Promoter Methylation and Silencing of the Retinoic Acid Receptor-β Gene in Lung Carcinomas

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**Background:** Retinoic acid plays an important role in lung development and differentiation, acting primarily via nuclear receptors encoded by the retinoic acid receptor-β (RARβ) gene. Because receptor isoforms RARβ2 and RARβ4 are repressed in human lung cancers, we investigated whether methylation of their promoter, P2, might lead to silencing of the RARβ gene in human lung tumors and cell lines. **Methods:** Methylation of the P2 promoter from small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) cell lines and tumor samples was analyzed by the methylation-specific polymerase chain reaction (PCR). Expression of RARβ2 and RARβ4 was analyzed by reverse transcription–PCR. Loss of heterozygosity (LOH) was analyzed by PCR amplification followed by electrophoretic separation of PCR products. Statistical differences were analyzed by Fisher’s exact test with continuity correction. **Results:** The P2 promoter was methylated in 72% (63 of 87) of SCLC and in 41% (52 of 127) of NSCLC tumors and cell lines, and the difference was statistically significant (two-sided P<0.001). By contrast, in 57 of 58 control samples, we observed only the unmethylated form of the gene. Four tumor cell lines with unmethylated promoter regions expressed both RARβ2 and RARβ4. Four tumor lines with methylated promoter regions lacked expression of these isoforms, but demethylation by exposure to 5-aza-2’-deoxycytidine restored their expression. LOH at chromosome 3p24 was observed in 100% (13 of 13) of SCLC lines and 67% (12 of 18) of NSCLC cell lines, and the difference was statistically significant (two-sided P = .028). **Conclusions:** Methylation of the RARβ P2 promoter is one mechanism that silences RARβ2 and RARβ4 expression in many lung cancers, particularly SCLC. Chemical demethylation is a potential approach to lung cancer therapy. [J Natl Cancer Inst 2000;92:1303–7]
ation of the RARβ gene promoter P2 in human lung cancers and cell lines.

MATERIALS AND METHODS

Cell Lines and Tumor Samples

All human lung cancer cell lines (66 SCLC lines and 78 NSCLC lines) and B-lymphoblastoid lines (n = 31) were established by us (23). The cells were grown in RPMI-1640 medium (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) supplemented with 5% fetal bovine serum and were incubated in 5% CO₂. Samples of tissue from 49 surgically resected primary NSCLC tumors, along with 24 samples of nonmalignant lung tissue from the same patients, were obtained from the Tumor and Tissue Repository at the Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas. Formalin-fixed, paraffin-embedded sections of 21 SCLC tumors were obtained from The University of Texas M. D. Anderson Cancer Center, Houston, or from Canisius Wilhelmina Hospital, Nijmegen, The Netherlands. Archival paraffin sections of tumor-negative lymph nodes from 12 of the NSCLC patients were utilized as controls for the paraffin-embedded tumors. Epithelial cells from buccal swabs (n = 12) and peripheral blood lymphocytes (n = 10) were collected from 22 healthy volunteers and served as negative controls for the frozen tissue samples. Appropriate Institutional Review Board permission was obtained from all participating centers, and written informed consent was obtained from all volunteers before usage of prospectively collected fresh samples. Institutional guidelines do not require written permission for the use of anonymous archival paraffin-embedded specimens.

Genomic DNA was obtained from cell lines, primary tumors, and nonmalignant cells by digestion with 200 μg/mL proteinase K (Life Technologies, Inc.) for 1 day at 37 °C, followed by two extractions with phenol–chloroform (1:1) (24). DNA was extracted from paraffin sections after precise laser-capture microdissection of the tumor cells or lymph nodes, as described previously (25).

Methylation-Specific Polymerase Chain Reaction

The methylation-specific polymerase chain reaction (PCR) employs an initial bisulfite reaction to modify the DNA. As a result, all unmethylated cytosines are deaminated and converted to uracils, while 5-methylcytosines remain unaltered. Subsequently, genomic DNA was subjected to bisulfite modification. As a result, all unmethylated cytosines are converted to uracils, while 5-methylcytosines remain unaltered. DNA was extracted from paraffin sections after precise laser-capture microdissection of the tumor cells or lymph nodes, as described previously (25).

DNA was treated with sodium bisulfite as described previously (26). Briefly, 1 μg of DNA was denatured by incubation with 0.2 M NaOH at 10 minutes at 57 °C. Aliquots of 10 μM hydroquinone (30 μM Sigma Chemical Co., St. Louis, MO) and 3 M sodium bisulfite (pH 5.0, 520 μL) (Sigma Chemical Co.) were added, and the solution was incubated at 50 °C for 16 hours. Treated DNA was purified by use of a Wizard DNA Purification System (Promega Corp., Madison, WI). Modified DNA was stored at −70 °C until used.

Amplification of bisulfite-modified DNA for the RARβ gene promoter P2 was performed by PCR as described by Côté et al. (27) with primers that were specific for either methylated or unmethylated RARβ sequences. Primers used to amplify the unmethylated RARβ gene were 5’-TCGAGACCGGAGCAGTGCC-3’ (sense) and 5’-GGAAATCCAAAAGAAGCAG-3’ (antisense). Primers used to amplify the methylated RARβ gene were 5’-TCTGAGATGGTGATGATGGTTGA-3’ (sense) and 5’-AACGCAATGCAACAAAGCAGTA3’-3’ (antisense). Normal lymphocyte DNA was treated with SsII DNA methyltransferase (New England Biolabs, Inc., Beverly, MA), subjected to bisulfite modification, and used as a positive-control DNA for each PCR reaction (28). Negative control samples without DNA were included for each set of PCR. PCR of DNA from nonmalignant tissues and samples from healthy volunteers served as negative (unmethylated) controls. PCR products were analyzed on 2% agarose gels containing ethidium bromide (Life Technologies, Inc.). Conversion of all unmethylated cytosines to thymidines was confirmed by sequencing eight individual PCR products.

Reverse Transcription-PCR Analysis

Four tumor cell lines in which RARβ P2 promoter had been identified as being methylated were incubated in culture medium with and without 5-aza-2-deoxycytidine (Aza-CdR) at a concentration of 2 μg/mL for 6 days, with medium changes on days 1, 3, and 5 (29). Cells were harvested at the end of the 6th day for extraction of mRNA with a polyadenylic acid tail [poly(A) RNA]. Reverse transcription (RT) was performed on poly(A) RNA with the SuperScript II First-Strand Synthesis System (Life Technologies, Inc.), with the use of RARβ2-gene-specific reverse primer (29). One microliter of the RT reaction mixture was used as template for PCR with primer set 1 (29) to produce a 256-base-pair (bp) RARβ2 gene product. A separate PCR was performed on the same RT product by use of primer set 2, which consists of forward primer 110-FF (30) and the above-described RT reverse primer, to amplify RARβ2 (623-bp product) and RARβ4 (264-bp product) simultaneously. One-step RT-PCR (Life Technologies, Inc.) was performed with primers for glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene (sense primer: 5’-ACAGTCGTCACCTGCTCCAC-3’; antisense primer: 5’-GGCTGTTCCACCAATCTTTCG-3’), to confirm the integrity of the poly(A) RNAs. PCR products were analyzed on 2% agarose gels.

Analysis of LOH

Fourteen polymorphic microsatellite markers (see Table 2) that are located in chromosome region 3p24 and flank the RARβ gene (31) were selected for LOH analysis. DNA from 13 SCLC and 18 NSCLC tumor cell lines and their corresponding B-lymphoblastoid lines (constitutional DNA) were analyzed as described previously (27). Briefly, 20 ng of genomic DNA was amplified by PCR in the presence of [α-32P]cytidine 5’-triphosphate by use of the microsatellite markers. The PCR products were separated by electrophoresis in 6% polyacrylamide gels containing 7 M urea and were visualized by autoradiography. Markers that identified two bands of different size but of similar intensity in the lane having normal DNA were termed “informative” (i.e., heterozygous). Markers that gave only a single band in the normal DNA lane were termed “noninformative.” LOH was defined as a loss of a band corresponding to an allele present in informative cases.

Statistical Analysis

Statistical differences between groups were examined by use of Fisher’s exact test with continuity correction. All P values are two-sided. Values of P<.05 were considered to be statistically significant.

RESULTS

Methylation-specific PCR was performed on bisulfite-modified control and tumor DNA samples by simultaneous use of primers for the methylated and unmethylated forms of the RARβ gene promoter P2. As detailed in Table 1, 72% (63 of 87) of SCLC and 41% (52 of 127) of NSCLC samples showed evidence of methylation of the RARβ gene promoter P2 (two-sided Fisher’s exact test) and for all samples (two-sided P<.05) were considered to be statistically significant.

Table 1. Incidence of methylation of the RARβ gene promoter P2 in lung tumors, tumor cell lines, and control tissues

<table>
<thead>
<tr>
<th>Sample</th>
<th>No.</th>
<th>No. of methylated samples (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraffin-embedded tumors</td>
<td>21</td>
<td>13 (62)</td>
</tr>
<tr>
<td>Cell lines</td>
<td>66</td>
<td>50 (76)</td>
</tr>
<tr>
<td>All SCLC samples</td>
<td>87</td>
<td>63 (72)</td>
</tr>
<tr>
<td>NSCLC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frozen tumors</td>
<td>49</td>
<td>21 (43)</td>
</tr>
<tr>
<td>Cell lines</td>
<td>78</td>
<td>31 (40)</td>
</tr>
<tr>
<td>All NSCLC samples</td>
<td>127</td>
<td>52 (41)</td>
</tr>
<tr>
<td>Total lung cancer samples</td>
<td>214</td>
<td>115 (54)</td>
</tr>
<tr>
<td>Nonmalignant tissues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral blood lymphocytes‡</td>
<td>10</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Epithelial cells from buccal swabs‡</td>
<td>12</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Paraffin-embedded tissues</td>
<td>12</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Frozen lung tissues</td>
<td>24</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total nonmalignant samples</td>
<td>58</td>
<td>1 (1.7)</td>
</tr>
</tbody>
</table>

‡SCLC = small-cell lung cancer; NSCLC = non-small-cell lung cancer.
†The differences in methylation incidences between SCLC and NSCLC cell lines were statistically significant for cell lines (two-sided P<.001). Fisher’s exact test and for all samples (two-sided P<.001) but not for tumor tissues (two-sided P = .19).
‡From healthy volunteers.
methylations of RARβ gene promoter P2. The difference in incidence of methylation of all 87 SCLC samples compared with all 127 NSCLC samples was statistically significant (P<0.001). The incidences of RARβ promoter gene methylation between adenocarcinomas (26 of 71 [37%]) and squamous cell carcinomas (13 of 24 [54%]), the major subtypes of NSCLCs, were not statistically significantly different.

Tumor cell lines are pure tumor populations that are free of nonmalignant cells, and 95% (137 of 144) of the tumor cell lines that we examined were homozygous for either methylated or unmethylated RARβ promoter gene sequences. Five percent (seven of 144) were heterozygous and showed amplification of both methylated and unmethylated RARβ promoter gene sequences. Sample gels are shown in Fig. 1, A.

In tumor samples, most of which consist of mixtures of tumor cells and nonmalignant cells, either the band that corresponds to unmethylated RARβ only or the bands that correspond to both methylated and unmethylated RARβ were present. In 57 of 58 control samples, we observed only the unmethylated form of the gene (Table 1). Only one of the microdissected samples from a histologically normal lymph node that was used as control for paraffin-fixed samples had both methylated and unmethylated DNA. The presence of unmethylated RARβ promoter sequences in all control tissues confirmed the integrity of the DNA in these samples.

Four each of methylation-positive and methylation-negative cell line mRNAs were selected for RARβ and RAR4 gene expression studies. NSCLC cell lines HCC44, HCC193, and HCC515 and SCLC cell line H209, in which the RARβ promoter was unmethylated, contained both RARβ and RAR4 transcripts (data not shown). SCLC cell line H1607 and NSCLC cell lines HCC15, H2087, and HCC1171, in which the RARβ promoters were methylated, lacked both transcripts (Fig 1, A). After demethylation with Aza-CdR, these four cell lines were positive for both transcripts (Fig 1, B), although the intensity of the bands varied in the different cell lines.

We analyzed a panel of 31 SCLC or NSCLC tumor cell lines that were paired with corresponding B-lymphoblastoid cell lines (Table 2) for LOH at or around the RARβ gene by use of 14 polymorphic markers. We found LOH in at least one of the markers in all 13 SCLC cell lines (100%) and in 67% (12 of 18) of the NSCLC cell lines. Marker D3S1567 had a low informative rate (i.e., was seldom heterozygous) but showed 100% loss in both SCLC and NSCLC cell lines. Markers D3S1583, D3S2336, D3S2335, and D3S2337, which are centromeric to D3S1567, showed statistically significant differences in the incidences of LOH in SCLC compared with NSCLC cell lines. Although the precise location of the RARβ gene relative to these loci (31) is not known, the region bounded by these markers may be within or close to the gene. While there was no apparent relationship between LOH for any specific marker and methylation status, LOH at one or more markers was present in 89% (16 of 18) of the tumor lines in which the RARβ gene promoter was methylated.

**DISCUSSION**

RARβ may be the member of the RAR receptor family that is primarily responsible for mediating the effects of retinoic acid (32). Reduced expression of RARβ has been reported in lung cancer (33) and in other solid tumors (29). Geradts et al. (17) reported loss of RARβ expression in 75% of SCLC tumor lines and in 53% of NSCLC lines. Xu et al. (15) observed reduced expression of RARβ2 in NSCLC tumors. A marked decrease in the expression of RARβ, as well as a high frequency of LOH at 3p24, was also observed in non-neoplastic lesions (5). Only two reports addressed expression of RARβ4. Sommer et al. (30) demonstrated that the ratio of RARβ4 to RARβ2 is elevated in breast tumor cell lines. In mice that carry an RARβ4-like trans-
gene, RARβ4-like expression showed tissue-specific variation (34) and was reduced in lung tissue. These data support the hypothesis that one or more isoforms of the RARβ gene may exert tumor-suppressive effects.

Aberrant methylation of the RARβ promoter gene has been observed previously in breast and colon cancers (29,35,36). Our observations demonstrate a high frequency of aberrant DNA methylation of the RARβ P2 promoter gene in lung cancers, particularly in SCLC. In the eight cell lines that we tested, there was complete concordance between aberrant methylation of the P2 promoter and silencing of both RARβ2 and RARβ4 transcripts. Furthermore, treatment with Aza-CdR restored transcript expression, indicating that methylation is one of the mechanisms responsible for loss of expression. Allelic losses at or around 3p24, the chromosomal location of RARβ, were more frequent in SCLC (100%) than in NSCLC (67%). The high frequency of LOH at 3p24, combined with the presence of only methylated sequences in most cell lines, fulfills the criteria for Knudson’s two-hit hypothesis for tumor suppressor gene inactivation (37). While strong circumstantial evidence exists for the role of inactivation of the RARβ gene in lung cancer pathogenesis, the possibility that other genes at 3p are responsible for or contribute to lung cancer pathogenesis must be considered.

Several genes are known to be inactivated in lung cancers by aberrant methylation (38). The frequencies of aberrant methylation of the RARβ gene reported herein are among the highest for any gene described to date in lung cancers (38). Ayoub et al. (39) have reported frequent repression of both RARβ2 and RARβ4 in the bronchial epithelium of smokers. Their finding and those of other investigators (33,40) suggest that alteration in RAR expression is an early event in lung cancer pathogenesis. Tumor cells may release their DNA into the circulation, which would allow detection of aberrant methylation in DNA from the sera of lung cancer patients (38). Chemical reversal of methylation-related gene silencing (an epigenetic phenomenon) is a potential therapeutic approach (41). Our findings indicate that aberrant methylation of the RARβ gene is a frequent abnormality in lung cancers and may have clinical applications for risk assessment, for diagnosis, and for novel therapeutic approaches.

REFERENCES


Notes

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