The Role of Retinoid X Receptor Messenger RNA Expression in Curatively Resected Non-Small Cell Lung Cancer

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ABSTRACT

Background: Retinoid X receptors (RXRs) have inhibitory effects on non-small cell lung cancer (NSCLC) cell growth, and RXRβ expression is reduced in NSCLC specimens compared with normal lung tissue. We hypothesized that suppressed RXR expression might be a prognostic factor of worse clinical outcome in patients with NSCLC.

Experimental Design: Using a quantitative real-time reverse transcription-PCR (TaqMan) method, we analyzed RXRα, RXRβ, and RXRγ mRNA expression in normal lung tissue and matching tumor samples from 88 patients with NSCLC.

Results: The median mRNA expression levels of all three RXR subtypes were frequently decreased in tumor tissues compared with matching normal lung tissue (RXRα, 67%; RXRβ, 55%; RXRγ, 89%). The RXRα (P = 0.001) and RXRγ (P < 0.001) median expression levels were significantly lower in the tumors. Patients whose tumors exhibited low RXRβ expression level had a statistically significant worse overall survival (P = 0.0005), whereas a trend toward worse survival was observed for patients with low RXRα expression. Multivariate analysis indicated that low RXRβ expression is an independent predictor of worse survival in patients with NSCLC (P = 0.017).

Conclusion: Suppressed mRNA expression of all three RXR subtypes is a frequent event in NSCLC. Reduced RXRβ expression might be an important biomarker for more aggressive disease in patients with NSCLC.

INTRODUCTION

Lung cancer is one of the most common malignancies in the world and is the leading cause of cancer-related deaths in the United States (1). Despite improvements in the detection and treatment of lung cancer in the past two decades, the 5-year survival rate remains <15% (2). New treatment strategies based on molecular classification of each patient's tumor may provide further improvements in outcome for patients with lung cancer.

Retinoids play an important role in regulating the morphogenesis, development, growth, and differentiation of cells by regulating gene expression through interactions with their cognate nuclear receptors (3, 4). Retinoid receptors belong to the greater steroid-receptor superfamily and are classified as RARs (3) and RXRs, with three subtypes each (α, β, and γ; Ref. 4). Each RAR and RXR subtype can be expressed in several isoforms, which differ in their NH2-terminal domain (5). RARs can form heterodimers with RXRs and recognize retinoid acid response elements that can activate transcription (5). RXRs can also form homodimers and activate retinoid X response element or form heterodimers with other members of the steroid-receptor family, thus providing opportunities for cross-talk among different signaling pathways (5).

Previous studies have demonstrated decreased expression of RARβ in lung cancer in vitro and in vivo, suggesting the possibility that RARβ might be functioning as a tumor suppressor gene in lung carcinogenesis (6–9). A recent publication reported that RARβ expression is also a prognostic factor for patients with NSCLC, although somewhat unexpectedly the association was between RARβ overexpression and worse clinical outcome in patients with stage I disease (10). Altered RXR expression has also been reported in various human tumors, including gastric (11), breast (12, 13), prostate (14), and lung cancer (8, 15, 16). Two studies have investigated RXR expression in malignant lung tissue (8, 15). Both studies reported decreased RXRβ expression in NSCLC, suggesting an association between loss of expression of this nuclear retinoid receptor and lung carcinogenesis (8, 15). For RXRα expression, one of these studies found frequent RXRα protein overexpression in NSCLC (15), whereas the other reported no change in RXRα mRNA expression (8).

The findings of these studies suggest that RXR expression might be associated with tumor behavior and hence clinical
outcome. This study was conducted to investigate whether RXR expression is a significant factor associated with clinical outcome in patients with curatively resected NSCLC.

MATERIALS AND METHODS

Patients and Specimens. Included in this study were tumor specimens and paired normal lung tissues from 88 patients with NSCLC that were available from a previous prospective tissue procurement trial of 103 consecutive patients with completely resected (R0 resection) NSCLC (17). There were 66 (75%) men and 22 (25%) women, with a median age of 64 years (range, 35–82 years). Forty-one (47%) patients had squamous cell carcinomas, 33 (37%) had adenocarcinomas, and 14 (16%) had large cell carcinomas. The primary tumors were graded histopathologically as well-differentiated (G1; 1 patient), moderately differentiated (G2; 19 patients), and poorly differentiated (G3; 68 patients). Tumor staging was performed according to the UICC TNM classification (18): Forty-four (50%) had stage I tumors, 17 (19%) had stage II tumors, and 27 (31%) had stage III tumors. All 88 patients underwent thoracic surgery. All tumors were radically removed by lobectomy (n = 57), bilobectomy (n = 10), pneumonectomy (n = 11), or extended pneumonectomy (n = 10), including mediastinal lymphadenectomy. Patients with histopathological stage IIIa tumors received postoperative radiotherapy. Informed consent was obtained from all patients.

The median follow-up was 85.9 months (range, 63–105 months), and no patient was lost to follow-up. Tissue for gene expression analysis was obtained intraoperatively immediately after lung resection and before starting mediastinal lymphadenectomy. The tissues were immediately frozen in liquid nitrogen and stored at −80°C. Tissues were analyzed from the following two locations: tumor and uninvolved lung tissue taken from the greatest distance to the tumor. Frozen sections (6 μm) were taken from blocks of tumor tissue, and starting with the first section, every fifth section was routinely stained with H&E and evaluated histopathologically. Sections were pooled for analysis from areas estimated to have at least 75% malignant cells.

mRNA Isolation. Total RNA was isolated by a single-step guanidinium isothiocyanate method using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer’s instructions. After RNA isolation, cDNA was prepared from each sample as described previously (19).

Real-Time RT-PCR Quantification. cDNAs and an internal reference gene (β-actin) were quantified using a fluorescence-based real-time detection method [ABI PRISM 7700 Sequence Detection System (TaqMan); Perkin-Elmer Applied Biosystems, Foster City, CA] as described previously (19–21). The PCR reaction mixture consisted of 600 nM each of the primers; 200 nM probe; 2.5 units of AmpliTaq Gold Polymerase; 200 μM each of dATP, dCTP, and dGTP; 400 μM dUTP; 5.5 mM MgCl₂; and 1× TaqMan Buffer A, which contains a reference dye, to a final volume of 25 μl (all reagents from Perkin-Elmer Applied Biosystems). Cycling conditions were 50°C for 10 s, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min.

The primers and probe sequences used were as follows:

For RXRa: primers, 5'-AAGGACCAGGAACGAGATGA-3' and 5'-ATCCCTCTCCACGCGATG-3'; probe 6FAM-5'-AGTCGACACGGCAGCCACG-3'-TAMRA

For RXRb: primers, 5'-CTCTGGATTCTAGGTACATTTGACTG-3' and 5'-GCCATCTGAACATCATTAGGA-3'; probe, 6FAM-5'-ACTCCTATGCTTCTCCTTTTCACACCCG-3'-TAMRA

For RXRγ: primers, 5'-GGGAGGTCTGAGGAAGAAAGAA-3' and 5'-TGGTAGACACATTGCTTCA-3'; probe, 6FAM-5'-TCAGCTGCTCTCGGCTTCCTCCTTG-3'-TAMRA

For β-actin: primers, 5'-TGAAGCAGCGTACTAGCT-3' and 5'-TCTCCAATGTCAAGCGACATT-3'; probe, 6FAM-5'-ACCACACGCCGACAGCGG-3'-TAMRA

For each sample, parallel TaqMan PCR reactions were performed for each gene of interest and the β-actin reference gene to normalize for input cDNA. The ratio between the values obtained provided relative gene expression levels for the gene locus investigated.

Statistical Analysis. Associations between two related variables were tested by using Wilcoxon signed rank test. The Mann-Whitney test and Kruskal-Wallis test were used to evaluate the associations between the expression of the RXRs (as continuous variables) and patients’ clinicopathological variables. Hazards ratios were used to calculate the relative risks of death. These calculations were based on the Pike estimate, with the use of the observed and expected numbers of events as calculated in the log-rank test statistic (22). The maximal χ² method of Miller and Siegmund (23) and Halpern (24) was adapted to determine which expression value best segregated patients into poor and good prognosis subgroups (in terms of likelihood of surviving), with the log-rank test used to measure the strength of the grouping. To determine a P that would be interpreted as a measure of the strength of the association based on the maximal χ² analysis, 1000 bootstrap-like simulations were used to estimate the distribution of the maximal χ² statistics under the hypothesis of no association (24). Multivariate analysis was performed with the Cox proportional hazards regression model. The level of significance was set to P < 0.05. All Ps reported were based on two-sided tests.

RESULTS

RXRa, RXRb, and RXRγ mRNA Expression in Nonmalignant and Tumor Tissues from NSCLC Patients. RXRa mRNA expression was detectable by quantitative real-time PCR (TaqMan) in 88 of 88 (100%) tumor specimens and 88 of 88 (100%) nonmalignant lung specimens. Fifty-nine (67%) patients showed equal or reduced RXRa mRNA expression levels in tumor tissues compared with matching nonmalignant tissue. The median RXRa mRNA expression was detectable in 88 of 88 (100%) tumor tissues and 87 of 88 (99%) nonmalignant lung tissues. Forty-eight (55%) patients had equal or reduced RXRb mRNA expression levels in tumor compared with matching nonmalignant tissue. The median RXRb mRNA expression was...
Role of RXR Expression in NSCLC

The median survival for patients with a RXR mRNA expression level of 12.9 was 32.4 months (95% CI, 22.5–42.4), whereas the median survival for those with a RXR mRNA expression level >12.9 was not reached. The observed log-rank test statistic was 12.05. To determine \( P \), we used bootstrap-like simulations to estimate the distribution of a maximal \( \chi^2 \) statistic because the cutoff point of 12.9 had been chosen after examining the data. The resulting adjusted \( P \) was 0.0005. The respective survival curves are shown in Fig. 1 and show 5-year survival rates of 34.4 ± 6.1% for patients with a RXR expression level ≤12.9 and 74.1 ± 9.9% for those with RXR expression >12.9 (\( P = 0.0002 \), Wilcoxon-Gahan).

The importance of RXR as a prognostic factor was next determined by the Cox proportional hazards model analysis. Two logistic regression models were tested. Model A included the parameters gender, age, histopathological type, UICC TNM tumor stage, grade of differentiation of the primary tumor, and RXR expression. Model B included the pT and pN categories instead of histopathological tumor stage. Significant independent prognostic factors were UICC TNM tumor stage (\( P < 0.001 \)) and RXR expression (\( P = 0.017 \)) expression in model A and the pN categories (\( P < 0.001 \)) and RXR expression (\( P = 0.021 \)) in model B. Table 3 shows the statistically significant parameters in the two regression models.

The median survival for patients with a RXR mRNA expression level ≤7.7 was 20.9 months (95% CI, 7.8–33.9), whereas the median survival for those with a RXR mRNA expression level >7.7 was 59.7 months. The observed log-rank test statistic was 1.84. Although this difference was not significantly different, a trend toward significance was observable (\( P = 0.17 \)). The 5-year survival rate was 33.3 ± 13.6% for patients with a RXR expression level ≤7.7 and 47.4 ± 5.7% for those with RXR expression >7.7 (\( P = 0.16 \)).

### Table 1: Association between the median RXR mRNA expression in tumor tissue and clinicopathological variables

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* TNM: T status, tumor infiltration; LN status, lymph node status.
* SSC, squamous cell carcinoma; AC, adenocarcinoma; LC, large cell; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

9.8 (range, 4.3–44.3) in tumor tissue and 10.4 (range, 0–22.7) in matching nonmalignant lung tissue (\( P = 0.72 \)). RXR mRNA expression was detectable in 81 of 88 (92%) tumor specimens and 81 of 88 (92%) nonmalignant lung specimens. Decreased RXR mRNA expression levels in tumors compared with matching normal lung tissues were detected in 78 (89%) patients. The median RXR mRNA expression was 0.046 (range, 0–6.2) in tumor and 0.49 (range, 0–5.9) in matching nonmalignant lung tissues (\( P < 0.001 \)).

The associations between patients’ clinicopathological data and RXRα, RXRβ, and RXRγ mRNA expression are shown in Table 1. No statistically significant associations were found.

**Association between RXRα, RXRβ, and RXRγ mRNA Expression Levels and Survival of Patients with Curatively Resected NSCLC.** With a median follow-up of 85.9 months for the 88 patients analyzed in this study, the median survival was 51.6 (range, 3.8–105.3) months. The effects of various clinical variables and RXRα, RXRβ, and RXRγ expression status on the median and 5-year survival rates are summarized in Table 2. The maximal \( \chi^2 \) statistic of Miller and Siegmund (23) and Halpern (24) was adapted to determine which RXRα, RXRβ, and RXRγ mRNA expression values best segregated patients into poor- and good-prognosis subgroups.

This method found that segregation for RXRβ mRNA levels was best achieved with a relative expression level of 12.9. The median survival for patients with a RXRβ mRNA level ≤12.9 was 32.4 months (95% CI, 22.5–42.4), whereas the median survival for those with a RXRβ mRNA level >12.9 was not reached. The observed log-rank test statistic was 12.05. To
Patients with RXRγ expression ≤0.09 showed a median survival of 43.2 months (95% CI, 10.3–76.1), whereas the median survival was not reached in the group with a RXRγ expression >0.09 (P = 0.35). The 5-year survival was 45.3 ± 6.8% for patients with low RXRγ expression levels and 51.4 ± 8.5% for patients with high RXRγ expression status (P = 0.278).

**DISCUSSION**

This study is, to our knowledge, the first to use a quantitative real-time RT-PCR (TaqMan) method to analyze the mRNA expression of all three RXR subtypes in surgical specimens from tumors of patients with NSCLC and paired normal lung tissue. We found decreased median mRNA expression of all three RXRs at high frequencies (RXRα, 67%; RXRβ, 55%; RXRγ, 89%) in tumor tissues, suggesting a fundamental role of decreased RXR mRNA expression in human lung tumorigenesis. Our results confirm previous publications reporting altered RXR expression in NSCLC (8, 15, 16). Picard et al. (15) demonstrated significantly decreased RXRβ protein expression, and Xu et al. (8) showed decreased RXRβ mRNA expression in NSCLC, suggesting an association between loss of RXRβ expression and lung carcinogenesis.
Our results concerning the role of RXRα and RXRγ in lung cancer differ from those reported in these other studies, however. Whereas Picard et al. (15) reported frequent RXRα protein overexpression by means of immunohistochemistry, Xu et al. (8) demonstrated unaltered RXRα and RXRγ mRNA expression by means of in situ hybridization in NSCLC. Interestingly, the latter study somewhat puzzling results concerning RARγ expression in the same tumor sample cohort when analyzed by different techniques. Whereas immunohistochemical RARγ expression analysis showed moderately decreased RARγ expression, RT-PCR showed a ≥50% decrease in RARγ expression in 41% of the tumors analyzed (15). These discordant findings may be explained by the fact that the different techniques for evaluating RARγ expression in these studies were semiquantitative, they investigated different parameters (protein and mRNA) and considered either tumor cells exclusively (immunohistochemistry) or all of the tumor specimen’s cell populations (RT-PCR). The reported 10-fold greater sensitivity of the quantitative TaqMan method (25) compared with conventional semiquantitative RT-PCR methods may additionally account for the different results obtained in our study.

The underlying mechanism responsible for decreased RXR expression observed in our study is not clear and was not the subject of this study. Other studies have reported frequent loss of heterozygosity at the RXR loci, but loss of heterozygosity was not always accompanied by altered RXR expression in lung cancer precursor lesions, suggesting inactivation of these genes by an alternative mechanism (16). The finding that complete loss of expression was detectable only for RXRβ in one tumor (1%) suggests that the changes are not attributable to structural damage to the genes, but may be modified at the transcriptional level. Abrupt methylation of CpG islands has been identified as an epigenetic mechanism for transcriptional silencing and has been reported in lung cancer (26, 27). A recent publication reported hypermethylation of the RARβ gene promoter P2 in 41% of investigated NSCLC specimens, suggesting that this may be one mechanism that causes RARβ gene silencing in many lung cancers (28). We speculate that aberrant RXR gene promoter hypermethylation might also contribute to the RXR expression silencing observed in the current study.

One of the critical questions is to identify molecular factors with prognostic importance in lung cancer. This is the first study investigating the prognostic importance of RXR expression in NSCLC. The most striking finding in our study is the association between RXR expression levels and survival in patients with NSCLC. Whereas decreased RXRα levels in tumors showed a trend toward inferior survival, low RXRβ expression was significantly correlated with poor survival in NSCLC. Furthermore, multivariate analysis showed that RXRβ expression is an independent prognostic factor for patients with curatively resected NSCLC. There are possible explanations for these findings. Decreased RXR mRNA expression would result in deficient RAR/RXR heterodimers and RXR/RXR homodimers. Thus, according to the recent model (29) in which RXR functions as a transcriptionally active partner in the context of RAR/RXR and RXR/RXR dimers, this could result in functional cellular retinoid deficiency. This defect may render retinoids unable to activate normal cellular programs and could allow cells to escape from normal homeostasis, thereby ultimately leading to a more aggressive cell clone. This hypothesis is indirectly supported by studies that showed that activation of RXR by receptor class-selective retinoids contributed to inhibition of lung cancer cell growth (5). Regardless of the actual mechanism leading to decreased RXR expression in NSCLC, our findings clearly indicate a significant role for RXR expression in the prognostic evaluation of patients with NSCLC. Further studies investigating the association between RXR expression and the outcome of retinoid treatment in patients with NSCLC will be important.

In conclusion, we demonstrated decreased mRNA expression of all three RXR subtypes in NSCLC, suggesting a fundamental role of these genes in lung carcinogenesis. RXRβ might be an important biomarker for a biologically aggressive disease in patients with NSCLC. These findings add another step toward the development of molecular classification of NSCLC and suggest that measuring RXR expression might help identify NSCLC patients at higher risk of early disease recurrence after operation. These patients may likely require additional postsurgery therapy to control their disease.

REFERENCES

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