Expression of Retinoid Receptor Genes and Proteins in Non-Small-Cell Lung Cancer

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Background: Retinoids can suppress carcinogenesis in high-risk non-neoplastic bronchial lesions and can reduce the risk of second primary non-small-cell lung cancer (NSCLC). The effects of retinoids are mediated by nuclear receptors, i.e., the retinoic acid receptors (RARα, RARβ, and RARγ) and the retinoid X receptors (RXRα, RXRβ, and RXRγ). We investigated whether abnormalities in the in vivo expression of retinoid receptors are observed in NSCLC. Methods: Expression of retinoid receptors in paired specimens of normal and cancerous tissues from the lungs of 76 patients with NSCLC was studied by use of anti-Retinoid receptor antibodies (except those against RXRγ) and immunohistochemistry. RAR messenger RNAs were analyzed by use of in situ hybridization and by reverse transcription–polymerase chain reaction (RT–PCR). Samples were also studied for loss of heterozygosity (LOH) at chromosome 3p24. All P values are two-sided. Results: All studied receptors were expressed in normal lung cells and in high-risk non-neoplastic lesions. In tumor cells, overexpression of RXRβ and RARα was frequently observed. In contrast, RXRβ expression decreased in 18% of the tumor specimens. Furthermore, there was a marked decrease in the expression of RARβ in 63% of the tumors (P<0.0001). Decreased expression of RARγ was observed by RT–PCR in 41% of the tumors (P<0.0001). LOH at 3p24 was observed in 41% of the tumor specimens from informative patients and in 20% of the non-neoplastic lesions. Conclusions: Expression of RARα and RXRα is either normal or elevated in NSCLC. In contrast, a large percentage of tumors show a marked decrease in the expression of RARβ, RARγ, and RXRβ as well as a high frequency of LOH at 3p24, which was also observed in non-neoplastic lesions. These data suggest that altered retinoid receptor expression may play a role in lung carcinogenesis. [J Natl Cancer Inst 1999;91:1059–66]

In spite of antismoking efforts and advances in therapy, lung cancer, because of its high incidence and its low 5-year survival rate (about 14%), is still the most common cause of death by cancer in developed countries (1). Therefore, new approaches, such as chemoprevention, need to be developed. During the last several years, a body of clinical and experimental studies has established retinoids as potent regulators of epithelial cell growth (2), with reported benefit in the prevention and treatment of different neoplastic conditions. Retinoids can suppress carcinogenesis in high-risk non-neoplastic oral and bronchial lesions (3,4) and can prevent the development of second primary cancers in patients with head and neck and lung cancer (4–6).

The regulation of normal and tumor cell growth and differentiation by retinoids results from their ability to control gene expression. These effects of retinoids are mediated by nuclear receptors, which belong to the steroid hormone receptor superfamily and function as ligand-activated transcription factors. Two families of receptors have been identified: the retinoic acid receptors (RARα, RARβ, and RARγ) and the retinoid X receptors (RXRα, RXRβ, and RXRγ) (7). While the three RAR types show strong affinities for both the all-trans and 9-cis isomers of retinoic acid, the RXRs show a marked specificity for the latter molecule. It is now well established that RXR/RAR heterodimers are the functional units that transduce the retinoid signal in vivo (8). These heterodimers activate transcription by binding to retinoic acid response elements located in the promoter region of retinoic acid-inducible target genes (7,9). Such genes include the RARβ2 gene (9,10), whose expression is enhanced by retinoic acid.

The mechanisms through which retinoids suppress carcinogenesis are complex and far from understood, in part because of the fact that a large number of genes involved in tumor cell differentiation and proliferation include retinoic acid response elements in their promoters (9,11,12). Retinoids also inhibit tumorigenesis and tumor growth through their ability to induce either apoptosis (i.e., programmed cell death) (13–15) or terminal differentiation (16). In addition, retinoids play a central role in tumor stroma production and thus in the control of tumor progression and invasion through their ability to regulate the expression of matrix metalloproteinases (17), transforming growth factor-β (12,18), and cell cycle regulator proteins, such as cyclin-dependent kinase I, p16, or p21 (16,19).

Up to now, the use of retinoids in clinical trials has been limited because of their pharmacologic effects and side effects (4). In addition, a majority of human NSCLCs or cell lines are resistant to all-trans-retinoic acid (20), and the mechanism of retinoic acid resistance has not been elucidated. However, more potent synthetic retinoids selective for an RAR or an RXR type and without the classic pattern of retinoid toxicity are now available (4,6,21). Moreover, novel synthetic retinoids that are able to overcome the retinoic acid resistance in NSCLC are now being developed (22,23).

The fact that the expression of RARβ2 messenger RNA (mRNA) is decreased or suppressed in a number of tumors, including lung carcinoma (24–28), squamous cell carcinoma of the head and neck (29), and breast carcinoma (30,31), suggests that it might be involved in cancer development. Such a deregulation could represent one of the mechanisms through which tumor cells escape from normal cellular homeostasis. In addition, the observation that transfectant lung cells re-expressing RARβ were less tumorigenic than the parental cells (32) and were res-

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cued for terminal differentiation (16) and retinoic acid sensitivity (33) raised the possibility that RARβ acts as a tumor suppressor. Thus, the analysis of RARs either in tumors or in bronchial noncancerous lesions is important for treatments and/or prevention of second primary tumors. Since no information is available concerning the in vivo expression of RAR and RXR proteins in NSCLC, this study was designed with the help of a panel of specific antibodies to evaluate the relative levels of expression of the different receptors and their relationship to mRNA expression and loss of heterozygosity (LOH) at chromosome 3p24, which includes the region coding for RARβ.

**SUBJECTS AND METHODS**

**Normal Subjects, Patients, and Surgical Specimens**

Patients with non-small-cell lung cancer (NSCLC) (n = 83 [75 males and eight females]; median age ± standard deviation, 61 years ± 9 years) not subjected to preoperative radiotherapy or chemotherapy were enrolled consecutively in this study after they gave written informed consent. The agreement for this study was given by the Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Nancy. All of these patients had a history of smoking. The histologic types of lung cancer were defined according to the criteria of the World Health Organization (34). There were 41 squamous cell carcinomas (SCCs) and 42 adenocarcinomas (ADCs). Disease staging and pathology were defined after surgery according to the recommendations for NSCLC staging given at the Fifth World Conference on Lung Cancer (35). There were 29 stage I tumors, 17 stage II tumors, 32 stage III tumors, and five stage IV tumors.

Normal lung specimens evaluated as controls were obtained (a) from each patient from a lobe free of any pathologic evidence of malignancy and (b) from nonsmokers undergoing lobectomy either for a coin lesion of the lung (n = 8) or for lung metastasis from primary cancers located in other sites of the body (n = 9).

Studies were conducted on paraffin-embedded tissues and/or the corresponding frozen samples. To determine whether the expression of retinoid receptors differs in tumors compared with distantly normal tissues and/or the corresponding frozen samples. To test the specificity of the signals, we performed negative control experiments either by omitting the primary antibody, by substituting the primary antibody with a nonimmune serum, or by omitting both the primary and the secondary antibodies.

**Immunohistochemistry and Semiquantitative Evaluation of Expression of RARs and RXRs and RARβ**

**Antibodies.** A panel of monoclonal and polyclonal antibodies was used. The mouse monoclonal antibodies MAB 9a(F) (36) and MAB 16RX3E8 (37) were used for the detection of RARα and RXRa proteins, respectively. Rabbit polyclonal antibodies were used for the detection of RARβ [RPβ(F) (38)], RARγ [RPy (F) (39)], and RXRα [RPRXα (A) (37,40)]. Because of the lack of antibodies recognizing RXRγ, the distribution of this receptor was not investigated. The specificity of all antibodies used in this study was previously checked by different techniques including western blotting (36–39). By this technique, the antibodies specifically recognize the cognate RAR not only in transected cells but also in a variety of cell lines and in mouse tissues. In addition, they do not reveal any signal, either by western blotting or by immunohistochemistry, when they are tested on knockout mouse embryos for the corresponding RARβ (40,41).

To appreciate the relative level of expression of each receptor in carcinoma cells compared with normal surrounding or distant lung tissue cells, we used each antibody at two optimized dilutions. For example, the RARβ antibody was used at either 1:1500 or 1:12,000. The former dilution was found to be the most sensitive for the nuclear detection of the protein and the identification of all tumors expressing RARβ, whereas the latter dilution allowed us to discriminate between tumors with high or low expression of RARβ. These dilutions were established from 21 tissue specimens with the use of geometric dilutions of the antibody. According to this method, at one geometric dilution standardized for normal tissue, RARβ labeling was clear in nuclei from normal tissue or stromal cells but was strongly decreased in most carcinoma cells. Two independent pathologists conducted the following semiquantitative evaluation of the signal intensity: The stromal cells (fibroblasts and endothelial cells) and the normal lung cells (pneumocytes, bronchiolar epithelial, and endothelial cells) expressing the RARs and RXRs at similar and constant levels in all samples studied were used as an internal standard, and the nuclear staining of these cells was scored as ++; in tumor nuclei, no expression was scored 0, a decreased expression was scored +, and an overexpression was scored ++.

**Immunohistochemical procedure.** Immunohistochemistry was performed on 5-μm paraffin-embedded sections (for RARβ and RARγ and for RXRa and RXRβ) or on frozen sections (for RARα). Paraffin-embedded sections were dewaxed and processed in a pressure cooker in citrate buffer (0.1 M, pH 6.0) for 15 minutes. Sections were incubated overnight at 4 °C with the specific polyclonal or monoclonal antibodies. After being washed in TBS–TWEEN (0.05 M Tris–HCl [pH 7], 150 mM NaCl, and 0.1% Tween 20), the bound antibodies were revealed by use of biotinylated goat anti-rabbit or anti-mouse antibodies (Dako, Copenhagen, Denmark). The sections were then incubated successively with the streptavidin–peroxidase complex (Dako) and the biotin–tyramide substrate solution (42) (1 mg/mL biotin–tyramide in 0.2 M Tris–HCl, 10 mM imidazole [pH 8.8], and 0.01% H2O2) for 10 minutes. Then, after a second incubation in the streptavidin–peroxidase complex, the sections were finally incubated in substrate solution (0.6 mg/mL, 3,3′-diaminobenzidine in 0.05 M Tris–HCl buffer containing 0.1% H2O2).

**Extracts and Immunoblotting**

Nuclear extracts were prepared from specimens of NSCLC or normal lung as described (39), fractionated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (12% polyacrylamide gel containing 1% SDS), and electrotransferred onto nitrocellulose filters. After blocking in phosphate-buffered saline (PBS) containing 3% nonfat powdered milk, the filters were immunoblotted with the specific rabbit polyclonal antibodies for RARα, RARβ, and RXRα (diluted 1:1000) for 2 hours at 37 °C, extensively washed in PBS containing 0.05% Tween 20, and then incubated for 30 minutes at room temperature with peroxidase-conjugated Protein A diluted 1:10,000 (Amersham, Les Ulis, France). Specific complexes were revealed by chemiluminescence detection according to the manufacturer’s protocol. The specificity of the reactions was checked by use of the same antibodies purified by affinity chromatography with sulfolink gel columns coupled with the synthetic peptide that was used for raising the antibodies (39).

**In Situ Hybridization**

The human RARα, RARβ, and RARγ riboprobes were prepared as previously detailed (43), according to Melton’s transcriptions protocol using 35 S-labeled nucleotides (Amersham). Probe length was reduced to an average of 150 nucleotides by limited alkaline hydrolysis.

Sections (5 μm thick) from tissue samples fixed in 4% paraformaldehyde were deparaffinized and acetylated in acetic anhydride (0.5% in 0.1 M triethanolamine [pH 8.0]) for 10 minutes. The 35 S-labeled sense or antisense probe diluted to 60,000 cpm/μL was applied to each section in 20 μL of the hybridization buffer—50% deionized formamide, 2× SSC (300 mM NaCl and 30 mM sodium citrate [pH 7.0]), 100 mM dithiothreitol, 1 mg/mL yeast transfer RNA, 1 mg/mL sonicated salmon sperm DNA, and 2 mg/mL bovine serum albumin. Hybridization was performed overnight at 60 °C. The sections were then rinsed with formamide (50%, 2× SSC) at 60 °C for 1 hour and digested with ribonuclease (RNase) (RNase A, 10 mg/mL; RNase T1, 500 U/mL; in 2× SSC) for 30 minutes at 37 °C. The sections were washed again in formamide for 1 hour at 50 °C, with a final wash in 1× SSC for 30 minutes at room temperature. Hybridized slides were autoradiographed with NTB2 emulsion (Kodak, Rochester, NY) and exposed at 4 °C. Triplicate sections from each specimen were developed at weekly intervals over a 3-week period with D19 Kodak developer.

Control experiments included pretreatment of sections with RNase for 1 hour at 37 °C (RNase A, 10 mg/mL; RNase T1, 500 U/mL; in 2× SSC) and hybridization with sense probes.
Multiplex Reverse Transcription–Polymerase Chain Reaction Analysis of RARα, RARβ, and RARγ mRNAs

Total RNA was isolated from the guanidium cesium-chloride procedure and reverse transcribed with the use of oligo-deoxithymidinie and avian myeloblastosis virus reverse transcriptase (La Roche, Meylan, France). For the comparison of the reverse transcription–polymerase chain reaction (RT–PCR) profiles obtained from normal lung and tumor specimens, an internal standard for RARα was constructed according to the procedure of Ferrari et al. (44), with minor modifications. This DNA fragment was 16 base pairs (bp) shorter than the RARβ sequence selected for amplification and coamplified with RAR mRNAs. The three sets of selected primers were as follows: 1) RARα, 5′-ACCCTCTCTACCCGCATCTC–AAGG and 5′-CATGCGCCTAGTAAACCTGCT–C; 2) RARβ, 5′-ATTCCAGTCTGCCATCAGTCC and 5′-CCTTCTCTGTGTCATCCATTCCC; and 3) RARγ, 5′-ATAGGAGCAGCTTTGCGC and 5′-CACACGAAGCATGTTGAGAC.

Antisense primers were 5′ labeled with a fluorescent dye (carboxyfluorescein). For each gene, 100 ng of complementary DNA was amplified by the amplification program corresponding to 25 cycles (94°C, 30 seconds; 70°C, 1 minute; and 72°C, 1 minute). Under these conditions, the PCR amplification was in a linear range. Samples were run in a 12% denaturing polyacrylamide gel on a model 373 automated fluorescent gel-scanning detection of molecular sizes and peak areas, allowing a semiquantitative evaluation of the PCR products.

PCR-Based LOH Analysis at Chromosome 3p24

The RARβ protein is encoded by a gene located at chromosome 3p24 (28,30). Because the complete genomic sequence at this locus is not available, the 3′ closest microsatellite dinucleotide (CA)n repeat polymorphism was selected (D3S1283; Genethon/GenBank Accession No. Z16798). The primers flanking the microsatellite used in the present study are as follows: 5′-GGGACGTACCCACCTG-TAGAAATG and 5′-GCTTGGTACAGGATC-GTGGTGATCC. PCR was performed from frozen tumor specimens, paired pieces of healthy parenchyma, and non-neoplastic lesions microdissected from paraffin-embedded sections. A hot-start PCR was performed. After an initial denaturation step at 94°C for 4 minutes, DNA was amplified through 28 cycles of PCR consisting of 30 seconds of denaturation at 94°C, 1-minute annealing at 65°C, and 1-minute extension at 72°C. The PCR products (150–160 bp) were separated by high-resolution electrophoresis in a 12% ultrathin-layer polyacrylamide gel rehydrated with 30 mM Tris–formate and with 1 M ribose added as a matrix modifier (45).

Statistical Analysis

McNemar’s test of symmetry was performed for each normal and tumor tissue pair. All P values were generated from two-sided statistical tests.

RESULTS

Expression of RARα, RARβ, RARγ, RXRα, and RXRβ Proteins in NSCLC and Paired Normal Lung Tissues

Seventy-six specimens of NSCLC (seven of the 83 paired specimens were rejected because they were unsuitable for analysis with the full set of antibodies) were analyzed by immunohistochemistry with specific antibodies. Controls included either normal lung parenchymal and bronchial tissues from normal patients or healthy lung tissue adjacent to the tumors of the patients with NSCLC. All control tissues expressed RARα, RARβ, RARγ, RXRα, and RXRβ proteins, and the same patterns of labeling were observed in all normal lung specimens. For all receptors, the labeling was completely restricted to nuclei. However, RARα and RXRα immunostaining was stronger than that of the other receptors. In addition, the intensity of the labeling of the nuclei differed, depending on the cell type: Bronchial epithelial cells, pneumocytes, fibroblasts, and endothelial cells reacted strongly with all antibodies, but chondrocytes from bronchial cartilage showed the strongest labeling. In contrast, interstitial lymphocytes, when present, reacted weakly, and alveolar macrophages were devoid of immunostaining.

It is interesting that stromal cells (fibroblasts, endothelial cells, and inflammatory cells) from tumors showed the same pattern of labeling with all of the tested antibodies as their counterparts from healthy tissues. This observation was made throughout all the specimens. Thus, fibroblast and endothelial cell nuclei from either stroma or surrounding normal lung could be used as internal standards for a semiquantitative evaluation of RAR and RXR expression in carcinoma cells.

Fig. 1 shows the expression of RARs and RXRs in carcinoma cells. Similar profiles were observed whether specimens with ADC or SCC were tested. Compared with RARα expression in stromal cells, RARα was strongly expressed in all carcinoma cells, with 26% of the samples showing an overlabeling of tumor cells nuclei (Fig. 1; panels j and k) and 74% showing a similar staining (data not shown). RXRα was also strongly expressed in carcinoma cells. However, in contrast to RARα, 85% of the tumor specimens showed an obvious overexpression of the receptor in carcinoma cells compared with stromal cells (Fig. 1; panels o and p) and normal lung cells, while 15% of the specimens only revealed an identical staining (data not shown). RARβ expression was markedly decreased in carcinoma cells: 57% of the specimens showed a decrease (Fig. 1; panel e), and 6% showed a complete absence of staining (Fig. 1; panel i). Only 37% of the specimens were labeled to the same extent as stromal cells (Fig. 1; panels a and g). The difference in RARβ protein expression between normal cells and carcinoma cells was statistically significant (P<0.0001). RARβ expression was decreased with a very similar frequency in ADC and SCC. However, no substantial relationship could be found between the level of RARβ expression and NSCLC histologic differentiation, disease staging, and smoking background (data not shown).

The expression of RXRβ was also decreased in carcinoma cells, compared with normal cells, in 18% of the specimens (Fig. 1; panel s). However, a moderate overlabeling could be seen in the remaining specimens (Fig. 1; panels q and r) (P<0.0002).

RARγ was present in all the tumor cells studied (Fig. 1; panels m and n). A moderate decrease in RARγ in tumor cells relative to normal cells, was observed in some tumor specimens, but the differences were not clear-cut enough to give statistical evaluations.

In all of the bronchial nontumorous lesions studied (n = 36), RARα, RARβ, RARγ, RXRα, and RXRβ proteins were also expressed, but they were not subjected to a semiquantitative evaluation.

The reliability of these immunohistochemical analysis results (and thus the specificity of RARs and RXRs antibodies used in this study) was checked by western blotting. Indeed, as Fig. 2 shows, when nuclear extracts are used from paired NSCLC and normal lung, the antibodies revealed only one protein corresponding to the respective RAR, without any additional nonspecific band. In addition, western blot analysis confirmed the decreased expression of RARβ in the tumor specimens.

Expression of RARα, RARβ, and RARγ mRNAs

Control specimens, normal lung tissue surrounding NSCLC, and tumor specimens were analyzed by in situ hybridiza-
Fig. 1. Differential *in vivo* expression of proteins and messenger RNAs (mRNAs) of retinoic acid receptors (RARα, RARβ, and RARγ) and retinoic X receptors (RXRα and RXRβ) in non-small-cell lung cancer—immunohistochemical (IHC) and *in situ* hybridization (ISH) investigation. **RARβ**, panels a, b, c, and d: squamous cell carcinoma (SCC) with a normal expression of RARβ protein (a) and mRNA (c); corresponding controls with IHC (b) and ISH (d). **RARβ**, panels e and f: SCC showing a strong decreased expression of RARβ protein (e) and mRNA (f) in carcinoma cells. **RARβ**, panels g, h, and i: adenocarcinoma (ADC) with a normal expression of RARβ protein (g) and mRNA (h) in tumor cells and stroma cells; ADC with no expression of RARβ protein in tumor cell nuclei (i). **RARα**, panels j, k, and l: strong staining of both ADC (j) and SCC (k) cell nuclei with RARα antibody associated with an intense mRNA expression (SCC) (l). **RARγ**, panels m and n: SCC (m) and ADC (n) specimens showing a normal labeling with RARγ antibody in carcinoma cells. **RXRα**, panels o and p: receptor overexpression in tumor cell nuclei from SCC (o) and ADC (p). **RXRβ**, panels q, r, and s: SCC (q) and ADC (r) specimens with moderate overexpression of RXRβ in carcinoma cells; SCC sample with a marked decreased expression of RXRβ in tumor cells (s). In all cases, the degree of expression in carcinoma cells was judged in comparison to that in stromal cells.
tion. Antisense probes for the three RARs (RARα, RARβ, and RARγ) hybridized to epithelial and mesenchymal cells in all sections. In all tumor specimens, the RARα mRNA signal was stronger in carcinoma cells than in either stromal cells (Fig. 1; panel I) or normal lung tissue. As shown in Fig. 1 (panels c, f, and h), the distribution of RARβ mRNA strictly paralleled that of the corresponding protein, thus allowing us to distinguish three classes of tumors: 1) tumors (39%) expressing similar levels of RARβ mRNA in carcinoma cells, stromal cells, and normal tissue (Fig. 1; panels c and h); 2) tumors (54%) expressing lower levels of RARβ mRNA in carcinoma cells than in stromal cells (Fig. 1; panel f); and 3) rare tumors (7%) without any detectable RARβ mRNA in carcinoma cells. With regard to RARγ mRNA, a moderate decrease in the signal was observed in many tumors compared with normal lung, but the results were not clear enough to reliably quantify the percentage of tumors with decreased expression, as already mentioned above for immunohistochemical analysis.

RARα, RARβ, and RARγ mRNAs were also detected in all paired samples studied by the semiquantitative multiplex RT–PCR. According to this technique, RARα transcripts were expressed at higher levels than were the RARγ and RARβ transcripts, in tumor as well as in normal lung (Fig. 3). This technique also confirmed the high frequency of decreased expression of the RARβ gene in tumors despite the presence of mRNAs relevant to stromal cells. Indeed, 59% of the tumor specimens showed a 50% or higher decrease in the RARβ RT–PCR product peak areas compared with normal lung (P < .0001). Strikingly, 41% of the tumor specimens also showed a 50% or greater decrease in RARγ RT–PCR product peak areas (P < .0001). This result contrasts somewhat with immunohistochemistry and in situ hybridization data that showed a moderately decreased expression of RARγ. This observation is probably relevant to the facts that the different techniques used to evaluate RARγ expression are semiquantitative, investigate distinct parameters (protein and mRNA), and consider either tumor cells exclusively (ISH) or all the tumor cell populations (RT–PCR). More extensive studies are necessary to accurately quantify the intensity of the decreased expression of RARγ. However, a simultaneously decreased expression of RARγ and RARβ was observed in 29% of the tumor samples (Fig. 3; panels a and c). Such results contrast with the very close values observed for the RARα complementary DNA peak areas from paired tumor and normal lung tissues.

3p24 LOH

RARβ is known to be encoded by a gene located at chromosome 3p24 (27, 29). Since an LOH has been reported to occur with a high frequency on chromosome 3p loci in NSCLC (45), LOH was studied at chromosome 3p24. At this locus, 37 (45%) of 82 patients were heterozygous. Among these informative patients, 15 (41%) of 37 showed an LOH, and one patient had a homozygous deletion. No substantial differences were observed between ADC (37%) and SCC (42%) (Fig. 4). It is interesting that 86% of the ADCs and 75% of the SCCs with LOH showed a decrease in the expression of RARβ protein and mRNA. In addition, two of three carcinomas in situ and two of nine bronchial metaplasias with dysplasia foci showed LOH at 3p24 (20% of the non-neoplastic lesions from informative patients). According to these results, there seems to be an association between LOH at chromosome 3p24, which includes the region coding for RARβ, and the decrease in RARβ expression.

DISCUSSION

Our study of in vivo expression is, to our knowledge, the first study in which immunohistochemistry was used in conjunction with in situ hybridization to analyze RARs and RXRs in surgical specimens from tumors of patients with NSCLC and paired normal lung tissue. The expression of RARs and RXRs had been studied earlier in specimens from lung cancer and normal tracheobronchial epithelial cells with the use of northern blotting (27) or in situ hybridization (25, 29) but not with immunohistochemistry. Our study confirms that, in NSCLC, the expression of RARα and RXRα is not substantially altered not only at the RNA level, but also at the protein level, despite a moderate overexpression of these receptors in some tumors. Although no reliable conclusion can be drawn about RARγ protein expression by immunohistochemistry, a frequent decrease in the expression of the mRNAs was observed in tumors, confirming a recent report (25).

The interesting feature of this study is the observation that the expression of the RARβ protein was markedly decreased in about 60% of the ADCs and SCCs and was rarely fully suppressed. In fact, RARβ was not detectable by immunohistochemistry in carcinoma cells from only 6% of the tested tumor specimens. Nevertheless, RARβ was normally expressed in the stromal cells and the surrounding normal lung tissue. These observations were confirmed at the mRNA level by RT–PCR and in situ hybridization by use of radioactive riboprobes with high specific activity. Our results differ from the results obtained by Xu et al. (25), who reported the absence of RARβ mRNA in 60% (instead of 6% under our experimental conditions) of the tumors by using nonradioactive probes. Such a discrepancy may result from the use in our study
of radioactive probes more sensitive than their nonradioactive counterparts and of highly sensitive biotin–tyramide conjugates for immunohistochemistry, thus allowing the detection of low levels of RARβ that are undetectable with the other techniques.

In addition, in agreement with a previous report (46), in our study 41% of the tumors showed LOH at chromosome 3p24, which contains the region coding for RARβ. It is interesting that 80% of the patients with LOH also showed a weak expression or the absence of the RARβ protein in tumor cells. According to these results, one may speculate that LOH is involved in the decreased expression of RARβ in tumor cells. Alternatively, these results may explain why the full suppression of RARβ is so rare. Such an hypothesis is consistent with studies by Mendelsohn et al. (47), who reported that heterozygous mice knocked-out for RARβ2 retain about 50%–30% of the wild-type amount of RARβ2, whereas homozygous mice do not express any detectable RARβ2. In fact, LOH at chromosome 3p24 probably occurs early in the carcinogenesis process, since it has been observed in four of 12 high-grade bronchial non-neoplastic lesions associated with some of the tumors. Thus, LOH may define a localized, predisposed region from which the cancer may arise (30). Nevertheless, although a number of studies have underlined the frequency of deletions at 3p24 in most lung carcinomas and non-neoplastic lesions (28,30), LOH at this loci is not the major mechanism responsible for the abnormal regulation of RARβ, since other deletions involving other loci also occur in the short arm of chromosome 3 (46,48,49).

The other main feature of our study is the observation of a decreased expression of RXRβ in 18% of the tumor samples, frequently associated with low expression of RARβ. Indeed, 29% of the tumors with a low RARβ expression also showed low levels of RXRβ. Such a simultaneously decreased expression of both RARβ and RXRβ in tumors would result in deficient RXRβ/RARβ heterodimers. Thus, according to the recent model (50) in which RXR functions as a transcriptionally active partner in the context of an RXR–RAR heterodimer, a defect in RARβ and to a lesser extent in RXRβ might play a role in lung cancer development. This defect may render retinoids unable to turn on normal cellular programs, especially those involving genes activated by RXRβ/RARβ heterodimers, including the RARβ2 gene itself. In this respect, the RARβ promoter has been found to be nonfunctional in a majority of lung cancer cell lines (20), and an association has been established between abnormal expression of RARβ and lung cancer development (16). However, other parameters should be considered, since the unresponsiveness of the RARβ gene has been demonstrated to result not only from nonfunctional RARs but also from other factors, such as an unbalanced equilibrium between the orphan receptors Nur77 and COUP-TF (51). Expression of Nur77 enhances ligand-independent transactivation of retinoic acid response elements and de-

Fig. 3. Reverse transcription–polymerase chain reaction multiplex analysis of messenger RNAs (mRNAs) of retinoic acid receptors (RARα, RARβ, and RARγ) in paired specimens of non-small-cell lung cancer and normal lung. Representative examples of an adenocarcinoma (a) and a squamous cell carcinoma (c) indicating decreased expression of RARβ and RARγ mRNAs in tumor specimens (a and c) compared with normal lung parenchyma (b and d).
sensitizes their retinoic acid responsiveness. Conversely, expression of COUP-TF sensitizes retinoic acid responsiveness of retinoic acid response elements by repressing their basal transactivation activity. Finally, other factors, such as the transcription-activating factors (52) and the coactivators and/or corepressors that interact with RARs to mediate target gene response (7), should also be considered. In this respect, aberrant chromatin remodeling by histone deacetylases and/or histone acetylases that are recruited to retinoic acid-target genes by nuclear corepressors and coactivators, respectively (7), has been associated with acute myeloid and promyelocytic leukemias (53–55). Thus, altered chromatin organization through deregulation of histone acetylation might also be an important event in the processes leading to cell transformation in lung cancer and should be considered.

In conclusion, our study demonstrates a sustained in vivo expression of RARs and RXRs in NSCLC and related preneoplastic bronchial lesions, associated with a frequent, markedly decreased expression of RARβ and to a lesser extent of RAR-γ and RARx in tumors. Such a study is a prerequisite for treatments of retinoic acid-sensitive lung cancers with retinoid derivatives. Direct aerosolization of the retinoids on bronchial epithelium providing the required concentration of the drug to activate receptors, with reduced toxicity, may greatly improve the chance of controlling early lung cancer (56). In this way, immunohistochemistry using biopsy specimens from tumors and preneoplastic lesions is a simple and reliable procedure to identify patients expressing RARβ in normal levels and who thus are the most susceptible to benefit from RA treatments. In addition, determining the relative levels of expression of the different RARs and RXRs in retinoid acid-resistant patients would be of great importance for the use of new RXR and/or RAR selective ligands (6,21–23).

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NOTES

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