Hypermethylation of cytosines in CpG-rich islands of the promoter regions of regulatory genes has been discovered as a common mechanism of gene silencing during carcinogenesis. We analysed 64 primary lung carcinomas for promoter methylation of the tumour suppressor genes (TSGs) p16 (p16\textsuperscript{INK4a}/CDKN2A) and p14 (p14\textsuperscript{ARF}) by methylation-specific PCR, in order to evaluate aberrant methylation as a potential biomarker for epigenetic alterations in tobacco-related lung cancer. Methylation of p16 was observed in 34% (22/64) of the lung tumours examined. In particular, p16 methylation occurred in nonsmall cell lung cancer (NSCLC) only, with 41% (22/54) of the tumours being positive. The highest frequency was found in large cell carcinoma (5/7, 71%), followed by adenocarcinoma (9/25, 36%) and squamous cell carcinoma (7/21, 33%). Methylation of the p14 gene was less frequent in lung cancer (4/52, 8%). When association with tobacco smoking was analysed, 42% (21/50) of NSCLC from ever smokers exhibited p16 methylation. Interestingly, the analysis revealed a significantly higher risk of p16 methylation in former smokers as compared to current smokers [odds ratio (OR) 5.1; 95% confidence interval (CI) 1.3–22]. The difference was retained after adjustment for age (OR 3.7; 95% CI 0.9–17). The promoter methylation results were then combined with data on genetic alterations determined previously in the same set of tumours. This data similarly showed that p16 methylation in parallel with p16 gene mutation or p14 methylation occurred more frequently in former smokers than in current smokers (44% vs. 14%; \(P = 0.035\)). Taken together, our data suggest that analysis of promoter methylation in TSGs may provide a valuable biomarker for identification of groups with an elevated risk of cancer, such as smokers and ex-smokers.

Key words: lung cancer; aberrant methylation; tobacco smoking; tumor suppressor gene

Hypermethylation of cytosines in CpG-rich islands of the promoter regions of genes is one of the mechanisms of gene silencing during the development in mammals.\(^1\) In cancer, hypermethylation of the promoter regions is associated with transcriptional inactivation and loss of expression of tumour suppressor and other regulatory genes, constituting an alternative, epigenetic way to the loss of gene function.\(^2,3\) Furthermore, recent data suggest that abnormal methylation of cancer-related genes occurs in human cancer in a tumour-type and gene-specific manner.\(^4\)

The tumour suppressor gene p16 (p16\textsuperscript{INK4a}/CDKN2A) is a cell cycle regulator that is frequently inactivated in many different types of malignancies, including lung cancer.\(^5,6\) It has been demonstrated that p16 gene may be transcriptionally inactivated by aberrant promoter methylation during tumorigenesis.\(^8–10\) The gene product, p16 protein, is a molecular component of the retinoblastoma protein (pRB) regulatory pathway, functioning as a specific inhibitor of cyclin-dependant kinases 4 and 6.\(^11\) Inactivation of p16 enables initial phosphorylation of pRB that releases it from transcription factor and allows subsequent progression of cells into S phase.\(^12,13\)

The p14 gene (p14\textsuperscript{ARF}/ARF), an alternative reading frame of the p16 gene locus using a separate promoter region, encodes an important regulatory protein functioning in the p53 pathway.\(^14,15\) p14 and p16 proteins are encoded by 2 different first exons and the encoding is continued in alternative reading frames through a common exon 2.\(^15\) Although encoded by the same locus, both proteins act in different cell cycle inhibition pathways.\(^16\) p14 neutralises MDM2-dependant p53 degradation, thereby increasing p53 stability.\(^16\) Methylation of p14 promoter has been shown to occur in human tumours.\(^3\)

Exposure to carcinogens from tobacco smoke is the primary cause of lung cancer, with occupational exposure to pulmonary carcinogens, such as asbestos, being the second major etiological factor.\(^17\) In addition to data that indicates that various types of genetic damage are associated with exposure to mutagenic and carcinogenic components from tobacco smoke,\(^18–20\) recent studies have suggested an association between p16 promoter methylation and tobacco smoking in lung cancer.\(^21–24\)

In our work, we determined the frequency of p16 and p14 promoter region methylation in primary lung tumours by methylation specific PCR (MSP), a method allowing sensitive detection of the methylation status of cytosine residues of CpG islands within a gene promoter.\(^25\) In addition, samples of nonmalignant peripheral lung tissue from the majority of the patients were also examined. We then analysed p16 tumour methylation data in regard to demographic, clinico-pathological and exposure characteristics of the cases. Finally, we analysed the promoter methylation data in combination with previously determined gene mutation data in current and former smokers.

MATERIAL AND METHODS

Patients

Primary lung tumour samples (\(n = 64\)) were obtained from lung cancer patients who underwent a surgical resection, as described previously.\(^26\) For 58 of the cases, a specimen of nonmalignant peripheral lung tissue was also available for investigation. In addition, leucocyte DNAs from cancer patients (\(n = 15\)) were examined. Ten of the lung cancers were diagnosed as SCLC, and 54 as NSCLC, including 23 adenocarcinoma, 21 squamous cell carcinoma, 2 adeno-squamous cell carcinoma and 7 large cell carcinoma cases. One case was a tumour carcinoid. The mean age

Abbreviations: CI, confidence interval; LCC, large cell carcinoma; MSP, methylation specific PCR; NSCLC, non-small cell lung cancer; OR, odds ratio; SCLC, small cell lung cancer. Sonata Jarmalaite’s former surname was Rudaitiene.

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*Correspondence: Finnish Institute of Occupational Health, Topeliuksenkatu 41 aA, FIN-00250 Helsinki, Finland. Fax: +358-9-47472110. E-mail: Kirsti.Husgafvel-Pursiainen@ttl.fi

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of the patients was 63.5 ± 8.7 years, 15 of them were females and 49 males. Five patients were lifelong nonsmokers, 21 former smokers (who ceased smoking at least 1 year before the diagnosis) and 38 current smokers. Smokers had consumed in average 21.3 ± 8.9 cigarettes per day and had a mean cumulative exposure of 40.4 ± 19.9 pack-years. Data on occupational asbestos exposure were available for 61 patient, with 22 cases exposed to asbestos at work.\(^{26}\)

**Methylation specific PCR**

Genomic DNA was extracted from frozen samples of lung tumour tissue, peripheral lung tissue and peripheral blood leukocytes using proteinase-K digestion and phenol/chloroform purification followed by ethanol precipitation. Methylation specific PCR,\(^{25}\) was used for analysis of the methylation pattern in 5′ region of the p16 and p14 genes. Briefly, 2 μg of DNA in a final volume of 50 μl were denatured with 2 M NaOH (Merck) for 10 min at 37°C, and after that exposed to bisulfite modification (converting unmethylated cytosines to uracils) with 3 M sodium bisulfite (Sigma Chemical Co., St. Louis, MO) plus 10 mM hydroquinone (Fluka, Milwaukee, WI) for 16 hr at 50°C. Modified DNA was purified with the Wizard DNA Clean-up System (Promega, Madison, WI) in vacuum manifold. DNA modification was completed by 3 M NaOH treatment for 5 min at room temperature. DNA was precipitated with 70% ethanol and dissolved in 40 μl of sterile water. Bisulfite modified DNA was analysed for methylation immediately or after the storage at −80°C. PCR primers specific for methylated (M) and unmethylated (U) sequences within the 5′ region of the p16 and p14 genes were synthesised according to published sequences\(^{25,27}\) and used to determine methylation in promoter region. A set of PCR primers for nonmodified DNA (W) was included in study to control the bisulfite conversion of DNA. Primer sequences for p16 gene analysis were as follows: M primers, 5′-CCACCTCAATACTGACCTCCGCCGACC (antisense); U primers, 5′-TTATAGGAGGTTGGGTGATTGT (sense) and 5′-CCAC-TAAAACTCAACCTCCCAA (antisense); W primers, 5′-CAGAGGGTGGGCGCGCGGC (sense) and 5′-CGGGCCGCGGCCGCG (antisense). Primer sequences for p14 gene analysis were as follows: M primers, 5′-GGTATAGGAGGTTGGGTGATTGT (sense) and 5′-AAAGCACCTCCTGACAC (antisense); U primers, 5′-TTTGTGTGTTAAAGGGGTGGTTAGT (sense) and 5′-CACAAACCCTCACTCCACA-AAC (antisense); W primers, 5′-CTGGTTGCAACAGGGCGGCCGCGC (sense) and 5′-CGAAAACCCTCACTCCAGGCGG (antisense). For p14 gene, PCR product amplified with M primers was 122 bp and that with U primers was 132 bp in length. The PCR mixture for 50 μl of total reaction volume contained 2–4 μl of modified DNA template, PCR buffer, 0.4 mM of each deoxynucleotide triphosphate, 2.5 mM of MgCl₂, 1% of dimethyl sulfoxide, forward and reverse primers at the final concentration of 6 ng/μl and 1.25 U of AmpliTaq Gold polymerase (Perkin-Elmer, Oak Brook, IL). Modified DNA from human urinary bladder cancer cell line T24 and from colorectal cancer cell line SW48 (both from American Type Culture Collection) were included in all the experiments as a positive control for methylation of p16 and p14, respectively. DNA from leukocytes of healthy controls (n = 2) was used to control unmethylated PCR product, and water controls were also included. DNA from T24 cell line was serially diluted with unmethylated leukocyte DNA from healthy controls to determine sensitivity of the detection. PCR was performed in a thermocycler at following conditions: 10 min at 95°C for the activation of polymerase, 35 cycles at 95°C for 45 sec, 62°C for 45 sec and 72°C for 45 sec and the final extension in 72°C for 10 min. Nine microliters of each of PCR product was loaded onto nonde-naturing 7.5% polyacrylamide gel and after ethidium bromide staining visualised under UV illumination. Nine microliters of each of PCR product was loaded onto nonde-naturing 7.5% polyacrylamide gel and after ethidium bromide staining visualised under UV illumination.

**Statistical analysis**

Odds ratios (OR) and the exact or Mantel-Haenszel 95% confidence intervals (CI) for 2 binomial samples were calculated for single variables using the computer software StatXact-4 for Windows (CYTEL Software Corporation, 1998). Owing to small sample sizes, exact logistic regression analysis was carried out to adjust for the effects of the statistically significant single variables. For this, the logistic procedure of the SAS statistical analysis system was used (SAS version 8.2). Two-sided Fisher’s exact test and Student’s t-test were used for comparison of categorical and continuous variables, respectively. P ≤ 0.05 was considered as statistically significant.

**RESULTS**

**Methylation in lung tumours and nonmalignant lung tissue**

To set up the methylation specific PCR, we analysed methylated sequences in the promoter regions of the p16 and p14 genes in DNAs from cancer cell lines T24 and SW48, respectively, as positive controls. Clear methylation signals were obtained for both genes by MSP. T24 DNA was diluted up to 20 times with unmethylated DNA from leukocytes, and p16 methylation was detected in the dilution series robustly and reproducibly (Fig. 1).

**FIGURE 1** Detection of promoter methylation in the p16 and p14 genes. M, PCR reaction with primers for the methylated sequence; U, PCR reaction with the primers for unmethylated sequence; T, tumour DNA; P, peripheral tissue DNA; H₂O, water control. (a) DNA from cancer cell line T24 (positive control for p16 methylation) serially diluted with leukocyte DNA from healthy controls (L, negative control). The amount of T24 DNA is presented in percentages. MSP detected p16 methylation in 20 times diluted (5%) T24 DNA. (b,c) MSP analysis of p16 methylation in tumour and lung peripheral tissue samples from lung cancer patients. Both methylation positive and negative samples are illustrated. The unmethylated PCR-product was seen in all samples. (d) MSP analysis of p14 promoter methylation. DNA from cancer cell line SW48 was used as the positive control in the p14 analysis.
cases, methylation was observed in peripheral tissue only. All leukocyte DNAs, whether from healthy controls (n = 2) or cancer patients (n = 15), were negative. In all tissue samples positive for methylation, the corresponding unmethylated PCR product from both genes was also detected.

The frequency of p14 promoter methylation was clearly lower in this series of lung tumours as compared to that of p16 gene. Four (8%) out of the 52 cases analysed for p14 exhibited methylation in the tumour (Table I). For 3 of those 4 with tumour methylation, a peripheral lung tissue sample was available for analysis, and 1 of them was methylated in p14 promoter region.

Methylation and clinico-pathological features

From the lung cancers analysed for p16 methylation, 54 cases were NSCLCs and 10 cases were SCLCs. No aberrant p16 methylation was observed in SCLCs (0/10), and these cases were excluded from further statistical analysis. In NSCLC, 41% (22/54) of the cases were positive for p16 promoter region methylation. Analysis of p16 methylation in relation to the demographic and clinopathological characteristics of the NSCLC patients is shown in Table II. Of the single variables, p16 methylation was statistically significantly associated with older age (≥65 years vs. <65 years; OR 3.6, 95% CI 1.0–13). Risk of p16 methylation also appeared higher among male patients (45% in men vs. 25% in women); the difference was, however, not statistically significant (OR 2.5, 95% CI 0.5–16). No difference was detected between adenocarcinoma and squamous cell carcinoma histologies (36% vs. 33%; OR 0.9, 95% CI 0.2–3.5). The highest rate of p16 methylation was found in large cell carcinomas (5/7, 71%; Fig. 2). The risk was clearly higher than that in adenocarcinoma (OR 4.2, 95% CI 0.6–53) but it did not reach statistical significance. Most of the cases that exhibited p16 promoter methylation in the non-malignant peripheral lung tissue were current smokers (4/5) and presented with squamous cell carcinoma of the lung (4/5).

In general, aberrant methylation of p14 gene promoter region was clearly less common than that of the p16 gene (8% vs. 34%, Table I). Similar to p16, all cases exhibiting p14 methylation were histologically NSCLCs (4/46, 9%). The low number of cases positive for p14 methylation did not allow statistical analysis.

p16 methylation and exposure

p16 methylation was determined in association with smoking habits among NSCLC cases (Table II). Totally, 42% (21/50) of ever smokers exhibited p16 methylation. Interestingly, the risk was significantly higher in ex-smokers as compared to current smokers (67% vs. 28%; OR 5.1, 95% CI 1.3–22). No significant associations with cumulative exposure, daily amount or duration of smoking were found in ever-smokers with NSCLC (data not shown). In life-long nonsmokers, p16 methylation was detected in 1 NSCLC case (1/4, 25%). The group was too small for meaningful statistical comparisons (Table II).

Logistic regression analysis was performed to adjust for the 2 variables (age and smoking status) that showed statistically significant associations. The increased risk of p16 methylation in ex-smokers as compared to current smokers was retained after adjustment for age (OR 3.7, 95% CI 0.9–17; p=0.08). The association

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**Table I**  Frequency of p16 and p14 promoter methylation in different tissue samples among the lung cancer cases studied

| Gene | Lung carcinoma | Peripheral lung tissue | Leukocytes |
|------|----------------|------------------------|------------|
|      | Total number | Methylated cases | Total number | Methylated cases | Total number | Methylated cases |
| p16  | 64 | 22 (34%) | 58 | 5 (9%) | 15 | 0 (0%) |
| p14  | 52 | 4 (8%) | 3 | 1 (33%) | n.d. | n.d. |

1All 3 cases methylated in the matching tumour tissues. 2n.d., not done.

**Table II**  Associations between p16 methylation and demographic, clinico-pathological, and exposure characteristics in non-small cell lung cancer (NSCLC)

| Characteristic | Total number of cases | Cases with methylated p16 (percent) | Cases with unmethylated p16 (percent) | OR (95% CI) |
|---------------|-----------------------|----------------------------------|------------------------------------|-------------|
| Sex           |                       |                                  |                                    |             |
| Female        | 12                    | 3 (25)                           | 9 (75)                             | 1 (Ref)     |
| Male          | 42                    | 19 (45)                          | 23 (55)                            | 2.5 (0.5–16) |
| Age, years    |                       |                                  |                                    |             |
| <65           | 27                    | 7 (26)                           | 20 (74)                            | 1 (Ref)     |
| ≥65           | 27                    | 15 (56)                          | 12 (44)                            | 3.6 (1.0–13) |
| Histology     |                       |                                  |                                    |             |
| Adenocarcinoma | 25                    | 9 (36)                           | 16 (64)                            | 1 (Ref)     |
| Squamous cell carcinoma | | 21 | 7 (33) | 14 (67) | 0.9 (0.2–3.5) |
| Smoking status|                       |                                  |                                    |             |
| Non-smokers   | 4                     | 1 (25)                           | 3 (75)                             | —           |
| Ex-smokers    | 18                    | 12 (67)                          | 6 (33)                             | 1 (Ref)     |
| Current smokers | 32                   | 9 (28)                           | 23 (72)                            | 0.2 (0.0–0.8) |
| Asbestos exposure | | 30 | 11 (37) | 19 (63) | 1 (Ref) |
| No            | 21                    | 10 (48)                          | 11 (52)                            | 1.6 (0.4–5.6) |
| Yes           |                       |                                  |                                    |             |
| p53 mutation  |                       |                                  |                                    |             |
| No            | 27                    | 11 (41)                          | 16 (59)                            | 1 (Ref)     |
| Yes           | 27                    | 11 (41)                          | 16 (59)                            | 1.0 (0.3–3.4) |
| K-ras mutation|                       |                                  |                                    |             |
| No            | 12                    | 5 (42)                           | 7 (58)                             | 1 (Ref)     |
| Yes           | 6                     | 1 (17)                           | 5 (83)                             | 0.3 (0.0–4.1) |
| p14 methylation |                      |                                  |                                    |             |
| No            | 42                    | 17 (40)                          | 25 (60)                            | 1 (Ref)     |
| Yes           |                       | 3 (75)                           | 1 (25)                             | 4.4 (0.3–241) |

1OR, odds ratio, and CI, confidence interval. 2Including 2 cases of adeno-squamous cell carcinoma. 3Data on asbestos exposure are missing for 3 patients, on K-ras gene mutation for 8 patients, and on p14 gene methylation for 8 patients.
We examined the frequency of promoter region methylation of the \( p16 \) and \( p14 \) genes in a series of lung tumours and the matching peripheral lung tissue specimens. Overall, 1/3 of the lung tumour samples exhibited \( p16 \) methylation. More than 40% of the NSCLCs exhibited \( p16 \) methylation in the tumour, whereas all SCLC tumours were negative. Risk of aberrant \( p16 \) methylation was elevated not only in current smokers but also in ex-smokers.

Our observation that showed no \( p16 \) methylation in SCLC is in agreement with a recent study by Toyooka and co-workers,\(^{28}\) where a very low rate of \( p16 \) methylation was detected in neuroendocrine lung tumours, including SCLC. In NSCLC, \( p16 \) methylation is more frequent, with 21% to 58% of the tumours being positive in different studies.\(^{21,22,29–34}\) Our data on NSCLC are well in keeping with those findings.

In NSCLC, positive associations have been found between \( p16 \) methylation in the tumour and squamous cell histology.\(^{22,28,34,35}\) Male sex,\(^{34}\) later stage\(^{31}\) and tobacco smoking.\(^{22}\) We did not detect predominance of \( p16 \) methylation in squamous cell carcinoma as compared to adenocarcinoma. In our series of lung tumours, the cell type showing the highest rate of \( p16 \) promoter methylation was large cell carcinoma, with more than 70% of the LCC tumours being positive. In addition, we found that \( p16 \) methylation frequently occurred in parallel with \( p53 \) mutation (3/5) in LCC, and there was 1 LCC case with alterations detected in all 3 genes studied, i.e., in \( p16, p14 \) and \( p53 \). Our finding fits well with literature data indicating promoter methylation as well as genetic alterations in multiple genes in LCC.\(^{24,34}\) Also, a meta-analysis on genetic changes in lung cancer reported the highest rate of \( p53 \) mutations in LCC.\(^{36}\)

In the present study, 42% of the NSCLC cases that had ever smoked had methylation in \( p16 \) in the tumour. From the 4 non-smokers with NSCLC studied, 1 exhibited \( p16 \) methylation in the tumour DNA. Several recent studies\(^{21–24,37}\) have reported associations between aberrant \( p16 \) methylation and smoking. Moreover, significant correlations between smoking characteristics, such as duration, pack-years or time since quitting smoking, and \( p16 \) methylation in NSCLC were found.\(^{22}\) In our study, ever smokers had increased frequency of \( p16 \) methylation, but we did not observe statistically significant associations between methylation and cumulative tobacco smoke exposure or duration of smoking. For occupational exposure to asbestos, the risk of \( p16 \) methylation was elevated in the exposed as compared to the nonexposed cases, but the difference was not statistically significant. A similar trend has recently been reported.\(^{22}\)

Interestingly, we discovered that former smokers with NSCLC had a significantly higher risk of \( p16 \) methylation than current smokers did (OR 5.1, 95% CI 1.3–22). This difference was retained after adjustment for age, the other variable found to be significantly associated with methylation (OR 3.7, 95% CI 0.9–17). Furthermore, when we combined our present data on \( p16 \) and \( p14 \) methylation with those on \( p53 \) mutations analysed earlier, we found that ex-smokers carried an alteration profile different from that in current smokers. All ex-smokers examined had at least 1 of the 3 alterations, whereas 34% of current smokers had none. In addition, \( p16 \) methylation occurred significantly more often in combination with alterations in the \( p53 \) pathway (i.e., \( p53 \) mutation and/or \( p14 \) methylation) in tumours from ex-smokers as compared to those from current smokers (44% vs. 14%; \( P = 0.035; \) Fisher’s exact test).

The occurrence of aberrant methylation in \( p16 \) gene in ex-smokers is in accordance with data from literature. A high prevalence of \( p16 \) methylation has been reported in adenocarcinomas from former smokers, with the detected level equalling to or even exceeding that detected in current smokers.\(^{38}\) In addition, promoter region methylation of several genes, including \( p16 \), has been detected at elevated frequencies in bronchial brush samples or bronchial epithelial cells from former smokers with or without...
liver cancer. The findings indicate recurrent abnormalities in smokers, who have quit smoking, are again in accordance with reports on high rates of p53 mutations observed in lung cancers from former smokers.

In summary, these observations suggest that smoking-related genetic and epigenetic abnormalities may persist in tumor cells after cessation of smoking.

We found p16 methylation in 9% (5/58) of the nonmalignant peripheral lung tissue samples, mainly from current smokers with squamous cell carcinomas. Similar rates of methylation (6–14%) in promoter regions of different cancer-related genes, including p16, APC, RASSF1A, RARB, DAPK, and FHIT, has been reported for noncancerous lung tissue from NSCLC patients. Methylation in p16 was shown to occur as an early event inpreneoplastic epithelial lesions of the lung and was detectable in bronchial epithelial cells and sputum samples from smokers. Also, smoking-related genetic damage, such as DNA adducts, has been demonstrated to occur in nonneoplastic lung tissue from cancer-free former smokers. These findings indicate the presence of various abnormalities in nontumour lung tissue in smokers suggest that larger areas of the histologically normal lung tissue suffer from tobacco-related damage that precedes neoplastic changes.

The finding that adjustment for smoking diminished the risk related to age suggests this notion.

In conclusion, we found that methylation of p16 gene was frequent in NSCLC tumours from both smokers and former smokers. Recent studies have suggested that, in lung cancer patients, aberrant methylation of genes regulating cell proliferation and growth is detectable in samples available through noninvasive sampling methods such as sputum or serum. Therefore, analysis of promoter methylation in such genes may provide a biomarker valuable for identification of groups with an elevated risk of cancer, such as smokers and ex-smokers.

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