreactivity was seen in 60% of SCCs. MDM2 and p14ARF expressions were each observed in about half of the cases of SCC and were not significantly associated with strong p53 immunoreactivity. Statistical analysis revealed that p14ARF expression was significantly correlated with both p16 and MDM2 expression. Moreover, strong p53 expression was not correlated with the expression of p21. In comparing clinicopathological status with the immunohistochemical results, lack of p16 immunoreactivity was observed in the elderly group (over 65 years) as compared with the younger group (less than 65 years). Strong p53 expression was frequently observed in higher stages of SCC, with the developing tumor located in the central field of the lung. Similarly, the frequency of p14ARF expression was high in centrally developed SCC, but low in SCC developed in the periphery. These results suggest that disruption of the RB and p53 pathways is a frequent event in SCC, and that abnormal expression of p16 and p53 plays a more critical role than that of pRB, p14ARF and MDM2 in the development of SCC of the lung.

Key words: pRB pathway — p53 pathway — Squamous cell carcinoma — Lung — Immunohistochemistry

Deregulation of cell cycle control is one of the most important mechanisms in the development of human tumors, including lung cancers. Most abnormalities of G1-S transition control in human tumors are attributed to abnormality of the p16-cyclin D1-CDK4-pRb cascade (the RB pathway).[1, 2] Moreover, at the G1 checkpoint, several other CDK inhibitors such as p15, p18, p21 and p27 strictly regulate cell proliferation.[3] However, mutation of tumor suppressor p53 has turned out to be the most frequent genetic abnormality in human cancers, and is significant because p53 plays an important role in cell cycle control, DNA repair and apoptosis.[4, 5] As regards cell cycle control, it is well established that p53 influences the RB pathway through the induction of p21.[3] Recent studies have shown that the inactivation and activation of p53 are regulated by MDM2 and the checkpoint kinase Chk2, respectively.[6] Chk2 causes G2 arrest by inhibiting cdc25C, which is independent of p53.

The *CDKN2A* gene has been isolated from hereditary malignant melanoma with chromosomal 9p deletion, and its transcript p16 is a recognized tumor suppressor, which can induce G1 arrest by inhibiting the phosphorylation of pRb through CDK4 and CDK6 inhibition.[3, 7] Abnormality of p16 expression is frequently observed in various human cancers including lung, pancreatic and esophageal cancers, glioblastomas, malignant melanomas and T-cell lymphoblastic leukemias.[2, 8–10] The abnormal p16 expression is due not only to genetic alterations such as homozygous deletion and point mutation of the *CDKN2A* gene, but also to epigenetic change in the methylation of the gene promoter.[11–13]

Recent advances in molecular biology have led to the identification of another protein encoded by *CDKN2A* gene and produced by alternative splicing.[14] This protein is named p14ARF, and acts by binding directly to MDM2, resulting in the stabilization of both p53 and MDM2.[15, 16] Therefore, the p14ARF-MDM2-p53-p21 cascade (the p53 pathway) is another important cell cycle regulatory system in G1 cell cycle arrest and may have a close connection to G2 arrest.[17, 18] Collaboration between the RB and p53 pathways in tumor suppression is evident from the observation that many cancers exhibit concomitant pRb and p53 abnormality.

Among human lung cancers, small cell lung cancers (SCLCs) are representative cancers genetically demonstrating the abrogation of both the RB and p53 pathways, as well as chromosomal 3p deletion.[19] On the other hand, p53 mutation and p16 abnormal expression are the most frequent changes in the non-small cell lung cancers (NSCLCs).[20, 21] However, our previous study revealed that the frequency of p16 abnormal expression differed

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between adenocarcinoma and squamous cell carcinoma (SCC), although they are also categorized as NSCLC.[22] This study aimed to elucidate abnormal cell cycle regulator protein expression in the RB and p53 pathways of SCC of the lung and to identify clinicopathological parameters closely related the abnormality of the RB and p53 pathways.

MATERIALS AND METHODS

Lung cancer tissues A total of 50 cases of SCC of the lung was randomly selected from our pathology files. After surgical operation, the resected lung tissues were fixed with 15% formalin and processed for routine pathological diagnosis. A paraffin block containing representative tumor histology in each case was selected and sections of 4 μm thickness were prepared. Subsequently, they were used for immunohistochemical study as well as for the reevaluation of tumor histology after hematoxylin and eosin staining.

Various clinicopathological data were obtained from our pathology files. All patients except two were male with ages ranging from 45–78 years (mean 66 years). Forty patients were heavy smokers with a Brinkmann index of more than 500. As regards tumor localization, 18 SCCs were in the central area of the lung field and the others were located in the periphery of the lung. According to the histological grade of tumor differentiation, 31 of the 50 SCCs were reevaluated as moderately differentiated and the remaining SCCs were composed of 12 poorly differentiated and 7 well-differentiated tumors. Pathological TNM classification revealed that 23 SCCs were evaluated as stage I, and stage II and III were allocated 8 and 19 tumors, respectively.

Immunohistochemistry for cell cycle regulatory proteins Immunohistochemical methods for pRb, p16, p14ARF, p53 and p21 were described in our recent papers.[23–25] The details of the primary antibodies were as follows; the antibody against pRb: 200 times diluted G3-245 mouse monoclonal IgG1 antibody (PharMingen, San Diego, CA), the antibody against p16: 100 times diluted JC8 mouse monoclonal IgG2a antibody (kindly provided by Dr. J. Koh, Massachusetts General Hospital Cancer Center, Boston, MA),[2, 22, 25] the antibody against p53: 100 times diluted DO-7 mouse monoclonal IgG2b antibody (Novocastra Laboratories, Newcastle, UK), the antibody against p14ARF: 100 times diluted C-18 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), the antibody against p21: 40 times diluted 4D10 mouse monoclonal IgG1 antibody (Novocastra Laboratories). After antigen retrieval treatment with the appropriate buffer, the sections were incubated with each primary antibody at 4°C overnight. For MDM2 immunohistochemistry, antigen retrieval was carried out by boiling in 0.01 *M* sodium phosphate-citric buffer, pH 8.0, for 10 min. Subsequently, the sections were incubated with a primary antibody against MDM2, which was 1000 times diluted SMP14 mouse monoclonal IgG antibody (Santa Cruz Biotechnology), at 4°C overnight. After treatment with biotinylated second antibody, the sections were incubated with streptavidin-biotin-peroxidase complex (Vectastain, Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature. Visualization was carried out with 0.02% 3-3′-diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 in 50 m*M* ammonium acetate-citric acid buffer, pH 6.0, as the chromogen. Slides were then counterstained lightly with hematoxylin.

Evaluation of immunohistochemistry For the evaluation of p16, pRb, p53, p21, p14ARF and MDM2 immunostains, the criteria used by previous investigators were partly followed in this study.[20, 21, 25] For the immunohistochemistry of pRb, p53 and p21, only nuclear staining was observed, and this was counted for immunoreactivity score. On the other hand, p16, p14ARF and MDM2 showed both nuclear and cytoplasmic immunostaining, but only nuclear staining, irrespective of cytoplasmic immunoreactivity, was considered as evidence of expressed proteins. Immunoreactivity for all cell cycle regulatory proteins investigated in this study was evaluated as strongly positive (++) when more than 50% of tumor nuclei were stained. Tumors were judged as moderately positive (+) when more than 10% of nuclei were stained, and negative (−) when less than 10% of tumor nuclei were stained or when the tumor cells completely lacked immunoreactivity.

Statistical analysis The χ^2 text and Fisher's exact probability test were used for statistical analysis of the results. *P* values less than 0.05 were considered significant.

RESULTS

Immunohistochemical analysis Immunoreactivity for pRb was exclusively located in the nuclei of SCC and almost all nuclei showed strong and diffuse immunoreactivity for pRb (Fig. 1D). Of the 50 SCC cases, only eight completely lacked pRb immunoreactivity, except for the nuclei of the normal cell component, and of them, six SCCs showed strong and diffuse immunoreactivity for p16 (Fig. 1, A and B). Of eight SCCs with pRb (−), five were p16 (++) and also positive for p14ARF (+/++) (Fig. 1, A, B and C). As shown in Table I, therefore, immunohistochemical pRb expression was inversely correlated with p16 immunoreactivity. The majority of SCCs (78%) was completely devoid of p16 immunoreactivity (Fig. 1E). Of the 50 SCCs, 24 (48%) were immunohistochemically positive for p14ARF, which showed diffuse immunoreactivity in the nuclei of SCC and in the nucleoli of some SCCs judged as (++) (Fig. 1F). When the immunohistochemical

status of p16 and that of p14ARF were compared, the expression patterns were significantly correlated with each other, although twice as many SCCs were positive for p14 ARF as for p16. Of 26 SCCs without any p14ARF immunoreactivity, 24 completely lacked p16 immunoreactivity (Table I). There were 14 SCCs with pRb (++), p16 (−) and p14ARF (+/++) (Fig. 1, D, E and F).

MDM2 immunoreactivity, usually limited to the nuclei and nucleoli, often with the cytoplasm, was observed in 27 SCCs, of which nine were scored (++). Table II summarizes the inverse relation between MDM2 and p14ARF immunoreactivity (Fig. 1, H and I). Strong and diffuse nuclear p53 staining judged as (++) was present at the frequency of 60%, while the remaining SCCs, except three, were judged as p53 (−) (Table III, Fig. 1, G and J). Of the 17 SCCs with p53 (−), 11 were immunohistochem-

Table I. Immunohistochemical Results of pRB, p16 and p14 in Lung Squamous Cell Carcinomas

	(%)	p16 (−) (78%)	p16 (+/++) (22%)	*P* value
pRB (++)	(84)	37	5	
pRB (−)	(16)	2	6	$P<0.05$
p14 (+/++)	(48)	15	9	
p14 (−)	(52)	24	2	$P<0.05$

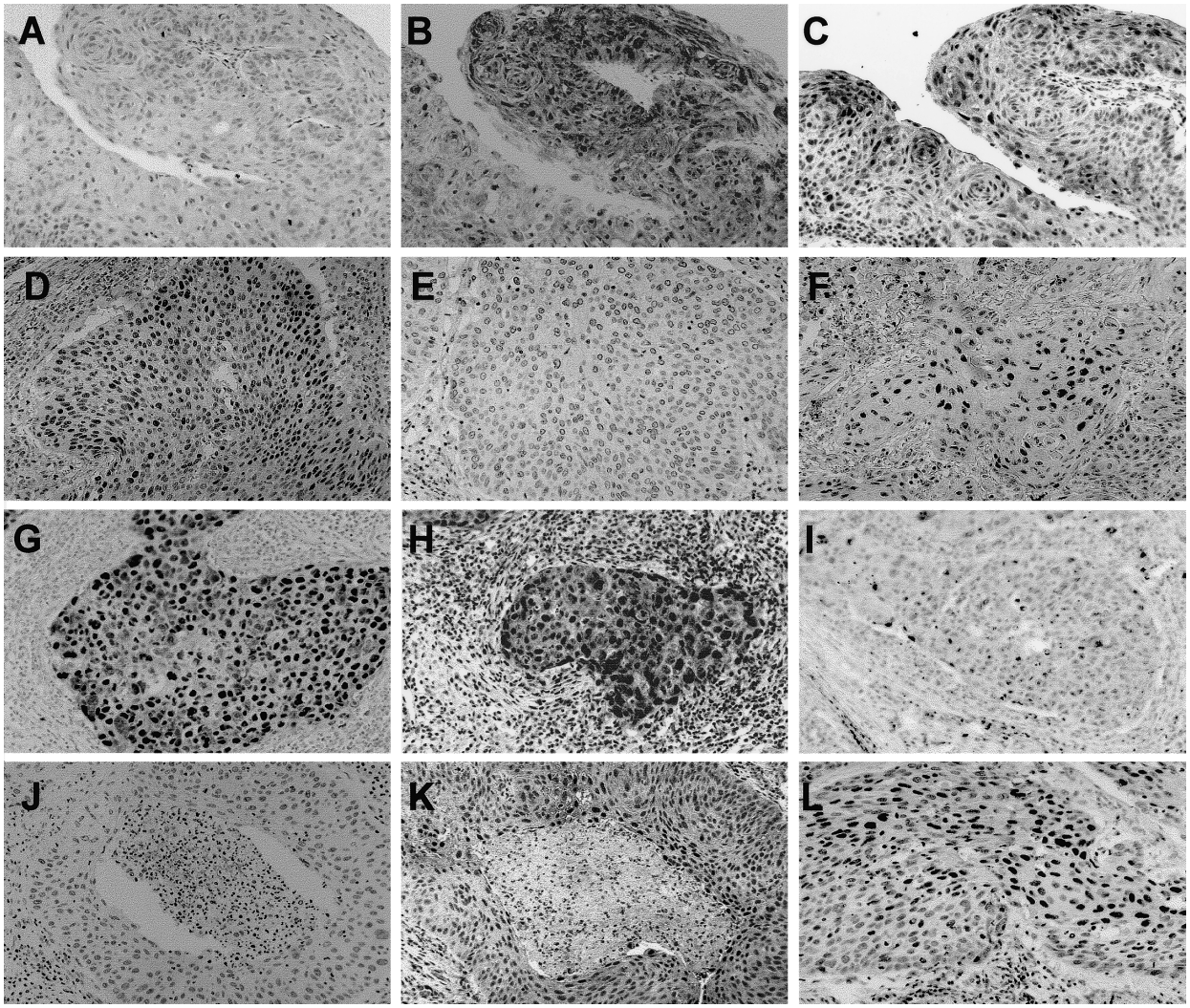

Fig. 1. Examples of immunohistochemical results for various cell cycle regulator proteins in SCCs. A SCC case lacked pRb immunoreactivity (A), but showed strong p16 (B) and p14ARF (C) staining. Another case with strong pRb immunoreactivity (D) was negative for p16 immunostaining (E), but positive for p14ARF (F). A SCC case with strong p53 positivity (G) showed MDM2 immunoreactivity (H) and lacked p14ARF (I). Another case without any p53 immunoreactivity (J) showed both MDM2 (K) and p14ARF (L) immunoreactivity.

Table II. Immunohistochemical Results of p14 and MDM2 in Lung Squamous Cell Carcinomas

	(%)	p14 (+/++) (48%)	p14 (−) (52%)	*P* value
MDM2 (+/++)	(54)	9	18	
MDM2 (−)	(46)	15	8	$P<0.05$

Table III. Immunohistochemical Results of p53, MDM2 and p21 in Lung Squamous Cell Carcinomas

	(%)	p53 (++) (60%)	p53 (+/−) (40%)	*P* value
MDM2 (+/++)	(54)	14	13	
MDM2 (−)	(46)	16	7	n.s.
p21 (+/++)	(50)	14	11	
p21 (−)	(50)	16	9	n.s

"n.s." means "not significant."

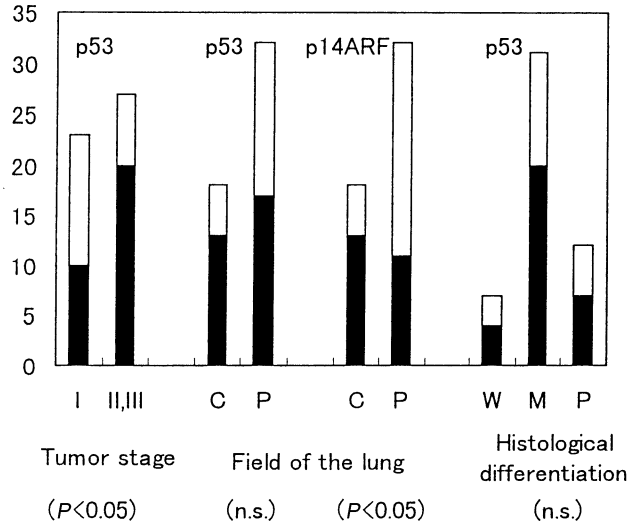

Fig. 2. Clinicopathological status and cell cycle regulatory protein expression. Open columns show the number of SCC cases. Black columns represent the number of positive cases. "C" and "P" mean "central" and "peripheral," respectively. "W," "M" and "P" mean "well differentiated," "moderately differentiated" and "poorly differentiated," respectively. "n.s." means "not significant."

ically positive for MDM2. However, no significant relationship between p53 and MDM2 immunoreactivity was observed in this study, and there was no relationship between p14ARF and p53 expression (Tables II and III). As shown in Table III, the results of MDM2 and p21 immunohistochemistry were very similar, usually overlapping each other. Half of the SCCs showed p21 immunoreactivity, while eight SCCs showed MDM2 (+/++), p53 (−) and p21 (+/++).

Clinicopathological relationship to immunohistochemical results The age distribution of all patients in this study (45–78 years of age) was compared with the immunohistochemical results. All patients positive for p16 except two were younger than 65 years of age ($P<0.002$). No other immunohistochemical results were related to the age distribution. As 40 patients were heavy smokers with a Brinkmann index of more than 500, no significant relation could be found between Brinkmann index and immunohistochemical results. Diffuse and strong p53 positivity judged as (++) was observed in higher-stage SCCs, with a frequency of 20 (74%) out of 27 stage II and III SCCs, compared with 10 (43%) of 23 stage I SCCs ($P<0.05$). Moreover, the presence of p53 (++) was remarkable in SCCs developed in the central area of the lung field (72%, 13 of 18 SCCs) as compared with SCCs located in the periphery of the lung (27%, 10 of 37 SCCs) (Fig. 2). Immunohistochemical positivity of p14 also revealed a similar tendency to that of p53 (++) ($P<0.05$). The numbers of well, moderately and poorly differentiated SCCs were counted as 7, 31 and 12, respectively. However, there was no significant relationship between histological differentiation and immunohistochemical results, including that for p53 (Fig. 2).

DISCUSSION

Cancer is caused by uncontrolled proliferation of cells, which is itself caused by abnormality of cell cycle regulatory mechanisms, mainly in the RB and/or p53 pathways. In SCLCs, both the RB and p53 pathways are completely disrupted at the *Rb* and *p53* gene level. However, NSCLCs are somewhat different from SCLCs in this respect.[26–29]

In the RB pathway, abnormality of pRb and p16 expression plays an important role in cell cycle regulation. In SCC of the lung, our study demonstrated immunohistochemical abnormality of pRb and p16 expression at frequencies of 16% and 78%, respectively. These results suggest that 90% of SCCs might have abnormality in the RB pathway, as shown in Table I. In human cancers, loss of p16 function can be attributed to homozygous deletion, point mutation or epigenetic promoter methylation of the gene. In NSCLCs, homozygous deletion and promoter methylation are the major causes of loss of p16 function, in contrast to SCLC.[22, 30] Previous immunohistochemical studies have shown frequent loss of p16 expression in 27–66% of NSCLCs.[31–33] Our result on p16 immunoreactivity was higher than that reported previously, possibly due to the fact that inactivation of p16/CDKN2A is more common in SCC compared to adenocarcinomas of the lung.[22, 31] In this study, strong and diffuse p16 immunoreactivity was observed in nine SCCs, of which six lacked

pRb immunoreactivity, showing inversely related expression levels of pRb and p16. Reciprocal pRb inactivation and p16 expression is a characteristic of SCLCs and cervical cancers.[18, 34, 35] In cervical cancers, it is well established that human papillomavirus has a key role in inactivation of pRb, through the viral E7 oncoprotein.[36]

Recently, the CDKN2A locus has been shown to encode two cell cycle regulatory proteins, p16 and p14ARF, which share an exon using different reading frames.[14] In lung cancers, p14ARF expression is lost at frequencies of 65% and 25% in SCLCs and NSCLCs, respectively.[37–39] Our study revealed that about half of SCCs lacked immunoreactivity for p14ARF, which is similar to the result for oral SCCs.[25] Moreover, our results revealed a significant relation between p14ARF and p16 expression and about half the cases lacked both p14ARF and p16 immunoreactivity. We speculate that the lack of both p14ARF and p16 immunoreactivity might be attributed to the homozygous deletion of the *CDKN2A* gene commonly seen in NSCLC. Conversely, the discrepancy between p14ARF and p16 immunoreactivity shown in this study might depend on different methylation status in the promoter region or point mutation of the *CDKN2A* gene. Although the discrepancy between mRNA and protein expression of p14ARF has been reported already,[38] further studies are required on the expression status of p14ARF and p16.

In the p53 pathway, p53 point mutation is the most frequent cause of disruption of cell cycle control in human cancers, including lung cancer. In this study, about 60% of SCC showed disruption of p53, in agreement with previous findings.[20, 21] Another possible cause of p53 pathway disruption is amplification of the *MDM2* gene or MDM2 overexpression. The amplification of the *MDM2* gene is frequent in soft tissue sarcomas, osteosarcomas, lymphomas and gliomas, but not in lung cancers.[40–42] In lung cancers, MDM2 expression in adenocarcinomas is still a controversial issue. So far, amplification of the *MDM2* gene or immunohistochemical overexpression has been reported in SCC of the esophagus and oral cavity, but not of the lung.[43, 44] In this study, MDM2 expression was immunohistochemically observed in about half of the SCC cases, of which nine showed strong immunoreactivity, suggesting overexpression of the *MDM2* gene. Overexpressed MDM2 promotes degradation of ubiquitinated p53, causing the disruption of the p53 pathway, as expected from the immunohistochemically negative results for p53. Of the nine MDM2 (++) SCCs, four were immunohistochemically negative for p53. However, no significant correlation between MDM2 and p53 immunoreactivity was observed in this study.

The disruption of the p53 pathway may be caused via the loss of p14ARF expression by homozygous deletion, mutation or methylation of the gene. In the p53 pathway, p14ARF can complex with MDM2, inhibiting the ability of MDM2 to induce degradation of p53. Concomitant p14ARF deletion and MDM2 overexpression have been reported in most diffuse large B-cell lymphomas, oral SCCs and malignant gliomas,[25, 45, 46] and our immunohistochemical data also show an inverse relationship between p14ARF and MDM2 expression. From data obtained in p14ARF-knockout mouse, p53 mutation is frequently associated with the occurrence of tumors.[47] The common occurrence of p53 mutation and p14ARF alterations suggests that p14ARF inactivation is not functionally equivalent to abrogation of the p53 pathway caused by p53 mutation. In addition, immunohistochemical inactivation of p14ARF concomitant with MDM2 overexpression and the alteration of p53 have recently been shown to be mutually exclusive events in primary oral SCCs.[25] However, no significant correlation has been found between p14ARF and p53 expression in NSCLC,[38, 39] and our results in SCC are consistent with previous results. The existence of a correlation between p14ARF alterations and p53 point mutation is still a controversial issue. In addition, no significant relation between strong p53 immunoreactivity and p21 expression was observed in this study. Several reports have suggested the induction of p21 via a p53-independent pathway.[48, 49]

Immunohistochemical expression of cell cycle regulatory proteins in the RB and p53 pathways was compared with various clinicopathological data of SCC investigated in this study. Previous reports have elucidated that p16-negative tumors are more frequent in SCCs than in adenocarcinomas and have a worse prognosis than p16-positive tumors.[31] In addition, the lack of p16 immunoreactivity was observed in the elderly group (over 65 years) as compared with the younger group (below 65 years) in this study. It is reasonable that genetic and epigenetic changes of the *CDKN2A* gene occur gradually during the aging process, stimulated further by tobacco smoking. In fact, another study has shown that the level of background anthracosis is closely associated with inactivation of p16 expression and also DNA methylation of the gene in pulmonary adenocarcinoma.[50] However, further studies are necessary before any conclusion can be reached.

A large amount of clinicopathological data concerning the p53 expression has been accumulated and it has already been shown that strong p53 immunoreactivity or p53 mutation is significantly correlated with tumor proliferative activity, aneuploid level, stage and metastasis, namely with poor prognosis, of NSCLC patients.[51] Consistent with previously published results,[52] p53 overexpression was more frequently observed in centrally developing tumors than in peripheral tumors of the lung in this study. In the central lung field, SCC may develop in large-sized bronchi, in which p53 alterations are usually an early event of multi-step carcinogenesis.[53] Similarly, p14ARF expression was significant among central and

peripheral SCC in this study. Frequent p53 overexpression with p14ARF expression in centrally developed SCC is quite similar to the features of oral SCCs.[25] In SCC of the lung, the oncogenic mechanism may be different between centrally and peripherally developing tumors.

In conclusion, disruption of the RB and p53 pathways might be frequent in SCC carcinogenesis in the lung, in which abnormal expression of p16 and p53 plays a more critical role than abnormal expression of pRB, p14ARF and MDM2.

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