

Retinoic Acid Receptor and Retinoid X Receptor Alterations in Lung Cancer Precursor Lesions¹

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ABSTRACT

Smoking prevention will decrease lung cancer incidence in time. However, early detection would improve lung cancer prognosis in subjects at risk provided that specific markers could be identified. We previously reported that retinoic acid receptor (RAR) and retinoid X receptor (RXR) expression was altered in lung tumors. *RAR-β* gene status could be derived from corresponding allelotyping and immunohistochemistry data. We now report the continued study on lung cancer precursor lesions. Fluorescence PCR-based assays were used for allelotyping at the *RAR/RXR* loci of: (a) 66 lung precursor lesions found at the free resection margins of 41 patients undergoing surgery for lung cancer (+ 31 paired tumors); and (b) bronchial cells also found at the free resection margins from 16 current and 8 never smokers operated on for noncancerous diseases. Three microsatellites located at *3p14-21* and *9p21* were also used for interwork comparison. Immunohistochemistry was additionally performed to evaluate *P53* and *RAR-β* expression in precursor lesions. χ^2 tests showed significant differences ($P < 0.05$) when comparing the results obtained from never smokers, smokers, squamous metaplasia, dysplasia + *in situ* carcinoma, and tumors. Microsatellite changes occurred frequently in all samples, but without specificity for any group ($P < 0.08-0.52$). They were globally correlated with tobacco exposure ($P < 0.04$), for which the *RAR-γ* marker appeared as a preferential target ($P < 0.004$). Few reparation error phenotypes were observed, mostly at the *RXR-α* and *RXR-γ* markers for which combined changes were also linearly increasing from never smokers to dysplasia + *in situ* carcinoma ($P < 0.05$ and $P < 0.03$). *RAR-β* marker losses also increased from the first to the last group studied ($P < 0.01$), with a concomitant decrease in *RAR-β* protein expression and correlated *p53* increased immunoreactivity ($P < 0.02$). Losses at *3p14*, *3p21*, and *P16* were frequent, but no significant differences between groups could be found. Unexpectedly, high constitutive homozygosity was observed near the *RAR-α* locus in squamous cell lung cancer cases. *RARs/RXRs* form homodimers or heterodimers involved in ligand binding. Their added alterations could result in a state of functional vitamin A deficiency in the affected bronchial cells. Further deletion events drawn from a limited repertoire of specific regions such as *3p14-21* and *9p21* could subsequently drive the deficient cells to invasive carcinoma.

INTRODUCTION

Stepwise modifications affecting the microscopic organization of the bronchial epithelium precede its malignant transformation. They are described as squamous metaplasia, dysplasia, and ISC³ and are

widely accepted as the natural history of squamous cell lung carcinoma. Metaplasia is the physiological repair process for the injured bronchial epithelium and is reversible, whereas dysplasia and ISC have been shown to be specific preneoplastic lesions (1–3). They are not believed to precede small cell lung cancer and proximal adenocarcinoma, although they are commonly associated with these tumors. Lung cancer precursor lesions demonstrate molecular alterations affecting different chromosome loci (for review, see Ref. 4). These alterations have also been observed in lung tumors and even in the normal bronchial mucosa of smokers (5, 6), where morphological changes caused by cigarette smoking were first noted years ago (7). It results in a “field cancerization” in which tobacco smoke carcinogens injure the entire lung. In the past, studies of premalignant lesions were difficult to perform due to technical limitations. However, new methods have been designed to visually improve identification of these lesions using fluorescence-based fibroscopy. Tissue microdissection has also been optimized to prepare specific DNAs (8) for genome screening for microsatellite changes such as LOH which are being used to locate specific lung cancer tumor suppressor genes (9–11).

Vitamin A and related retinoids are known to regulate normal lung development, maturation, and maintenance of the bronchial epithelium. Chronic vitamin A deficiency in hamsters results in the replacement of normal tracheal epithelium by a pseudostratified squamous epithelium. Reversal occurs when vitamin A is restored to the diet. A similar phenomenon occurs *in vitro* in retinoid-deprived bronchial cells (12). The control of gene expression by retinoids is complex and depends on the nature of the ligand, the type of ligand-binding proteins, and the interacting nuclear retinoid receptor genes. They include two different families: the *RAR* and *RXR*, with three subtypes for each (α , β , and γ) and several isoforms arising from promoter usage and alternate splicing. In addition, the *RARs/RXRs* form homodimers and/or heterodimers that bind to *cis*-acting response elements of retinoid target genes and interact also with varied coactivators or corepressors. *RXRs* are unusual because they bind to their response elements as homodimers. They also associate with many other hydrophobic ligand receptors such as the peroxisome proliferator-activated receptors. Thus, the retinoids extend their function to cross-modulate specific cell surface receptor signaling pathways (for review, see Ref. 13).

Retinoids have been tested in cancer prevention, but with somewhat puzzling results (14, 15). *RAR-β* is the best-studied member of the *RAR* family in the lung cancer process. It is believed to function as a tumor suppressor gene (16, 17). By studying nucleic acids as well as the encoded proteins (18), we reported that *RAR/RXR* had modified combined expression in lung tumors. The extension of the study to lung precursor lesions was difficult because tissue fixation is necessary for reliable identification and results in nucleic acid degradation. Moreover, the small size of the lesion precludes extensive immunohistochemistry screening. However, a microsatellite located near the *RAR-β* gene (D3S1283) was found to be very useful in reflecting the gene status in lung tumors. Therefore, *RARs/RXRs* were targeted for

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³ The abbreviations used are: ISC, *in situ* carcinoma; RAR, retinoic acid receptor; RXR, retinoid X receptor; LOH, loss of heterozygosity; RER, replication error phenotype.

more allelotyping studies testing RAR- β protein expression in pre-malignant lesions in an effort to further define the early roles of RAR/RXR in lung cancer.

MATERIALS AND METHODS

Patient Population. The patient population (lung cancer patients, $n = 41$) included patients who underwent lung surgical resection with a curative intent between 1989 and 1998 at the Fédération de Pneumologie, Center Hospitalier Universitaire (Nancy, France). pTNM (tumor-node-metastasis) tumor staging and histological grading were performed following the WHO guidelines. Examination of H&E-stained sections of the free resection margins detected a lung cancer precursor lesion that was graded by consensus as squamous metaplasia, dysplasia, or ISC as described previously (6). These samples were added to specimens left over from our previous study (2) of six subjects for whom more than one lung cancer precursor lesion was found by serial sections of the bronchial tree.

A control population without lung cancer was recruited from the archived samples of consecutive patients operated on at our institution between 1997 and 1999. It included patients who were either current smokers ($n = 16$) or lifelong never smokers ($n = 8$). As described above, an examination of the free resection margin was conducted.

Other sets of patients recruited at our institution were tested only for their constitutional status at the RAR- α and RAR- γ markers as described in the DNA section. Set 1 consisted of 50 current smokers who were enrolled successively in 1999 for an ongoing smoking cessation program. They were paired in sex, age (± 2 years), and tobacco consumption (± 2 pack/year) with the smoker population described above. Set 2 included the 40 patients with squamous cell lung carcinoma who were described previously (2, 18). Set 3 included 62 cases of pleural mesothelioma diagnosed between 1988 and 1998 at our institution that have been confirmed by the French Mesopath Panel. The patient and control populations are described in Table 1.

All graded resection free margins were serially cut (4- μ m sections) and laid on slides for tissue microdissection and immunohistochemistry testing.

DNA Purification. Five to ten consecutive sections of each lung cancer precursor lesion ($n = 66$), tumor ($n = 4$), and free resection margins from never smokers ($n = 8$) and smokers ($n = 16$) were used to separately collect all types of bronchial cells and paired normal control cells whenever necessary ($n = 44$) under the basal membrane by microdissection as described previously (8). Normal healthy tissue and paired tumor (macrodissected) obtained during surgery and kept frozen at -80°C were also used for DNA preparation ($n = 31$) with tissue proteinase K digestion for 24–72 h, phenol/chloroform extraction, and further ethanol precipitations.

For the set 1 subjects, blood DNA was prepared with the Nucleon BACC3 kit from Pharmacia (Orsay, France). For sets 2 and 3, frozen normal lung tissue from patients with squamous cell lung carcinoma or microdissected (mesothelioma) normal tissue provided constitutional DNA.

DNA Amplification. All of the reagents and the apparatus were from Pharmacia unless otherwise specified. All sense primers were labeled in 5' with CY5. The microsatellites were CA repeats chosen on the Genethon site,⁴ where the sequences of the flanking primers were also found. They were as follows: (a) at 17q12, D1751804 (RAR- α); (b) at 3p24.2, DS1283 (RAR- β); (c) at 12q13.13, D12S368 (RAR- γ); (d) at 9q34.3, D9S158 (RXR- α); (e) at 6p21, D6273 (RXR- β); (f) at 1q23, D1S2635 (RXR- γ); (g) at 3p14.2, D3S1300; (h) at 3p21, D3S1582; and (i) at 9p21, D9S171 (P16). Microsatellite amplifications were performed in a minithermocycler from MJ Research (Watertown, MA). Duplex PCRs were accomplished in a 10- μ l final volume including 0.20 μ l of Taq polymerase, 0.8 μ l of PCR mix, 0.8 μ l of the four deoxynucleotide triphosphate mixture (2 mM), 0.8 μ l of each primer pair (10 pM), and 1 μ l of DNA (10–50 ng). The PCR cycles were as follows: (a) a 10-min hot start at 95°C ; (b) 38 cycles of 94°C for 35 s, 65°C for 1 min, and 70°C for 1 min; and (c) a final extension step at 70°C for 10 min. At the end of the PCR, 4 μ l of loading dye (Dextran 2000; 5 mg/ml in deionized formamide) were mixed with each reaction, and 9 μ l of the mixture were heat-denatured and further electrophoresed on 7 M urea-acrylamide (6%) gels using an Alf express sequencing machine fitted with short plates. CY5-labeled molecular weight

Table 1 Patient population

Tumor histology	No. of patients	No. of squamous metaplasias	No. of dysplasias	No. of ISCs	Total no. of lung precursor lesions
SQCLC ^a	26	20	16	6	42
ADC	12	8	10	1	19
SCLC	3	2	0	1	3
Total	41	32	26	8	66
Controls ^b	24 ^c	2	1	0	3

^a SQCLC, squamous cell lung cancer; ADC, adenocarcinoma; SCLC, small cell lung carcinoma.

^b No cancer, 16 smokers and 8 never smokers.

^c This number includes 21 histologically normal epithelia.

markers (100, 150, and 200 bp) were deposited on each side of each gel. Duplex PCRs were always performed using the following primer sets together: (a) 3p14/3p21; (b) RAR- α /RXR- γ ; (c) RAR- γ /P16; and/or (d) RAR- β /RAR- γ . PCRs for only one microsatellite at a time were also performed with the same protocol. The results were analyzed online with the Allele Links software. LOH was defined as a complete disappearance of either microsatellite alleles in the bronchial cells or in the tumors when compared with the heterozygous paired normal sample. All patients were first screened for their constitutional status for a given microsatellite before proceeding to allelotyping of the sample. RER was present when, in a constitutional heterozygous sample, the size of the paired lung cancer precursor lesion or tumor alleles shifted. Whenever LOH or RER was observed, the experiment was repeated for confirmation. Representative data are shown on Fig. 1.

Immunohistochemistry. p53 antibodies (DO7; Novacastra, Newcastle-upon-Tyne, United Kingdom; 1:200) were used on lung cancer precursor lesions as described previously (2–18). Using an optic grid fitted onto the ocular ($\times 480$), the results were quantified by counting all of the premalignant cells and the number of those cells that were positively stained for p53. Positive specimens contained 30% stained cells. The RAR- β antibodies [Rp β (F)] were generous gifts from Dr. C. Rochette-Egly and P. Chambon (Institut de Génétique et Biologie Moléculaire de Strasbourg, Strasbourg, France). They were used only on the larger lung cancer precursor lesions (12a, 12e, 14, 24, 25, and 35, as described in Ref. 18).

Microsatellite Changes. The allelotyping results were broken down into five sample “progression” groups: (a) never smokers; (b) smokers; (c) squamous metaplasia; (d) dysplasia + ISC; and (e) tumors. Because DNA was not available for all experiments, the number of observed abnormalities or microsatellite changes in each group and for a given microsatellite is expressed as a percentage and as a ratio: LOH + RER number in the samples: number of heterozygous paired samples. All of the data are shown in Table 2, in addition to the immunohistochemistry results of our previous studies (2, 18).

Microsatellite Informativity. Because one patient can have several precursor lesions, the overall microsatellite informativity status in each group was expressed as a percentage and as a ratio of the total number of heterozygous patients: total number of homozygous + heterozygous patients in the group (Table 3).

Statistical Analysis. All of the computations were performed using BMDP software. All results were statistically analyzed by two-sided tests. Significance of difference for contingency tables (2×2) was assessed by Fisher's exact test; as for contingency tables ($n \times m$) by Pearson's χ^2 test. ANOVA was realized with the Kruskal-Wallis test, and mean comparisons were performed using the Mann-Whitney test. Microsatellite changes from never smokers to tumors and from never smokers to dysplasia + ISC were also tested with a χ^2 test for trended linear progression. The association of any microsatellite change with smoking, age, p53 immunohistochemistry results, and differences in the repartition of constitutional heterozygous samples was considered for each microsatellite and between groups.

RESULTS

Tobacco exposure and age were quite similar in all groups (40 ± 20 pack/years with $P < 0.74$ and 62 ± 10 years and $P < 0.08$). As seen in Fig. 1, microdissection was very efficient to prepare separate specific DNAs with consequently complete rather than near complete

⁴ ftp://ftp.genethon.fr/pub/Gmap.

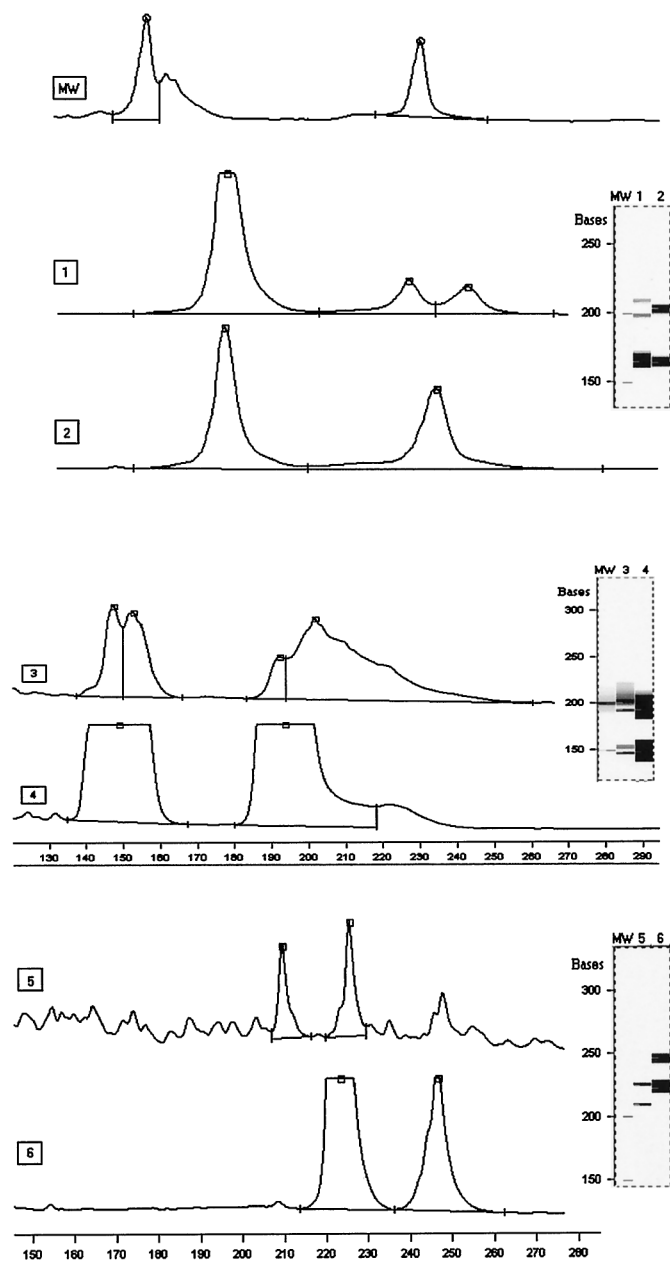


Fig. 1. Allelotyping results including specific gel aspects. *MW*, molecular weight standards (100, 150, and 200 bases). Constitutive DNA (1) and DNA purified from the normal bronchial epithelium (2) of patient 44; duplex PCR for the P16 and *RAR-γ* markers with LOH for *RAR-γ*. Constitutive DNA (3) and DNA from the premalignant lesion (4) of patient 8; duplex PCR for the *RAR-β* and *RAR-γ* markers with concomitant LOH for both markers. Constitutive DNA (5) and tumor DNA (6) from patient 4 with a RER for the *RAR-α* marker.

LOH in the specimens. The amplicon peak was often larger for the remaining allele. Although tumors were carefully macrodissected (70% of tumor cells), it is possible that LOHs have been hidden by the stromal cells. RER by allele expansion (Fig. 1, panel 6) was observed once at the *RAR-α* marker in sample 23P, three times at the *RXR-γ* marker in samples 4T, 39T, and 18(g)P, once at 3p14 in 57 but 7 times at the *RXR-α* marker in samples 7T, 10T, 20P, 24P, 30T, 37P, and 61 with DNA prepared from either fixed or frozen tissue (underlined sample numbers). There were neither global ($P < 0.63$) nor individual ($P < 0.08-0.52$) microsatellite change differences between the five sample groups (Table 3). The overall number of microsatellite changes was correlated to the level of tobacco consumption

($P < 0.04$) but not to the age of the patients ($P < 0.82$). Microsatellite changes at the *RAR-α* marker were rare in most specimens, but the microsatellite informativity was low in precursor lesions (22%) and tumors (23%). LOHs at the *RAR-β* marker were present in all types of precursor lesions (35%) and tumors (60%) and were even seen in smokers (22%). There was an increasing number of LOHs at this marker from group 1 to group 5 ($P < 0.01$), with no significant difference in the repartition of heterozygous samples in all groups ($P = 0.3$). LOH at the *RAR-γ* marker was common, but the microsatellite informativity was low in premalignant specimens (28%) and tumors (29%). LOH at this locus was strongly correlated with tobacco consumption expressed in pack/years ($P < 0.004$). LOH or, more often, RER for D9S158 (*RXR-α*) was rarely observed in precursor specimens (11%) and in smokers (29%) but was more frequently seen in tumors (35%). However, the repartition of heterozygous samples in the five groups was nonhomogenous ($P = 0.2$). LOH at the *RXR-β* marker was frequent, but with a low overall microsatellite informativity in all groups. There was a significant increase in the microsatellite changes for the *RXR-γ* marker from groups 1 through 4 ($P < 0.03$). Microsatellite changes for D3S1300 (*3p14*) were frequent in all specimens, but with a linear decrease in the number of heterozygous samples from groups 1 to 5. LOH at the *3p21* locus was also common, with a higher but not significant prevalence seen in the dysplasia + ISC group. LOH at the *P16* microsatellite was present in 40% of smokers, 44% of precursor lesions, and 71% of tumors. Six patients had several premalignant lesions for which the allelic losses were not similar. Moreover, different microsatellite alleles were also lost in the different specimens (patients 1, 6, and 12).

Lung cancer precursor lesions analyzed in this study (Table 2) were paired with nine tumors that were screened previously by semiquantitative immunohistochemistry for the expression of *RAR-α*, *RAR-β*, *RXR-α*, and *RXR-β* and by quantitative reverse transcription-PCR for *RAR-γ* expression in specimens 27T and 29T. The allelotyping data are fully consistent with the immunohistochemistry results: a decrease in *RAR-β* is accompanied by LOH in heterozygous samples; whereas increased or normal *RAR-α* and *RXR-α* expression did not coincide with any microsatellite change. With regard to the *RXR-β* and *RAR-γ* testing, the data are inconsistent. In premalignant specimens 12a, 14, and 35, LOH at the *RAR-β* marker and decreased *RAR-β* immunostaining were concomitant (Fig. 2), whereas no decrease in *RAR-β* expression and no change for D3S1283 were seen in premalignant samples 12e, 23, and 24. P53-positive staining was observed for only 35% of dysplasias but in all ISCs and in all paired tumors (except for one case) and therefore strongly correlated with the higher grade of lung cancer precursor lesion ($P < 0.001$). *RAR-β* LOH was also correlated with P53-positive staining ($P < 0.02$).

For D17S1804 and D12S368, an unexpectedly high rate of constitutional homozygosity was found in premalignant and tumor samples. Based on these results, we proceeded with genotyping for the *RAR-α* and *RAR-γ* markers: 40 more subjects (test 1 population), for a total of 66 squamous cell lung carcinoma cases; 48 more smokers (test 2 population) without lung cancer, for a total of 64 subjects and for comparison: 62 mesothelioma (test 3 population). Patients with squamous cell lung carcinoma were less heterozygous than expected for both the *RAR-α* (36%) and *RAR-γ* (29%) markers. Smokers were more heterozygous for the *RAR-α* microsatellite (58%) but were equally as homozygous for the *RAR-γ* microsatellite (27%). The low rate of heterozygosity at both loci persisted when the 18 additional nonsquamous cell lung tumors were included (36% and 29%, respectively). By contrast, in mesothelioma, 70% and 44% of subjects, respectively, were found to be heterozygous.

Table 2 Study results; panel A for squamous metaplasia; panel B for dysplasia + ISC with paired lung tumors (T); panel C for smokers and never smokers (bronchial epithelium)^a

A: SQUAMOUS METAPLASIA

MS	RAR α		RAR β		RAR γ		RXR α		RXR β		RXR γ		3p14		3p21		P16	
	N	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T
1c	□	□/nd	○	○/nd	●	○/nd	○	○/nd	□	□/nd	□	□/nd	○	○/nd	□	□/nd	□	□/nd
1e	□	□/nd	○	○/nd	●	○/nd	○	○/nd	□	□/nd	□	□/nd	○	○/nd	□	□/nd	□	□/nd
1f	□	□/nd	○	○/nd	○	○/nd	○	○/nd	□	□/nd	□	□/nd	○	○/nd	□	□/nd	□	□/nd
2	○	○↑	□	□=	□	□	□	□↑	□	□=	□	□	●	●	□	□	□	□
3	○	○	○	●	□	□	□	□	□	□	●	●	□	□	○	●	□	□
4	□	□	○	●	□	□	○	○	□	□	○	◆	□	□	○	○	○	●
5	□	□	●	○	□	□	○	○	□	□	□	□	□	□	□	□	□	□
6a	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	●	○/nd	○	○/nd	●	○/nd	□	□/nd
6b	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	●	○/nd	□	□/nd
6c	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	●	○/nd	□	□/nd
6d	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	●	○/nd	□	□/nd
6e	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	●	○/nd	□	□/nd
6f	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	●	○/nd	□	□/nd
6g	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	○	○/nd	□	□/nd
6h	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	○	○/nd	□	□/nd
7	□	□	●	○	□	□	○	◆	□	□	○	○	□	□	□	□	○	○
8	○	○	●	○	●	○	○	●	□	□	●	○	●	○	□	□	□	□
9	□	□	○	●	●	○	○	○	□	□	□	□	□	□	●	○	□	□
10	○	○	○	○	□	□	●	◆	●	●	●	○	●	●	□	□	●	○
11	□	□	○	○	●	●	□	□	□	□	□	□	□	□	□	□	□	□
12c	□	□	●	●	□	□	○	●	□	□	○	●	□	□	○	●	●	●
13	□	□=	○	●↓	□	□	□	□↑	□	□=	○	○	□	□	□	□	○	●
14	●	○	●↓	●	□	□	○	●	□	□	○	●	□	□	●	○	○	○
15	□	□/nd	○	○/nd	○/nd	○/nd	□	□/nd	●	○/nd	□	□/nd	●	○/nd	●	○/nd	□	□/nd
16	□	□	○	○	□	□	○	○	□	□	○/nd	○	□	□	○	○	○	●
17	○/nd	○/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	●	○/nd	□	□/nd
18d	□	□=	○	●↓	□	□	□	□↑	□	□=	●	○	□	□	○	○	○	○
19	○/nd	○/nd	□	□/nd	□	□/nd	○	○/nd	□	□/nd	□	□/nd	○	○/nd	○	○/nd	□	□
20	□	□	○	○	●	●	◆	○	○	○	○	□	□	□	□	□	□	□
21	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	●

B: DYSPLASIA

MS	RAR α		RAR β		RAR γ		RXR α		RXR β		RXR γ		3p14		3p21		P16	
	N	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T
22d	○	○/nd	□	□/nd	●	○/nd	○	○/nd	□	□	●	○/nd	□	□/nd	○	○/nd	●	○/nd
22e	●	○/nd	□	□/nd	●	○/nd	○	○/nd	□	□	●	○/nd	□	□/nd	○	○/nd	●	○/nd
23	◆	○/nd	○=	○/nd	□	□/nd	○	○/nd	○	○/nd	●	○/nd	□	□/nd	○	○/nd	○	○/nd
24	□	□/nd	○=	○/nd	□	□/nd	◆	○/nd	□	□/nd	□	□/nd	□	□/nd	○	○/nd	□	□/nd
25	○	○=	□	□↓	□	□	□	□↑	●	○=	●	○	□	□	□	□	□	□
26	□	□/nd	□	□/nd	●	○/nd	○	○/nd	○	○/nd	□	□/nd	□	□/nd	□	□/nd	●	○/nd
6i	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	○	○/nd	□	□/nd
6j	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	○	○/nd	□	□/nd
6k	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	○	○/nd	□	□/nd
6l	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	○	○/nd	□	□/nd
6m	□	□/nd	□	□/nd	□	□/nd	□	□/nd	□	□/nd	○/nd	○/nd	○	○/nd	○	○/nd	□	□/nd
27	□	□=	□	□↓	□	□↓	○	○	○	○	●	○	□	□	○	●	●	●
28	□	□	□	□	□	□=	○	○	□	□	●	○	□	□	○	○	□	□
29	●	○=	○	●↓	□	□↓	○	○↑	□	□	□	□	□	□	○	○	○	□
30	□	□	□	□	□	□	○	◆	□	□	○	●	□	□	○	○	●	●
12a	□	□	●↓	●	□	□	○	●	□	□	○	●	□	□	○	●	●	●
12e	□	□	○=	●	□	□	○	●	□	□	○	●	□	□	○	●	○	●
31	□	□=	□	□=	●	●	□	□	●	●=	●	○	□	□	□	□	○	●
32a	□	□	□	□	●	●	○	○	○	○	□	□	□	□	□	□	□	□
32c	□	□	□	□	●	●	○	○	○	○	□	□	□	□	□	□	○	○/nd
18a	□	□=	○	●↓	□	□	□	□↑	□	□=	○	○	□	□	●	○	●	○
18c	□	□=	○	●↓	□	□	□	□↑	□	□=	●	○	□	□	○	○	●	○
18e	□	□=	○	●↓	□	□	□	□↑	□	□=	●	○	□	□	○	○	○	○
18g	□	□=	○	●↓	□	□	□	□↑	□	□=	◆	○	□	□	○	○	○	○
33	□	□/nd	●	○/nd	□	□/nd	○	○/nd	○	○/nd	○/nd	○/nd	□	□/nd	●	○/nd	□	□/nd

B: In Situ CARCINOMA

MS	RAR α		RAR β		RAR γ		RXR α		RXR β		RXR γ		3p14		3p21		P16	
	N	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T	P	T
34	□	□	●	●	□	□	○	○	□	□	□	□	●	○	○	●	□	□
35	□	□=	●↓	●↓	□	□	○	□=	□	□=	□	□	□	□	□	□	□	□
36	●	○/nd	□	□	○	○/nd	○	○	○	○	□	□	○	○	○	●	□	□
37	□	□↑	□	○=	●	●	◆	○=	□	□=	●	●	□	□	●	●	●	●
38	□	□	●	●	●	○	○	○	□	□	●	○	□	□	□	□	○	●
39	□	□	○	●	○/nd	○	○	●	□	□	○/nd	◆	○	○	○	○	○	□
40	○	○/nd	□	□	□	□	○	○	□	□	○	○	□	□	□	□	□	□
41	nd	nd	●	○/nd	nd	nd	□	□/nd	□	□/nd	nd	nd	●	○/nd	○	○/nd	nd	nd

Table 2 Continued

C: SMOKERS										C: NEVER SMOKERS												
N	MS	RAR α	RAR β	RAR γ	RXR α	RXR β	RXR γ	3p14	3p21	P16	N	MS	RAR α	RAR β	RAR γ	RXR α	RXR β	RXR γ	3p14	3p21	P16	
42		○	□	●	○	○	●	□	□	□	58	●	□	□	●	□	□	□	○	□	□	□
43		●	□	●	○	○	○	●	○	□	59	○	○	□	○	○	□	□	○	○	□	□
44		□	○	●	●	●	□	●	□	●	60	○	□	□	□	□	□	□	□	○	□	□
45		□	○	○	●	○	○	○	●	○	61	○/nd	□	□	◆	□	□	□	○	○	□	□
46		□	○	□	□	□	○	□	○	□	62	○	○	□	○	□	□	□	●	□	□	□
47M		●	○	□	□	□	□	○	●	□	63	□	□	□	□	□	○	●	□	□	●	□
48		□	○	□	○	○	●	○	○	○	64	□	□	□	□	□	○/nd	□	□	□	□	□
49		○	□	○	○	□	○	□	□	□	65	○	□	□	□	□	□	○	□	□	□	●
50D		○	□	●	□	□	□	○	○	●												
51		□	□	□	□	□	●	●	○	□												
52		□	○	●	□	○	●	●	□	□												
53M		○	□	□	□	□	○	○	□	□												
54		○	●	●	□	□	○	●	□	□												
55		○	□	●	□	□	□	●	□	□												
56		○	○	□	□	□	○	□	□	○												
57		○	●	○	○	○	◆	□	□	□												

^a MS, microsatellite name; N, patient number (fixed or frozen tissue (bold); P, precursor lesion; T, tumor; □, homozygous; ○, heterozygous; ●, LOH; ◆, RER; nd, not done; ↑, RAR/RXR expression increased; ↓, RAR/RXR expression decreased; =, RAR/RXR expression normal.

DISCUSSION

We observed combined RAR/RXR expression changes in lung tumors (18) and undertook a new study to further define the status of RAR/RXR in lung precursor lesions. Within the technical restrictions detailed above, allelotyping for microsatellites located near the RAR/RXR gene loci was performed. The microsatellites chosen were the closest possible microsatellites to the concerned genes, except for the more distant RAR- γ marker, which is, in fact, close to the RAS gene, an oncogene known to play a role in lung cancer, and to the fragile site FRA 12A. Interestingly, losses of this marker were correlated with tobacco exposure. However genetic losses were not consistent with our previous results in tumors 27 and 29. D6S273 has just been moved on the Genethon map from 6p11 to 6p22, near to the keratin gene cluster. High levels of homozygosity were found in all studied samples, and hemiallelic losses did not match the previous immunohistochemistry results. However, Virmani *et al.* (19) reported that 6p21 is a region of frequent allelic losses in non-small cell lung cancer. Unfortunately, there were no reliable RXR- γ antibodies to match the

RXR- γ marker allelotyping results. As shown in Table 2, the allelotyping data of the other microsatellites were mostly consistent with the previous tumor immunohistochemistry screening. For the RAR- α marker, there was neither tumoral LOH nor decreased gene expression, but there were too many homozygous samples. Immunohistochemistry performed on five lung cancer precursor lesions with or without LOH for D3S1283 demonstrated decreased expression of the RAR- β gene when there was also a LOH. A similar concordance was found previously in 75–86% of 76 lung tumors (18). Allelic losses were already present in smokers, but not in lifelong never smokers. However, from so few cases, it is difficult to conclude any tobacco carcinogen-specific action. Decreased RAR- β quantity would impair heterodimerization with RXR partners. In combination with other RAR/RXR disruptions (promoter methylation), this could result in a functional cellular retinoid deficiency. Similar events have been described recently for RAR- γ and RXR- α in the skin as a consequence of UV irradiation (20). Lung tumors were found to overexpress the RXR- α protein in combination with RAR- β underexpression (18),

Table 3 Allelotyping results

Microsatellite names and loci	D17S1804 RAR- α 17q12	D3S1283 RAR- β 3p24.2	D12S368 RAR- γ 12q13.13	D9S158 RXR- α 9q34.3	D6S273 RXR- β 6p21-3	D1S2635 RXR- γ 1q23	D3S1300 3p14.2 3p14	D3S1582 3p21 3p21	D9S171 p16 9p21
Heterozygous vs. homozygous ^a									
NS ^b	6 vs. 2	2 vs. 6	0 vs. 8	4 vs. 4	0 vs. 8	3 vs. 5	5 vs. 3	3 vs. 5	2 vs. 6
S	10 vs. 6	9 vs. 7	10 vs. 6	7 vs. 9	7 vs. 9	12 vs. 4	12 vs. 4	7 vs. 9	5 vs. 11
P	12 vs. 48	34 vs. 29	17 vs. 44	35 vs. 28	13 vs. 50	36 vs. 18	27 vs. 36	42 vs. 21	24 vs. 38
T	7 vs. 20	20 vs. 11	9 vs. 22	22 vs. 7	8 vs. 24	19 vs. 12	8 vs. 23	14 vs. 17	14 vs. 17
Specimen microsatellite changes (%) ^c									
NS	16	0	0	50	0	0	40	0	100
S	20	22	70	29	14	33	58	29	40
P	31	35	88	11	45	57	30	40	44
T	0	60	56	35	56	44	38	43	71
Microsatellite informativity (%) ^d									
NS	75	33	0	50	0	37	62	37	25
S	63	56	63	44	44	75	75	44	31
P	20	54	28	56	20	67	43	67	63
T	25	65	29	75	25	61	25	45	45
Expected informatives (%)	82	68	86	70	85	86	72	82	72

^a In number of tested specimens.

^b NS, nonsmokers; S, smokers; P, precursor lesions; T, tumors.

^c LOH + RER in the specimens/number of heterozygous paired specimens.

^d Number of heterozygous subjects/total number of subjects. Results are underlined when they concern at least 50% of heterozygous subject.

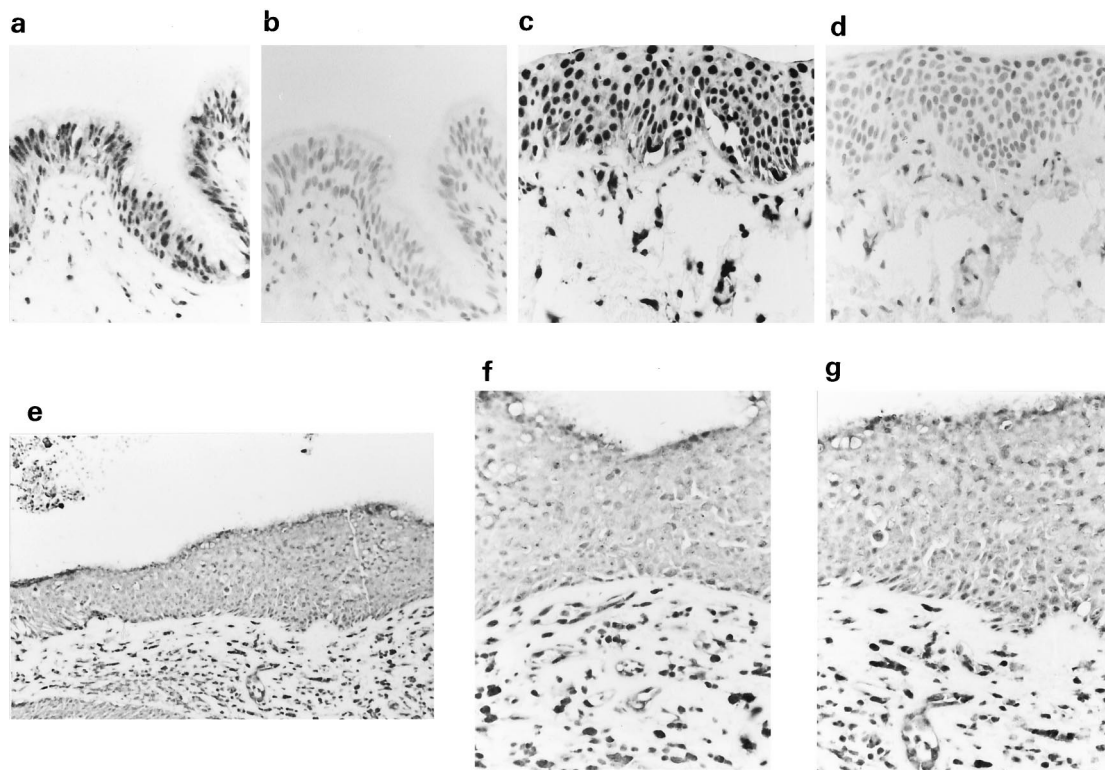


Fig. 2. Immunohistochemical investigation of RAR- β protein in normal bronchial epithelium and preinvasive lesions. Normal bronchial epithelium (a) and squamous metaplasia lesion (c) show a normal and similar expression of RAR- β protein in nuclei from both epithelial cells and submucosal cells; corresponding controls, b and d. Squamous metaplasia (e), squamous metaplasia with dysplasia (f), and ISC (g) lesions show a strong decrease in expression of RAR- β , as judged in comparison to the degree of expression in submucosal cells.

possibly as an adaptation to retinoid deficiency. Normal expression or overexpression of RXR- α has also been observed in breast tumors and mouse skin tumors (21). Conflicting results placing microsatellite instability in lung tumors between 0% and 60% have been published previously (22, 23). We recently participated in extensive lung tumor allelotyping (18, 24), and although different methodologies were used, the results were consistent: RERs were rare. For the present study, fixed or unfixed tissues were used, and, as found previously, RERs were rare. The discrepancies might depend on: (a) the type of polymorphic marker used (CA repeats *versus* trinucleotides or tetranucleotides, for which the spontaneous rate of mutation can be very high); (b) the criteria used to define RER as a shifting of one or both alleles; and (c) microsatellite instability as RER at one or several markers. Low polymorphism of microsatellites at certain loci may be explained by the rather conservative structure of the genes lying at such loci. In squamous cell lung carcinoma, the frequency of homozygosity at D17S1804 (RAR- α , 17q12) was unusual and was not found in smokers or in patients with mesothelioma. However, higher rates of homozygosity at D12S368 (RAR- γ , 12q13.13) were found in both squamous cell lung carcinoma and smokers by comparison with mesothelioma cases. The clinical and biological significance of such homozygosities needs to be investigated carefully, even in comparable ethnic populations, to eliminate a founder effect. Indeed, homozygosities could favor altered DNA recombinations.

In general, it is thought that molecular damage incidence increases as histopathological lung cancer precursor lesions progress from hyperplasia to ISC. Identifying the genes targeted at each step would reconstitute the natural history of squamous cell lung carcinoma. However, comparisons between investigations are difficult because of differences in methodology and in criteria for studying and scoring the molecular disruptions. The systematic screening of lung tumors for

allelic losses led to the identification of multiple distinct regions of recurrent deletions at 3p, 17p, 9q, 5q, 13q, 8p, and 11p (approximately ranked by decreasing frequency), suggesting that these regions contain unidentified genes involved in lung cancer (24). Based on these findings, allelic losses have been reported in lung cancer precursor lesions mainly on 3p and 9p regions (4, 9–11, 25). Four hot spots have been identified in lung tumors at 3p12, 3p14, 3p21, and 3p25, and a candidate tumor suppressor gene at 3p14, fragile histidine triad (FHIT), has been cloned (26). Whereas LOH at 3p14 has been associated with the presence of aberrant transcripts that involve partial deletions of this gene, other studies have suggested that LOH can occur without abnormalities and is influenced by the proximity of the *FRA 3B* region on which tobacco exposure may be causal (27). This might also be the case for the RAR- γ marker used here. Transfer of DNA fragments from 3p21.3 into tumor cell lines suggested that the region has tumor suppressor gene activity (28). The protein tyrosine phosphatase gene and a mitogen-activated protein kinase are potential candidates located on 3p21, whereas others have been eliminated (25). *P16/CDKN2* is located on 9p21; a high percentage of alterations of this gene has been observed in many tumors types, but the frequency of LOH found in lung tumors is higher than the frequency of mutations, suggesting that other tumor suppressor genes reside on 9p (25). The size of the lung cancer precursor lesion prevents fine mapping, but the 3p21 region remains a good candidate for tumor suppressor gene localization.

Allelotyping data have been obtained in current smokers, former smokers, and nonsmokers for normal and abnormal bronchial epithelium. Wistuba *et al.* (6) studied several microsatellites at the 3p14–21-24 loci and the retinoblastoma, *P53*, and *P16* (D9S171) regions. No molecular changes were found in nonsmokers, but interpretation of the findings was potentially limited by the different age distribution

of the nonsmokers, who were significantly younger than the smokers. Among the smokers, there was a modest correlation between the number of molecular changes/subject and smoking exposure, but the variation between current and former smokers was not significant. LOH occurred mainly at *3p* (38%) and *P16* (23%). At *3p21*, LOH was detected in histologically normal epithelium, whereas *3p14* losses were detected only in dysplasia. RER was detected in smokers (64%), even in normal histological specimens. In addition, there was a loss of the same allele of a polymorphic marker in the same patient. By contrast, Mao *et al.* (5) found few *3p14* LOHs in nonsmokers (20%) but found more in current smokers (85%) than in former smokers (45%); premalignant lesions were also more frequent in current smokers. *P16* losses were found in 23% of the specimens. *P53* losses at *17p13* reached 18%, but no RER was reported. The alleles lost in the different biopsies for the studied polymorphic markers were different in the same patient. It is difficult to reach a consensus from these studies, but our results concerning the smokers are more in accordance with the latter work.

Several studies have repeatedly shown p53 increased immunoreactivity usually reflective of *P53* mutations in premalignant lung lesions (2, 29, 30). Other abnormalities, including an increased cellular proliferation rate (2) and c-myc (2) and bcl-2 overexpression with changes in the keratin pattern toward squamous epithelia, have also been described previously (31, 32).

Smoking prevention is the first tool against lung cancer prevention. For former smokers, early detection is necessary because lung cancer prognosis remains poor, despite several therapeutic improvements. Many efforts have been directed toward the identification of biomarkers for early detection of lung cancer (33) and chemoprevention (14, 25). This study and our previous study (18) suggest that retinoid deficiency may be among the first events contributing to lung tumorigenesis and imply that retinoids could be used in lung cancer chemoprevention. Aerosolized early on site, they could reverse the deficiency in stabilizing *RAR/RXR* expression for increased ligand binding to restore normal cellular differentiation. Early lung cancer detection could associate yeast functional assays of *RAR-β* and *P53* in shed bronchial cells and complementary testing of microsatellites, such as D3S1283, whose loss is correlated with decreased *RAR-β* expression.

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