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Methylation of Cytochrome P4501A1 Promoter in the Lung Is Associated with Tobacco Smoking

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ABSTRACT

Cytochrome P4501A1 (CYP1A1), which is involved in the metabolic activation of polycyclic aromatic hydrocarbon procarcinogens derived from tobacco smoke, is induced in the lung up to 100-fold because of tobacco smoking. Our aim was to study whether promoter methylation has any role in the smoking-associated expression of CYP1A1 in human lung. Methylation of CpG sites up to 1.4 kb upstream of *CYP1A1* gene was studied first by sequencing. Because methylation was observed between nucleotides −1400 and −1000, a methylation-specific single-strand conformational polymorphism method was designed for the region between nucleotides −1411 and −1295 that contains five potential methylation sites, one of them at the xenobiotic responsive element core sequence. Single-strand conformational polymorphism was used on DNA from normal lung samples and peripheral WBCs of smokers and nonsmokers, and on human lung adenocarcinoma (A549) and bronchial epithelial (Beas-2B) cell lines. In lung tissue complete or partial methylation occurred in 33% of heavy smokers (>15 cigarettes/day, $n = 30$), 71% of light smokers (≤15 cigarettes/day or quitted 1–7 days earlier, $n = 42$), and in 98% of nonsmokers (never and ex-smokers, $n = 49$). Methylation was found to increase in 1–7 days after quitting smoking. In active smokers the lack of methylation in the studied region of *CYP1A1* promoter was associated with a slightly higher pulmonary 7-ethoxyresorufin *O*-deethylase activity in the regression models allowing for the daily tobacco consumption and age. No association was observed in WBC between methylation and tobacco smoking. In lung-derived cell lines the methylation remained stable regardless of induction with benzo(*a*)pyrene, but a higher induction was observed in Beas-2B cells, which also had less methylation than A549 cells. The association of tobacco smoking with *CYP1A1* methylation in the lung suggests that promoter methylation is involved in the regulation of CYP1A1 induction *in vivo*.

INTRODUCTION

Cytochrome P4501A1 (CYP1A1) is involved in the metabolic activation of polycyclic aromatic hydrocarbons into mutagenic and carcinogenic derivatives that bind to DNA and may subsequently initiate the neoplastic transformation (1–3). Polycyclic aromatic hydrocarbon compounds together with nitrosamines are considered the most important species of carcinogens in tobacco smoke (4). The CYP1A1 enzyme activity is induced by tobacco smoking up to 100-fold as compared with the very low constitutive expression in human lung (5, 6). Wide interindividual variation in the enzyme activity has been observed that is not explained by the most common polymorphisms of the *CYP1A1* gene or its regulating enzymes (7). A high inducibility of CYP1A1 has been connected with increased susceptibility to smoking-associated lung cancer (8, 9).

The induction of CYP1A1 expression is regulated by an intracellular aryl hydrocarbon (AH) receptor to which the polycyclic aromatic hydrocarbon chemicals bind with high affinity. In the cytoplasm AH receptor forms a complex with a heat shock protein of M_r 90,000, the X-associated protein 2 (XAP2, AIP, or ARA9) and a cochaperone protein of M_r 23,000. After ligand binding, the AH receptor complex translocates into the nucleus, dissociates from the protein complex, and binds to a nuclear protein called AH receptor nuclear translocator (ARNT). Formation of the aryl hydrocarbon receptor (AHR):ARNT heterodimer converts the complex into its high-affinity DNA-binding form, which subsequently binds to its specific DNA recognition site, the xenobiotic responsive element (XRE) located upstream of the *CYP1A1* gene, leading to chromatin and nucleosome disruption, increased promoter accessibility, and an increase in transcription of *CYP1A1* gene (10, 11).

DNA methylation regulates the expression of several genes, *e.g.*, tissue-specific genes often show a higher level of methylation in the tissues where these genes are silent than in those where they are expressed (12). Furthermore, promoter hypermethylation is a well-known silencing mechanism for several tumor-specific genes with a CpG-rich promoter (13, 14). Cell type- and tissue-specific differences in the expression of CYP1A1 are thought to be due to cell-specific coactivators and corepressors possibly via a direct interaction with the AHR:ARNT complexes bound to XREs (15). To our knowledge, the occurrence of methylation in the promoter of human *CYP1A1* has not been studied previously *in vivo*. Our aim was to study whether promoter methylation has any role in the smoking-associated expression of CYP1A1 in the lung and if any part of the interindividual variation in the response of CYP1A1 to smoking is attributable to promoter methylation.

MATERIALS AND METHODS

Study Subjects. To assess whether methylation occurs in the *CYP1A1* promoter in human lung tissue *in vivo*, lung DNA was first studied in four active smokers with low CYP1A1 enzyme activity and in two long-term ex-smokers (Table 1). After the site of methylation was localized, a single-strand conformational polymorphism (SSCP) assay designed for this region was applied to examine lung DNA from 72 smokers, 45 ex-smokers, and 4 never-smokers. Eleven of the smokers had quitted 1–7 days before surgery. All of the subjects were from a collection of patients operated on for a tumorous lung lesion between 1988 and 1996 at the Helsinki University Hospital. Detailed information of the study population is given in Table 2. The patients were interviewed personally about their smoking and occupational history, and their informed consent for participation in the study was obtained. The study protocol was approved by the local Ethical Review Board.

Tissue Samples. The normal lung tissue samples used in this study were obtained from lobectomy or pulmonectomy specimens. For comparison, cancer tissue was studied from 10 individuals and peripheral blood leukocyte (WBC) DNA from 17 individuals. Cancer tissue was identified in the pathological examination of the specimens. Samples were stored at −80°C until use.

Methylation Analysis by Sodium Bisulfite Sequencing. Genomic DNA was isolated from frozen tissue powder using QIAamp DNA Mini kit (Qiagen). Sodium bisulfite treatment converts unmethylated cytidine into thymidine. Methylation of CpG sites can thus be detected by recognition of unmodified cytidine at CpG sites by sequencing. Bisulfite modification was performed with standard methods (16). Modified DNA was purified with the QIAquick PCR Purification kit (Qiagen). Modification was followed by NaOH (0.3 M

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Table 1 *Characteristics of individual lung samples studied for methylation by means of sequencing of cloned fragments of CYP1A1 promoter*[a]

Case no.	Smoking[b]	Tissue	EROD[c]	No. of clones sequenced				
				I	II	III	IV	V
40	S, 10 c/d	Normal lung	2.00	6	10	8	5	3
249	S, 20 c/d	Normal lung	3.76	—	—	4	—	—
264	S, 20 c/d	Normal lung	4.18	8	—	—	5	9
294	S, 10 c/d	Normal lung	8.82	6	—	9	4	—
173	ES, 24 y	Normal lung	3.30	9	2	8	8	6
191	ES, 20 y	Normal lung	0.30	7	6	9	10	8

[a] See Fig. 1 for cloned PCR products numbered I–V from 5′ to 3′.
[b] Smoking: S, smoker; c/d, cigarettes per day; ES, ex-smoker; y, years since quitting smoking.
[c] 7-Ethoxyresorutin *O*-deethylase (EROD) activity in pmol/min/mg microsomal protein.

final concentration) treatment for 5 min at room temperature, ethanol precipitation, and elution into 30 μl of water. Five fragments of *CYP1A1* promoter were amplified. The amplified fragments contain four XRE core sequences: −1385. . . −1381, −1058. . . −1054, −985. . . −981, and −501. . . −497. The CpG sites up to 1400 bp upstream of *CYP1A1* gene and the location of PCR-amplified and cloned fragments I-V (numbered from 5′ to 3′) are shown in Fig. 1. The sense primer sequences for fragments III and IV, and antisense primer sequences for fragments II and IV contained one CpG dinucleotide each. For amplification of these fragments a mixture of primers containing C or T for sense and G or A for antisense primers were used. Primer sequences for segments I-V were 5′-GTTAGTTGGGGTTAGGTTGAG-3′ (sense) and 5′-CATAACCTAACTACCTACCTCC-3′ (antisense), 5′-GTTAGTTGGGG-TTAGGTTGAG-3′ (sense) and 5′-AACCACCCAACCG/AACCCATT-3′ (antisense), 5′-AATGGGTC/TGGTTGGGTGGTT-3′ (sense) and 5′-AAA-CCCCCACCCTACCCCC-3′ (antisense), 5′-GGGTTTTGGGGGATAGGT-TT-3′ (sense) and 5′-CG/ATACAAATACCTCCCCAAC-3′ (antisense), and 5′-GGAAGGAGGTTATTAC/TGGGG-3′ (sense) and 5′-CACCTAAAA-ATCCCAATTCCAA-3′ (antisense), respectively. In the PCR reaction, 5 μl (10 μl for fragment III) of bisulfite-modified DNA was amplified in the reaction mixture containing 0.25 mM deoxynucleoside triphosphate, 1 μM of each primer, and 2.5 units of HotStarTaq DNA polymerase (Qiagen) in a total volume of 50 μl at 95°C for 15 min, 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by elongation at 72°C for 10 min. The amplified PCR products were purified from low melting agarose gels and cloned by using the pGEM-T Easy Vector System II (Promega). Plasmids containing inserts were verified by restriction digestion. Several clones of each PCR product were sequenced with the ABI PRISM DNA Sequencing kit (PE Applied Biosystems, Warrington, United Kingdom) in the ABI PRISM 377 DNA sequencer (Table 1).

Methylation Analysis by SSCP. Sequencing of the cloned fragments of *CYP1A1* promoter demonstrated methylation both in active smokers and in long-term ex-smokers only at the 5′ end of the studied region, covering two XRE core sequences (−1385. . . −1381 and −1058. . . −1054; Table 3). One of these two XREs (−1385. . . −1381) was methylated in five clones (Fig. 1). Therefore, SSCP according to Suzuki *et al.* (16) was applied to fragment I, which contains five potential CpG methylation sites, one of them at the XRE core sequence. After bisulfite treatment, this 117-bp fragment was amplified by PCR using primers with a fluorescent dye label at the 5′ end of either sense or antisense primer. Because detection of the sense strand gave better separation of methylated and unmethylated DNA, the FAM$^{5'}$-labeled sense primer

Table 2 *Characteristics of the study population*

Group	M/F[a]	Age[b]	Years of smoking[b]	Squamous/ adeno/others[c]
Smokers				
>15 c/day	28/5	58 ± 10	41 ± 10	14/15/4
≤15 c/day	27/12	65 ± 7	44 ± 8	16/16/7
Ex-smokers				
≤1 year	9/2	61 ± 7	40 ± 8	8/2/1
>1 year	31/3	66 ± 7	35 ± 13	18/7/9
Never-smokers	1/3	61 ± 8	0	0/0/4

[a] M/F, number of males/females; c, cigarettes.
[b] Mean years ± SD.
[c] Histological tumor type: squamous, squamous cell carcinoma; adeno, adenocarcinoma; others, other types including small cell carcinoma, large cell carcinoma, adenosquamous, and pleomorphic carcinoma (never-smokers: leiomyosarcoma, carcinoid tumor and two inflammatory tumors).

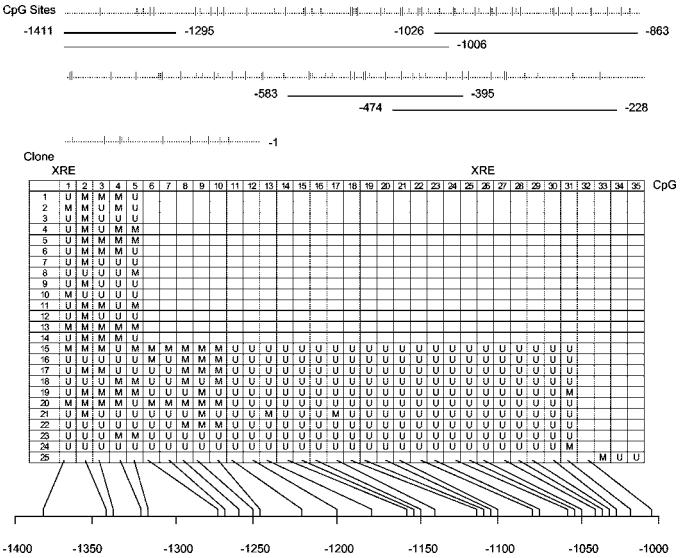

Fig. 1. CpG sites and methylation of the *CYP1A1* promoter region 1400-bp upstream of the gene. The density of CpG sites, and the location of amplified and cloned fragments I-V from 5′ to 3′ are shown. The CpG sites between −1400 and −990 are numbered from 1 to 35, and all of the clones with methylation are listed below. Clones 1–14 represent fragment I, clones 15–24 fragment II, and the clone 25 fragment III. CpG sites are indicated as follows: M, methylated; unmethylated, U.

was used in subsequent analyses. The PCR reaction was performed as before except for the final elongation step of 90 min. For SSCP analyses, fluorescent PCR products were diluted with formamide containing GeneScan-500 Size Standard (6-carboxytetramethylrhodamine), heat-denatured, and loaded onto 5% GeneScan Polymer (PE Applied Biosystems) gel containing 10% glyserol. Electrophoresis was carried out at 29°C for 30 min in the ABI PRISM 310 Genetic Analyzer. Data were analyzed by using the GeneScan 3.7 software (PE Applied Biosystems). The results were visualized as peak images.

The SSCP analysis was first applied to the cloned fragment I. Various combinations of methylation in the five CpG sites at this region were identified by sequencing, and these clones were subsequently used as standards for migration of differentially methylated DNA (Fig. 2). Serial dilutions from 1:5 to 5:1 of cloned methylated and unmethylated DNA were used to examine the relation of the peak height to the amount of DNA. The heights of the peaks given by the GeneScan 3.7 software were found roughly to correspond to the percentages of methylated and unmethylated DNA in a sample.

When uncloned PCR products were studied, the peak was accepted to represent partially or completely methylated DNA, if it was recognized as a peak by the computer software, and if the peak was located within the area between the peaks given by the cloned unmethylated and completely methylated DNA strands, which were run as controls in each series of samples. Examples of images given by the cloned DNA strands representing different methylation patterns and by uncloned PCR products from human lung samples are shown in Fig. 3. The distance of a peak from the peak of unmethylated DNA was not directly proportional to the number of methylated CpG sites (Fig. 2). However, none of the peaks of the partially methylated clones overlapped the peak of the completely methylated DNA.

For statistical analyses, the heights of the peaks were converted into per-

centages of total DNA in a sample, and the peaks were interpreted to represent unmethylated DNA, partially methylated DNA (all of the peaks between complete methylation and no methylation), or completely methylated DNA. The SSCP analysis was repeated two or three times from 30 samples with good reproducibility. The best visual image was chosen for statistical analyses.

Analysis of 7-Ethoxyresorufin *O*-Deethylase (EROD) Activities in Lung Samples. EROD activity in lung microsomal protein fractions was measured kinetically at room temperature by a fluorometric method (17). The assay mixture (2.1 ml) contained 50 μg of microsomal protein, 0.05 mM NADPH, 1 μM 7-ethoxyresorufin, 1% DMSO, and 0.1 M Tris-HCl buffer (pH 7.8). For calibration, 10 μl of 5.0 μM resorufin was added to each assay. The protein determinations and preparation of lung microsomes have been described elsewhere (18).

EROD activity as repeated from individual microsomal samples showed a relative SD of $\leq$7%. The reproducibility of determinations of EROD activity was studied also in a series of two separate lung tissue samples from eight individuals. The intraclass correlation coefficient for EROD activity in paired lung samples was 0.41.

Analysis of *CYP1A1* Genotypes. The *CYP1A1* alleles ascribed to the presence of *Msp*I site at the 264th base downstream from the additional polyadenylation signal (*CYP1A1*2A*), and to the substitution of isoleucine to valine at residue 462 in the haem-binding region (*CYP1A1*2B*) were determined as described earlier (19, 20).

Statistical Analyses. The differences between proportions were assessed by 2-tailed Fisher's exact tests. The evaluation of the relationship between the numeric variable EROD activity, the number of cigarettes smoked per day, age, methylation of *CYP1A1* promoter, and *CYP1A1* polymorphisms was carried out by ordinary least-squares multiple regression (21). To fulfill the assumption of normality, the outcome variable EROD activity was subjected to a logarithmic transformation. Methylation of *CYP1A1* promoter was entered into the models using an indicator variable for no methylation. For the *Msp*I and Ile^{462}Val polymorphisms indicator variables for variant genotypes were applied.

Analyses on A549 and Beas-2B Cell Lines. The human lung adenocarcinoma cell line A549, originally from the American Type Culture Collection (Rockville, MD), was cultured in Ham's F12 medium with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), and 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The virus transformed human bronchial epithelium Beas-2B cell line (originally from American Type Culture Collection) was cultured in DMEM nutrient mixture F-12 Ham (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen), and 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The cells were cultured at 37°C in 5% CO_2 and saturated humidity. Nearly confluent cells were incubated in the presence of benzo(*a*)pyrene [(B(*a*)P; Sigma] or vehicle only (DMSO) for desired time, A549 cells for 24 and 96 h, and Beas-2B cells for 24 h. Fresh medium and B(*a*)P were replaced every 24 h. Both cell lines were incubated with three different concentrations of B(*a*)P (Fig. 4). After incubation, the cells were washed with PBS (pH 7.4), lysed in guanidine thiocyanate solution, and subjected to RNA extraction by cesium chloride centrifugation (22). Five μg of total RNA was electrophoretically resolved and transferred to Nylon filter. The filters were air dried and cross-linked by UV exposure. The filters were hybridized with [α^{32}P]dCTP-labeled probes using the standard procedures. *CYP1A1* promoter methylation in A549 and Beas-2B cells was analyzed by SSCP in B(*a*)P-treated and untreated cells.

Table 3 *Percentage of methylation at CpG sites in CYP1A1 promoter of smokers[a] (S) and long-term ex-smokers[a] (ES)*

Fragment no.[b]	No. of clones sequenced, S/ES	% of methylated CpG sites, S/ES	Methylation of XRE[c]
I	20/16	19/14	Yes (−1385. . .−1381)
II	10/8	10/16	Yes (−1385. . .−1381)
III	21/17	0/<1	No
IV	14/18	0/0	No
V	12/14	<1/0	—

[a] See Table 1.
[b] See Fig. 1 for cloned PCR products numbered I–V from 5′ to 3′.
[c] XRE, xenobiotic responsive element.

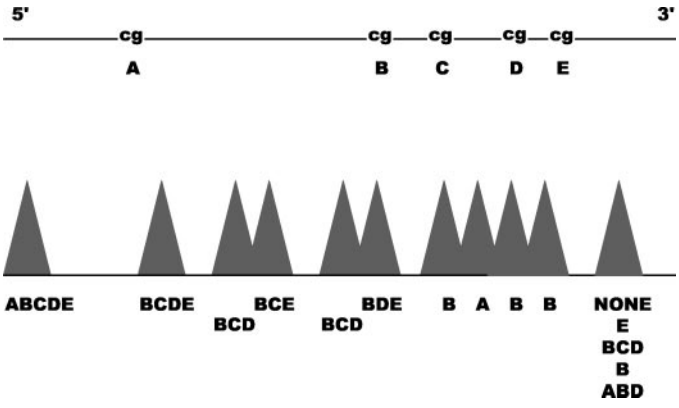

Fig. 2. Schematic presentation of the migration of differentially methylated cloned fragments (nucleotides from −1411 to −1295) of *CYP1A1* promoter in single-strand conformational polymorphism. The fragment contains 5 potential methylation sites marked from A to E.

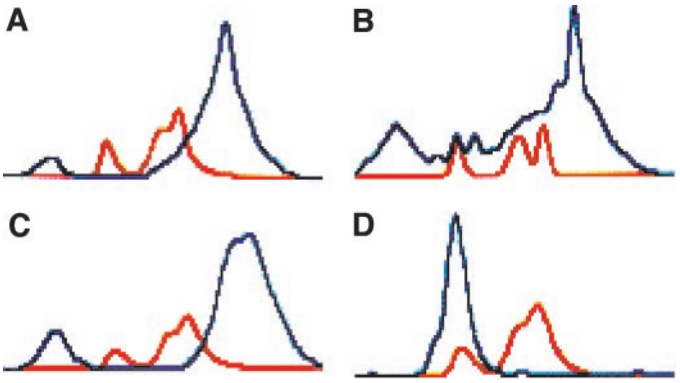

Fig. 3. Methylation-specific single-strand conformational polymorphism of a fragment (nucleotides from −1411 to −1295) of *CYP1A1* promoter from normal lung DNA of a smoker (*A*) and a never-smoker (*B*), and cloned fragments with no methylation (*C*), and complete methylation (*D*). The smoker sample (*A*) migrates as the unmethylated clone (*C*), whereas the never-smoker sample (*B*) has additional peaks demonstrating partially and completely methylated DNA. *Red line*, size marker.

RESULTS

Methylation of *CYP1A1* Promoter. The possible occurrence of the methylation of *CYP1A1* promoter in human lung was first studied in four active smokers with low pulmonary EROD activity and in two long-term ex-smokers (Table 1), because it was hypothesized that methylation may inhibit *CYP1A1* transcription. The regions of the promoter covering the XRE core sequences between nucleotides −1411 and −228 were amplified and cloned after sodium bisulfite treatment (Fig. 1). It was observed that both in active smokers and in nonsmokers the methylation of CpG sites occurred only at the 5′ end of the studied region between nucleotides from −1400 to −1000 (Fig. 1; Table 3). At this region on average 9% (5 of 54 clones) of the first XRE (−1385. . . −1381) and 12% of all of the CpG sites were methylated. The first XRE core sequence from the 5′ end of the studied region was the only XRE that was found to be methylated (Fig. 1; Table 3). Complete, partial, and no methylation was found in 3%, 33%, and 64% of the clones of fragment I and in 0%, 89%, and 11% of the clones of fragment II, respectively.

The methylation of *CYP1A1* promoter was studied in lung DNA from 121 individuals by SSCP applied for fragment I. The peaks in the SSCP images that represented completely and partially methylated DNA corresponded up to 20% and 40% of DNA, respectively.

To study cell-type differences in the degree of methylation SSCP was also performed on 10 lung cancer samples that represented all of

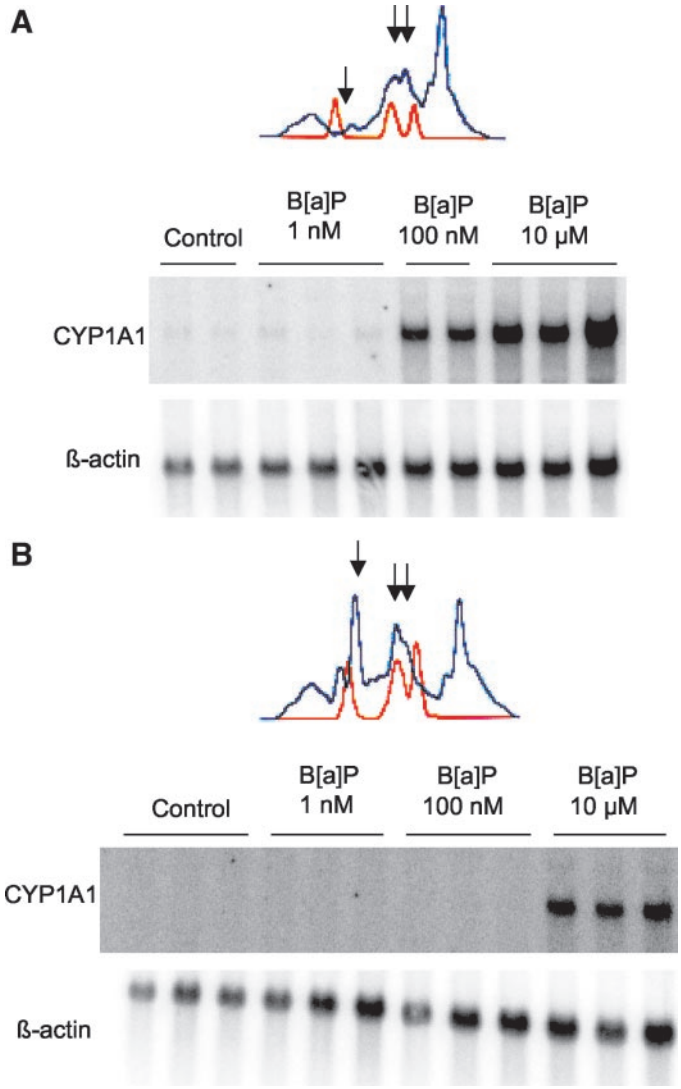

Fig. 4. *CYP1A1* promoter methylation and CYP1A1 expression in virus transformed human bronchial epithelial cells (*Beas-2B; A*), and in human lung adenocarcinoma cells (*A549; B*). Beas-2B cells were induced with a lower concentration of benzo(*a*)pyrene than A549 cells. In single-strand conformational polymorphism images the peak of complete methylation (*arrow*) and partial methylation (*double arrow*) are lower in Beas-2B (*A*) than in A549 cells (*B*). *Red line*, size marker.

the major histological types and on 17 peripheral WBC samples. Similar percentages of completely and partially methylated DNA as in normal lung DNA were observed in lung tumor (data not shown) and in WBC DNA (Table 4).

***CYP1A1* Promoter Methylation and Smoking.** The methylation of *CYP1A1* promoter was dependent on smoking so that part of lung DNA of the promoter region between nucleotides −1411 and −1295 was completely methylated in 94% of nonsmokers (never smokers and ex-smokers for >7 days), but in only 46% of smokers ($P < 0.000$; Table 5). Eleven of the smokers had quitted smoking 1–7 days before surgery. An increase in the occurrence of complete methylation from 41% of active smokers to 73% of those who had quitted 1–7 days earlier was observed ($P = 0.097$; Table 5). Moreover, in active smokers a dose-dependence between the occurrence of methylation and the number of cigarettes consumed per day was observed. Complete or partial methylation was observed in 71% of the light smokers (≤15 cigarettes/day), whereas it was found only in 33% of the heavy smokers (>15 cigarettes/day, $P = 0.005$; Table 5). Age and gender had no effect on the methylation of *CYP1A1* promoter (data not shown). In peripheral WBC no association was found between smoking and methylation of *CYP1A1* promoter (Table 4).

***CYP1A1* Promoter Methylation and CYP1A1 Enzyme Activity.** The CYP1A1 dependent EROD activity ranged from 1 to 184 (median 30.5) pmol/min/mg microsomal protein in smokers and from 0.3 to 3.3 (median 0.7) pmol/min/mg in nonsmokers. The number of cigarettes smoked per day accounted for the pulmonary EROD activity better in the active smokers with no methylation than in the smokers with methylation between nucleotides −1411 and −1295 upstream of *CYP1A1* gene according to SSCP. However, the regression models allowing for the effect of age and the daily tobacco consumption explained only 26% and 37% of the variation in EROD activity in the active smokers with and without methylation, respectively. The lack of methylation was associated with a slightly higher pulmonary EROD activity (data not shown). For example, the estimated mean level of EROD activity was 34 pmol/mg/min for the subjects with no methylation, age of 60, and smoking 20 cigarettes/day, whereas it was 26 pmol/mg/min for similar subjects with methylation of *CYP1A1* promoter (not statistically significant difference). There was no correlation between EROD activity and the percentage of methylated DNA in a sample either in smokers or in nonsmokers (data not shown).

***CYP1A1* Promoter Methylation and *CYP1A1* Polymorphism.** Because the *CYP1A1*2A* and **2B* alleles have both been linked to the increased risk of lung cancer in epidemiological studies, we studied whether either of these polymorphisms associate with the degree of methylation of *CYP1A1* promoter. The frequencies of the variant *CYP1A1*2A* and **2B* alleles were 0.12 and 0.04, respectively, in our study population, and neither of them had any association with the methylation of *CYP1A1* promoter in the lung (data not shown).

***CYP1A1* Promoter Methylation and CYP1A1 Expression in A549 and Beas-2B Cells.** *CYP1A1* promoter methylation in human lung adenocarcinoma cells (A549) and in virus transformed human bronchial epithelial cells (Beas-2B) was studied by SSCP. A549 cells were treated with three concentrations of B(*a*)P for 24 and 96 h. In altogether 23 experiments with A549 cells the mean percentages of partially and completely methylated DNA were 28 (5 ± SD) and 35 (9 ± SD), respectively. Beas-2B cells were exposed to three concen-

Table 4 *Methylation of the region between −1411 and −1295 upstream of CYP1A1 gene in lung tissue compared to peripheral WBC*

			Methylation (Partial/ Complete in % of DNA)	
Case no.	Smoking[a]	EROD[b]	Lung	WBC
200	S, 20 c/d	82	0/0	26/17
202	S, 20 c/d	142	0/0	16/6
207	S, 20 c/d	100	0/0	22/7
185	S, 15 c/d	75	0/0	19/7
201	S, 12 c/d	133	0/0	17/7
285	S, 10 c/d	2	20/8	36/8
288	S, 7 c/d	9	0/0	20/8
292	S, 2 c/d	3	8/13	22/5
191	ES, 20 y	0.3	11/0	0/9
310	ES, 21 y	0.7	7/8	27/0
246	ES, 22 y	1	11/10	11/7
153	ES, 28 y	1	6/7	22/0
309	ES, 30 y	2	7/6	9/8
319	ES, 34 y	0.7	0/16	22/6
102	ES, 48 y	1	5/6	9/6
84	NS	0.5	26/11	9/8
86	NS	2	23/9	21/7
51	NS	3	14/7	NA[c]
76	NS	2	20/6	NA

[a] Smoking: S, smoker; ES, ex-smoker; NS, never-smoker; c/d, cigarettes per day; y, years since quitting smoking.
[b] 7-Ethoxyresorutin *O*-deethylase (EROD) activity in pmol/min/mg microsomal lung protein.
[c] WBC not available (NA).

Table 5 *Occurrence of methylation between −1411 and −1295 upstream of CYP1A1 gene in smokers and nonsmokers according to SSCP*

		Time since smoking			Active smokers[a]	
Methylation	Never-smokers % (n/N)	>1 year % (n/N)	≤1 year[b] % (n/N)	1–7 days % (n/N)	≤15 c/d[c] % (n/N)	>15 c/d % (n/N)
Complete[d]	100 (4/4)	94 (32/34)	91 (10/11)	73 (8/11)	55 (17/31)	27 (8/30)
Partial[d]	100 (4/4)	88 (30/34)	82 (9/11)	73 (8/11)	71 (22/31)	33 (10/30)
No methylation	0 (0/4)	0 (0/34)	9 (1/11)	27 (3/11)	29 (9/31)	67 (20/30)

[a] Smoking daily until the date of surgery.
[b] Time since smoking 2–52 weeks.
[c] c/d, cigarettes per day.
[d] Most subjects with methylation had both complete and partial methylation in single-strand conformational polymorphism.

trations of B(*a*)P for 24 h. In Beas-2B cells the mean percentages of partially and completely methylated DNA in 12 experiments were 37 (5 ± SD) and 11 (6 ± SD), respectively. In both cell lines the degree of methylation remained stable regardless of the exposure to B(*a*)P or duration of induction (data not shown). However, in Beas-2B cells, which had a lower degree of complete methylation than A549 cells ($P < 0.001$), CYP1A1 expression was induced with a lower concentration of B(*a*)P than in A549 cells (Fig. 4).

DISCUSSION

The occurrence of methylation in the promoter of *CYP1A1* in a region covering 1.4 kb 5′ upstream of the gene was studied. About 10–20% of CpG sites were methylated in human lung in a region between nucleotides −1400 and −1000 upstream of *CYP1A1*. The only XRE core sequence that was found to undergo methylation is located at −1385. . . −1381. A methylation-specific SSCP was applied to a region between nucleotides −1411 and −1295. Smoking was observed to influence the methylation so that peaks in the SSCP image demonstrating complete or partial methylation occurred in 98% of nonsmokers in contrast with in 56% of smokers in the SSCP analyses on lung tissue of 72 smokers and 49 nonsmokers. Also, there was an inverse correlation between methylation and the number of cigarettes smoked daily. After quitting smoking the methylation of *CYP1A1* promoter was increased already 1–7 days because the last cigarette. These findings suggest that methylation takes part in the regulation of CYP1A1 induction *in vivo* in human lung.

Several binding sites for AHR:ARNT complex (XREs) are located in the upstream sequence of human *CYP1A1*. A specific recognition sequence has been identified consisting of a central core, 5′-GCGTG-3′, and the appropriate flanking nucleotides that are necessary for the binding and functionality of the AHR:ARNT complex (23–25). We chose to amplify the part of *CYP1A1* promoter, which according to the literature carries functional XREs. Five fragments of *CYP1A1* promoter covering four XREs that fulfill the criteria of the consensus sequence and are located within 1400 bp upstream of *CYP1A1* gene, were amplified and cloned after sodium bisulfite treatment of lung DNA. Methylation of CpG sites was localized at the most 5′ part of the studied region.

To investigate whether the observed promoter methylation had any influence on *CYP1A1* transcription, the methylation between nucleotides −1411 and −1295 was analyzed with SSCP, and the CYP1A1-related EROD activity was determined in 121 human lung samples. EROD activity was higher in the active smokers with no methylation than in those with methylation of the *CYP1A1* promoter, but the difference was not statistically significant after adjustment for age and for the daily tobacco consumption. If *CYP1A1* promoter methylation in human lung is directly dependent on exposure, *e.g.*, on ligand binding to AH receptor, the effects of smoking and DNA methylation on EROD activity are not separable, and, thus, the association between EROD activity and methylation cannot be studied by regression analysis allowing for the daily tobacco consumption.

The methylation of one XRE may, at least in part, be compensated by binding of the AHR:ARNT complex to the remaining unmethylated XREs, and by the function of coactivators and repressors. Furthermore, *CYP1A1* promoter methylation may extend 5′ upstream beyond the studied region and affect other XREs that are necessary for the smoking-associated induction of CYP1A1. Kubota *et al.* (26) performed deletion analyses of the human *CYP1A1*-chloramphenicol acetyltransferase (CAT) expression plasmid and observed that the expressed CAT activity decreased progressively with the elimination of three regions, −7.6 to −4.2, −1296 to −988, and −988 to −532. The deletion from −1292 to −988 caused the largest decrease in the induced CAT activity (26). It is interesting that the methylation we observed in the *CYP1A1* promoter was localized in this same region.

Two negative regulatory elements are located in the 5′-upstream region between the positions −794 and −558 of human *CYP1A1* (27). According to the present study DNA methylation is not likely to participate in the function of negative regulatory elements. We did not study the methylation specifically in this region, but the DNA methylation that occurred in the 5′ end of the region we studied did not extend to the region of negative regulatory elements.

There has not been much evidence supporting the possible role of methylation in the regulation of CYP1A1 induction. Cytocine methylation at CpG dinucleotides of the XRE core sequence has been found to diminish the AHR:ARNT-DNA interaction in gel shift assays (28, 29) and to inhibit the enhancer function in the CAT assays (28). A gender-specific expression of mouse Cyp2d9 is regulated by DNA methylation (30). In the study of Kress and Greenlee (15) DNA methylation was not involved in cell line-specific 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced expression of human *CYP1A1* and *CYP1B1* genes.

The connection between methylation and CYP1A1 induction is supported by our findings from human lung-derived cell lines. In immortalized bronchial epithelial cells (Beas-2B) the peak of complete methylation in SSCP images was remarkably lower and the B(*a*)P-induced CYP1A1 expression higher than in lung adenocarcinoma (A549) cells. However, it cannot be ruled out completely that the difference in CYP1A1 induction between the two cell lines might be attributable to cell type-specific factors other than methylation. The smoking-associated CYP1A1 expression in human lung takes place mainly in distal bronchial, bronchiolar, and type II alveolar epithelium (31), suggesting that the cells from which Beas-2B and A549 cell lines originate were highly inducible for CYP1A1.

DNA methylation is a common silencing mechanism for tumor-specific genes with a CpG-rich promoter (14). Similarly to carcinogenesis, immortalization of cells may cause disturbances in DNA methylation. Thus, cell lines may not be a good surrogate for normal cells and tissues in studies of DNA methylation. In contrast to the finding about the dose-dependent association of smoking with *CYP1A1* promoter methylation in human lung *in vivo*, the methylation in lung-derived cell lines remained stable regardless of the induction. The inverse correlation between smoking and *CYP1A1* promoter

methylation in lung tissue but not in peripheral WBC favors an organ-specific regulation of methylation.

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