Journal of Cellular Biochemistry 64:185–190 (1996)

Examination of p53 Alterations and Cytokeratin Expression in Sputa Collected From Patients Prior to Histological Diagnosis of Squamous Cell Carcinoma

Marshall Anderson,[1,2]* Sue Sladon,[1] Ruth Michels,[1] Lois Davidson,[1] Kenneth Conwell II,[1] John Lechner,[3] Wilbur Franklin,[2] Geno Saccomanno,[1] and Jonathan Wiest[1,2]

[1]St. Mary's Hospital and Medical Center, Cancer Research Institute, Grand Junction, Colorado
[2]University of Colorado Health Science Center, Cancer Center, Denver, Colorado
[3]Inhalation Toxicology Research Institute, Albuquerque, New Mexico

Abstract Mutations in the p53 gene are detected in greater than 50% of squamous cell carcinomas of the lung and to a lesser extent in adenocarcinomas. The p53 protein is also overexpressed in a relatively high percentage of preinvasive lesions of the bronchial epithelium. However, unlike tumor tissue, immunoreactivity does not necessarily imply that cells in preinvasive lesions carry a mutant p53 allele. In some cases, overexpression may result from a cellular checkpoint reaction to a toxic or mutagenic substance such as exposure to tobacco smoke. In any case, p53 overexpression in preinvasive lesions may serve as a biomarker for high risk assessment of lung cancer and other tumors in the aerodigestive tract. A study was designed to retrospectively analyze p53 overexpression in cells from sputum samples collected prior to histological tumor diagnosis. The rationale was based on the observation that both preinvasive and tumor cells from the bronchial epithelium are exfoliated into the airways and can be detected based on morphology in sputa. Two sets of cases were chosen: 1) patients whose first primary tumor was a squamous cell carcinoma containing a mutant p53 allele with overexpression observed in most of the tumor cells; and 2) patients whose squamous cell tumor did not contain a mutant p53 allele. Cells which stained positive for p53 expression were observed in sputum samples collected from all six patients whose tumors were positive for a mutant p53 allele. Also p53 positive cells were detected on sputum slides for two of the five cases where the tumor DNA did not contain a mutation and/or tumor cells which overexpress p53 were not detected in tissue sections. Although cells which stained positive for p53 were present in sputum from patients whose tumors contained a missense mutation, the presence of p53 overexpression was not specific for tumors which contain an altered p53 allele since overexpression was detected in sputum cells from patients whose tumor DNA did not contain a p53 mutation and/or tumor cells which stained positive for p53 were not observed in tissue sections. However, the p53 positive cells in sputa collected from the latter group of patients could have been exfoliated from other lesions which contained a mutant p53 allele. The accumulation of p53 in some sputum cells was concomitant with expression of simple epithelial type cytokeratins (CK) 8 and 18 or at least one of the other cytokeratins detected by a broad spectrum (PAN) CK antibody mixture. These data imply that most of the sputum cells which overexpress p53 are epithelial cells. Moreover, our results are consistent, at least in part, with other observations that cells which overexpress p53 in dyplasias and hyperplasias express CK 8, 18. We will continue to explore the possibility that expression of cytokeratins 8, 18 and/or other cytokeratins in conjunction with p53 overexpression and/or morphological criteria could define a new class of atypical cells which are predisposed to cancer development. J. Cell. Biochem. 25S:185–190.

Key words: cytokeratins; lung cancer; p53 overexpression; sputum

Mutations in the p53 gene are detected in greater than 50% of squamous cell carcinomas of the lung and to a lesser extent in adenocarcinomas. Previous studies have demonstrated p53 overexpression and/or p53 mutations in preinvasive lesions of the bronchial epithelium [1–4], epithelium of the head and neck [5–9] and esophageal epithelium [10,11]. Approximately 80% of the observed alterations in the p53 gene

Contract grant sponsor: Department of Energy, contract grant number DE-FG03-94ER61842/A000; Contract grant sponsor: The University of Colorado SPORE in Lung Cancer grant, contract grant number 58187.

*Correspondence to: Dr. Marshall Anderson, Department of Environmental Health, Office of Director, University of Cincinnati, P.O. Box 670056, Cincinnati, OH 45267-0056.

Received 27 November 1995; Accepted 11 March 1996

are missense mutations [12] which impart a much longer half-life to the mutant protein than the wild-type protein. Thus, the mutant protein can be detected by immunocytochemical staining with an antibody to p53 whereas the normal protein can not usually be visualized. Occasionally, positively staining nuclei are observed in a tumor which does not contain a mutant p53 gene. Wild-type p53 can be stabilized by binding to cellular proteins, i.e. mdm-2, and to some oncogenic viral proteins. Also, response to toxic insults such as ionizing radiation induces the expression of p53 and the wild-type protein can be detected by immunocytochemical staining. However, in general, immunoreactivity is a good predictor of a tumor containing a mutant p53 allele. Of course, tumors with a p53 allele inactivated by a nonsense mutation or by deletions/insertions will probably not stain with a p53 antibody since these types of mutations usually lead to the expression of a truncated or unstable protein.

In several studies, overexpression of the p53 protein was observed in a relatively high percentage of preinvasive lesions. For example, p53 overexpression was detected in 30% of mild and moderate dysplasia and 60% of severe dysplasia of the bronchial epithelium [2]. Similarly, 11 of 15 and 9 of 20 preinvasive dysplastic lesions of head and neck, were shown to overexpress p53 [5,6]. However, unlike tumor tissue, immunoreactivity does not necessarily imply that these cells in the preinvasive lesions carry a mutant p53 allele. In some cases, the overexpression may be a result of a cellular checkpoint to the toxic exposure of smoking. Boyle et al. [7] examined 37 noninvasive lesions of the head and neck for a p53 mutation and detected mutations in only 19% of these lesions, i.e., 5 of 24 in CIS lesions and 2 of 13 dysplasias. We also analyzed a small set of preneoplastic lesions for the presence of p53 mutations and detected a mutation in two of nine samples examined (unpublished data). Additional studies are required to ascertain the relationship between p53 overexpression and the presence of mutations in preinvasive lesions. In any case, p53 overexpression in preinvasive lesions may serve as a biomarker for high risk assessment of lung cancer and other tumors in the aerodigestive tract.

With this background, a study was designed to retrospectively analyze for p53 overexpression in cells present in sputum samples collected prior to the final tumor diagnosis. The rationale was based on the observation that preinvasive and tumor cells in the bronchial epithelium are exfoliated into the airways and therefore, can be detected in sputa based on morphological criteria. Two sets of cases were chosen: 1) patients for which the first primary tumor was a squamous cell carcinoma containing a mutant p53 with overexpression observed in most of the tumor cells; and 2) patients for which the squamous cell tumor did not contain a mutant p53 allele. The first phase of this project was the analysis for p53 overexpression in sputum samples collected prior to histological tumor confirmation. This report describes the results of these analyses.

ANALYSIS OF SPUTUM SAMPLES FOR CELLS WHICH OVEREXPRESS P53

Sputum samples collected from 11 patients diagnosed with a squamous cell carcinoma of the lung were analyzed for cells which overexpress p53. In this study, the sputum samples analyzed were collected within several months prior to histological tumor diagnosis and these sputum specimens were cytologically diagnosed as suspicious for carcinoma, CIS or carcinoma (Table I). Some of the cases presented in Table I also have sputum samples available one or more years prior to tumor diagnosis. The results presented in this report will be extended to include the analysis of cytologically non-malignant sputum samples collected at earlier time points prior to the final tumor diagnosis.

A missense mutation in p53 was detected in the tumor DNA for 6 of 11 patients presented in Table I, and overexpression of p53 was observed in their tumor tissue. For the other five cases, p53 overexpression was not observed in the tumor tissue and no mutation was detected in two of the cases examined.

The following is a brief description of the procedure used to analyze sputum specimens for cells which overexpress p53: 1) a monolayer of sputum cells was prepared on a glass slide utilizing a large format cytofunnel (megafunnel) supplied by Shandon Lipshaw, Inc. (Pittsburgh, PA). A manuscript in preparation will describe the procedure for preparation of Shandon megafunnel slides from sputum samples. The Shandon megafunnel slides can be generated from a sputum slurry or sputum cells removed from previously prepared Saccomanno

TABLE I. Analysis of Sputa Collected Prior to Diagnosis of Squamous Cell Carcinoma for p53 Overexpression

Patient number	Tumor stage[a]	p53 mutation[b]	Time of sputum collection[c]	Sputum diagnosis	p53 immunocytochemistry stain	
					Fixed tissue[d]	Sputum
03B	I	codon 249	1.0	carcinoma	A	positive
22	I	exon 5	1.0	CIS[e]	A	positive
27	I	exon 5	1.0	carcinoma	A	positive
58	I	codon 245	0.25	carcinoma	A/B	positive
61	I	exon 7	7.0	carcinoma	A/B	positive
62	II	exon 5	1.0	carcinoma	A	positive
45	IIIA	none	1.0	carcinoma	negative	negative
63	II	none	0.5	carcinoma	negative	positive
67	ND[f]	ND[f]	3.5	carcinoma	negative	positive
68	I	ND[f]	2.0	suspicious	negative	negative
69	I	ND[f]	0.25	carcinoma	negative	negative

[a]All tumors are moderately differentiated squamous cell carcinoma except for number 68 which is a well differentiated squamous carcinoma.
[b]p53 mutations in exons 5–9 were detected by SSCP analysis.
[c]Time in months prior to final tumor diagnosis.
[d]Overexpression was determined by immunocytochemical staining and defined as follows: A = greater than 75% of the nuclei stained positive for p53; B = between 50–75% of the nuclei stained positive for p53; and negative = no nuclei stained positive for p53.
[e]CIS = carcinoma *in situ*.
[f]ND = not determined.

smear slides [13]. The monolayer of sputum cells is framed in a 1 × 2 cm rectangle located in the center of a specially designed glass megafunnel slide; 2) Megafunnel slides were stained for p53 gene overexpression using the monoclonal DO-7 antibody (DAKO) and diaminobenzidine (DAB) as a chromogen. The detection system consisted of an avidin-biotin peroxidase method using a Vectostain Elite ABC kit (Vector); 3) x,y coordinates were determined for each of the nuclei which stained positive for p53 and photomicrographs were taken; and 4) the samples were then stained with a modified Papanicolaou (Pap) stain to determine if the cells that stained for p53 were morphologically atypical based upon standard diagnostic criteria.

Cells which overexpress p53 were detected on sputum slides for each of the six cases where the tumor contained a missense mutation and the tumor cells stained positive for p53 on a corresponding tissue section (Table I). Also, positive p53 staining cells were detected on sputum slides for two of the five cases where the tumor did not contain a mutation and/or tumor cells which overexpress p53 were not detected on a tumor section (Table I, cases 63 and 67). The number of cells which stained positive for p53 on the megafunnel slide ranged from 0–23 as shown in column 3, Table II. Although cells which stained positive for p53 were present in sputum from patients whose tumors contained a missense mutation, the presence of p53 overexpression was not specific for tumors which contain an altered p53 allele since overexpression was detected in sputum cells from patients whose tumor DNA did not contain a p53 mutation and/or tumor cells which stained positive were not observed in tissue sections. However, the p53 positive cells in sputa collected from the latter group of patients could have been exfoliated from other lesions which contained a mutant p53 allele.

The Shandon megafunnel slides containing cells which overexpress p53 were then stained with the modified Pap stain. The exfoliated cells in the sputa which stained with p53 demonstrated atypical cytological morphology and did not stain orangophilic with orange G-6 (Surgipath). Many of the sputa cells which overexpressed p53 were morphologically consistent with undifferentiated tumor cells (Fig. 1a) whereas others had atypical features such as an altered nuclear to cytoplasmic ratio, and irregular nuclear and cytoplasmic features (*Fig.* 1b) but did not display malignant cytological criteria. Our observation for p53 positive cells in the sputa was consistent with the pattern of p53 staining in tumors. The positive staining nuclei in the tumor sections were seen in the less-well-differentiated proliferative zones while

TABLE II. Immunocytochemical Analysis of Sputa for Cells Which Overexpress p53 and Express Either Cytokeratins 8, 18 or a PAN* Cytokeratin

Patient number	Slide number	Immunocytochemical analysis		
		p53[a]	CK 8,18[b]	PAN[c] CK
03B	03B-T1	23	11	11
22	22-T1	3	1	
27	27-T1	3	0	
	27-T2	5	2	2
58	58-T1	7	5	1
61	61-T1	3	1	2
	61-T2	3	1	
62	62-T1	3	2	
	62-T2	1	1	
45	45-T1	0	0	
63	63-T1	3	1	
	63-T2	1	1	
67	67-T1	2	2	
	67-T2	6	4	
68	68-T1	0	0	
69	69-T1	0	0	

*A broad spectrum cytokeratin (CK) antibody consisting of a mixture of monoclonal antibodies to CK 1, 4, 5, 6, 8, 10, 13, 18 and 19.

[a]The number of cells on a Shandon megafunnel slide which overexpressed p53.

[b]The number of p53 positive cells that also expressed CK 8, 18. There were numerous other cells which stain with CK 8, 18 which did not overexpress p53.

[c]The number of p53 positive, CK 8, 18 negative cells that stained with the PAN CK antibody. The p53 positive cells which did not express CK 8, 18 did express at least one of the other cytokeratins detected by the PAN CK antibody. Numerous other cells stained with the PAN CK antibody which did not overexpress p53.

positive staining was not observed in the more differentiated areas and within the keratin whorls. This observation has been reported several times for squamous cell carcinomas [5,8,9]. Based on our data and previous reports in the literature, overexpression of p53 was only observed in the more undifferentiated cells in dysplastic and hyperplastic lesions [5,6,8,9]. In the study by Nees et al. [8], p53 accumulation was concomitant with expression of at least one of the simple epithelial type cytokeratins 8 and 18.

CONCOMITANT EXPRESSION OF P53 AND CYTOKERATINS 8 AND 18 IN SPUTUM CELLS

The intermediate filament proteins selectively expressed in epithelial cells are the cytokeratins (CK) which consist of a family of more than 20 proteins. The expression of cytokeratins in epithelial tissue is tightly correlated with the differentiated state of the cells. The pattern of expression of cytokeratins are often altered in pathological conditions such as preneoplastic lesions [14–16] and cytokeratin antibodies may be utilized in tumor diagnosis [17–19].

The cytokeratins 8 and 18 are expressed not only in simple squamous epithelial cells but also in some stratified epithelia such as the endocervical columnar and basal cells [18,20]. These cytokeratins are expressed in all adenocarcinomas and very often in squamous cell carcinomas [17–19]. In addition, CK 8, 18 are also extensively expressed in cervical intraepithelial neoplasia [18] and in the epithelia of the head and neck [8]. As mentioned above, Nees et al. [8] detected concomitant expression of CK 8, 18 and p53 in the more undifferentiated cells in dysplastic and hyperplastic lesions of the head and neck.

The megafunnel slides which had been previously stained for cells which overexpress p53 were stained with an antibody to CK 8, 18 (NCL-5D3, Novocastra Laboratory) to analyze for concomitant expression of these simple cytokeratins and p53. The detection system for CK 8, 18 is the Vectorstain Elite ABC kit (Vector) using the Vector VIP chromogen which elicits a purple color which contrasts with the brown DAB stain used for p53 detection. Figure 2 illustrates two atypical squamous cells which express both p53 and CK 8, 18. As shown in Table II, 53% of the cells which overexpressed p53 stained positive for CK 8, 18 (32 of 60 cells). We then stained the slides with a broad spectrum (PAN) CK antibody mix (C2562, Sigma) using the Vector VIP chromogen to determine if the p53 positive cells which were CK 8, 18 negative expressed different cytokeratins. For the cases examined, most of the p53 positive cells were positive for either the CK 8, 18, or at least one of the other cytokeratins detected by the PAN CK antibody. These data imply that most of the sputum cells which overexpress p53 are of epithelial origin. Moreover, our results are consistent, at least in part, with the observation of Nees et al. [8] that cells which overexpress p53 in dysplasia and hyperplasia also express CK 8, 18.

There were also numerous cells on the sputum slides which stained with the CK 8, 18 antibody and did not overexpress p53. These Pap stained CK 8, 18 positive cells were all basophilic and some cells exhibited atypical morphological features such as a basaloid shape

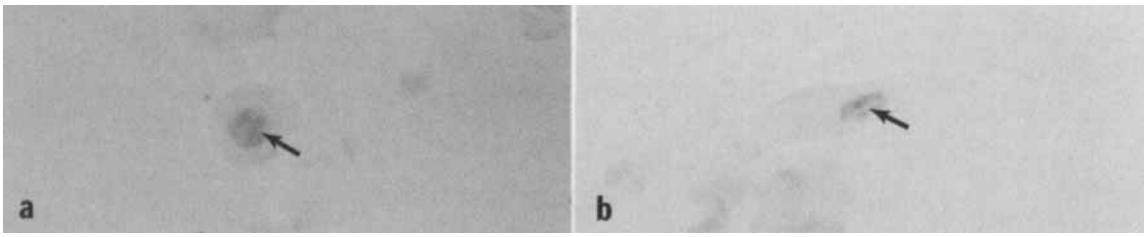

Fig. 1. Immunocytochemical staining of sputa cells which overexpress p53. **a** and **b** show cells which have DAB positive stained nuclei (arrow) with the p53 monoclonal antibody DO-7. The cell shown in a is a moderately differentiated squamous tumor cell and the cell depicted in b is a moderately atypical squamous cell, X600.

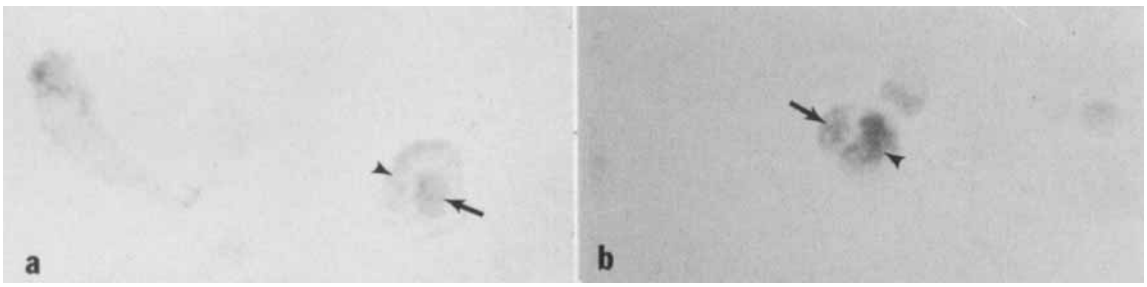

Fig. 2. Immunocytochemical analysis of atypical squamous cells from sputum which concomitantly express p53 and cytokeratin 8, 18. **a** and **b** show cells which have DAB positive stained nuclei (arrow) with the p53 monoclonal antibody, DO-7, and Vector VIP positive stained cytoplasm (arrowhead) with the CK 8, 18 monoclonal antibody, NCL-5D3, ×600.

and an altered nucleus to cytoplasmic ratio. Since these sputa were diagnosed as suspicious, CIS or carcinoma (Table I), some of these CK 8, 18 positive, p53 negative cells were morphologically consistent with tumor cells. However, many of these cells did not exhibit malignant criteria but depicted atypical morphological features which suggest that they were exfoliated from preinvasive lesions. In future studies to examine for p53 overexpression in cytologically non-malignant sputa samples collected before histological tumor diagnosis, we will also analyze for atypical cells which express CK 8, 18 and other cytokeratins. These studies may help identify a class of premalignant cells in sputa which are not presently diagnosed in the clinical screening of sputa slides.

ACKNOWLEDGMENTS

This study was supported by a Department of Energy grant DE-FG03-94ER61842/A000 and the University of Colorado SPORE in Lung Cancer grant No. CA 58187. The authors thank Sue Hainer for her assistance in the preparation of this manuscript.

REFERENCES

1. Sozzi G, Miozzo M, Pastorino U, Pilotti S, Donghi R, Giarola M, De Gregorio L, Manenti G, Radice P, Minoletti F, Della Porta G, Pierotti MA (1995): Genetic evidence for an independent origin of multiple preneoplastic and neoplastic lung lesions. Cancer Res 55:135–140.
2. Bennett WP, Colby TV, Travis WD, Borkowski A, Jones RT, Lane DP, Metcalf RA, Samet JM, Takeshima Y, Gu JR, Vähäkangas KH, Soini Y, Pääkkö P, Welsh JA, Trump BF, Harris CC (1993): p53 protein accumulates frequently in early bronchial neoplasia. Cancer Res 53:4817–4822.
3. Sozzi G, Miozzo M, Donghi R, Pilotti S, Cariani CT, Pastorino U, Della Porta G, Pierotti MA (1992): Deletions of 17p and *p53* mutations in preneoplastic lesions of the lung. Cancer Res 52:6079–6082.
4. Sundaresan V, Ganly P, Hasleton P, Rudd R, Sinha G, Bleehen NM, Rabbitts P (1992): p53 and Chromosome 3 abnormalities, characteristic of malignant lung tumours, are detectable in preinvasive lesions of the bronchus. Oncogene 7:1989–1997.
5. Ahomadegbe JC, Barrois M, Fogel S, Le Bihan ML, Douc-Rasy S, Duvillard P, Armand JP, Riou G (1995): High incidence of p53 alterations (mutation, deletion, overexpression) in head and neck primary tumors and metastases; absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesions. Oncogene 10:1217–1227.

6. Shin DM, Kim J, Ro JY, Hittelman J, Roth JA, Hong WK, Hittelman WN (1994): Activation of *p53* gene expression in premalignant lesions during head and neck tumorigenesis. Cancer Res 54:321–326.
7. Boyle JO, Hakim J, Koch W, van der Riet P, Hruban RH, Roa RA, Correo R, Eby YJ, Ruppert JM, Sidransky D (1993): The incidence of *p53* mutations increases with progression of head and neck cancer. Cancer Res 53:4477–4480.
8. Nees M, Homann N, Discher H, Andl T, Enders C, Herold-Mende C, Schuhmann A, Bosch FX (1993): Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: A possible molecular basis for the development of multiple tumors. Cancer Res 53:4189–4196.
9. Gusterson BA, Anbazhagan R, Warren W, Midgely C, Lane DP, O'Hare M, Stamps A, Carter R, Jayatilake H (1991): Expression of p53 in premalignant and malignant squamous epithelium. Oncogene 6:1785–1789.
10. Wang L-D, Hong J-Y, Qiu S-L, Gao H, Yang CS (1993): Accumulation of p53 protein in human esophageal precancerous lesions: A possible early biomarker for carcinogensis. Cancer Res 53:1783–1787.
11. Bennett WP, Hollstein MC, Metcalf RA, Welsh JA, He A, Zhu S-M, Kusters I, Resau JH, Trump BF, Lane DP, Harris CC (1992): *p53* Mutation and protein accumulation during multistage human esophageal carcinogenesis. Cancer Res 52:6092–6097.
12. Harris CC, Hollstein M (1993): Clinical implications of the p53 tumor-suppressor gene. N Engl J Med 329: 1318–1322.
13. Ferrell L, Saccomanno G (1988): Diagnostic cytology techniques including fine needle aspirations: Practical considerations. In Thurlbeck WM (ed): "Pathology of the Lung." New York: Thieme Medical Publishers, Inc., pp 105–114.
14. Sundberg JP, Erickson AA, Roop DR, Binder RL (1994): Ornithine decarboxylase expression in cutaneous papillomas in sencar mice is associated with altered expression of keratins 1 and 10. Cancer Res 54:1344–1351.
15. Smedts F, Ramaekers F, Troyanovsky S, Pruszczynski M, Robben H, Lane B, Leigh I, Plantema F, Vooijs P (1992): Basal-cell keratins in cervical reserve cells and a comparison to their expression in cervical intraepithelial neoplasia. Am J Pathol 140:601–612.
16. Lindberg K, Rheinwald JG (1989): Suprabasal 40 kd keratin (k19) expression as an immunohistologic marker of premalignancy in oral epithelium. Am J Pathol 134:89–98.
17. Ivanyi D, Groeneveld E, van Doornewaard G, Mooi WJ, Hageman PC (1990): Keratin subtypes in carcinomas of the uterine cervix: Implications for histogenesis and differential diagnosis. Cancer Res 50:5143–5152.
18. Smedts F, Ramaekers F, Troyanovsky S, Pruszczynski M, Link M, Lane B, Leigh I, Schijf C, Vooijs P (1992): Keratin expression in cervical cancer. Am J Pathol 141:497–511.
19. Broers JLV, Ramaekers FCS, Rot MK, Oostendorp T, Huysmans A, van Muijen GNP, Wagenaar SS, Vooijs GP (1988): Cytokeratins in different types of human lung cancer as monitored by chain-specific monoclonal antibodies. Cancer Res 48:3221–3229.
20. Bosch FX, Leube RE, Achtstätter T, Moll R, Franke WW (1988): Expression of simple epithelial type cytokeratins in stratified epithelia as detected by immunolocalization and hybridization in situ. J Cell Biol 106: 1635–1648.