Similar DNA methylation pattern in lung tumours from smokers and never-smokers with second-hand tobacco smoke exposure

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Tobacco smoke causes lung cancer in smokers and in never-smokers exposed to second-hand tobacco smoke (SHS). Nonetheless, molecular mechanisms of lung cancer in SHS-exposed never-smokers are still elusive. We studied lung cancers from current smokers (n = 109), former smokers (n = 56) and never-smokers (n = 47) for promoter hypermethylation of five tumour suppressor genes—p16, RARB, RASSF1, MGMT and DAPK1—using methylation-specific polymerase chain reaction. Lung tumours from ever-smokers suggested an increased risk of p16 hypermethylation as compared to never-smokers (P = 0.073), with former smokers having the highest frequency of p16 hypermethylation (P = 0.044 versus current smokers and P = 0.009 versus never-smokers). In the never-smoking group, p16 hypermethylation was seen in lung tumours from SHS-exposed individuals (4/33; 12%) but in none of the non-exposed individuals (0/9). The overall occurrence of hypermethylation (measured both as methylation index and as number of genes affected) was similar in those ever exposed to tobacco smoke (smokers, SHS-exposed never-smokers) and differed from non-exposed never-smokers. In multivariate analysis, p16 hypermethylation was more prevalent in lung tumours from male than female patients (P = 0.018) and in squamous cell carcinomas than in adenocarcinomas (P = 0.025). Occurrence of TP53 mutation in the tumour was associated with hypermethylation of at least one gene (P = 0.027). In all, our data suggest that promoter hypermethylation pattern in SHS-exposed never-smokers resembles that observed in smokers. Association between TP53 mutation, a hallmark of smokers’ lung cancer, and methylation of one or more of the lung cancer-related genes studied, provides further evidence for common tobacco smoke-related origin for both types of molecular alterations.

Introduction

Globally, ~22% of adult populations are current smokers. Cancer deaths account for some 7–8 million deaths worldwide each year, with lung cancer as the leading cause of cancer death in men, and the second most common cause of cancer death in women (1,2). The risk of developing lung cancer is ~10 to 20 times higher for smokers than for never-smokers and depends on the age at starting smoking and smoking duration (1,3). After smoking cessation, the risk of developing lung cancer decreases, but the relative risk in former smokers never reaches the baseline levels of never-smokers (4). From 10 to 15% of all lung cancers occur in subjects with no history of smoking (3,5). Recent estimates for the USA indicate that 10 000–15 000 US lung cancer deaths annually occur among those who have never smoked (6). This makes non-smokers’ lung cancer, if considered as a distinct disease entity, 1 of the 10 most common cancer types in terms of fatalities (6).

Tobacco smoke, including second-hand tobacco smoke (SHS; also known as environmental tobacco smoke or ETS), contains well >60 recognised carcinogens and procarcinogens, with polycyclic aromatic hydrocarbons (e.g. benzo (a) pyrene [BaP]), N-nitrosamines (e.g. tobacco-specific nicotine-derived 4-(methylhNitrosamino)-1-(3-pyridyl)-1-butanol [NNK]), aro\-matic amines, benzene, aldehydes and ethylene oxide among the most prevalent and potent (3,5). Chronic exposures to the complex mixture of tobacco smoke constituents induce genetic and epigenetic alterations that may, in turn, lead to malignant growth and cancer development (3,7). In line with such data, there is sufficient evidence that tobacco smoking can cause >15 different types of human cancer (3,8).

SHS has also been classified as a human carcinogen; it causes lung cancer, with an average excess risk of lung cancer among never-smokers regularly exposed to SHS in the order of 20–30% (3,5). In addition, there is now limited evidence for an association between SHS and cancers of the larynx and pharynx (8). Despite extensive tobacco control programmes in Europe and the USA, SHS continues to represent the most significant single source of indoor air exposure to carcinogens (3,5).

Promoter hypermethylation represents an epigenetic hit, which inactivates gene expression by extensive methylation of cytosines in CpG dinucleotide-rich islands in the promoter-enhancer region of a gene. Aberrant promoter methylation has commonly been observed to affect tumour suppressor genes (TSGs) in human neoplasia. It is considered one of the major mechanisms of tumourigenesis, operating instead of or in addition to gene mutations (9). Significant associations have
been established between smoking and promoter hypermethylation of TSGs in lung tumours from smokers and, similarly, such associations have been reported for DNA from plasma, serum or sputum from cancer-free smokers (9–11). The extent of gene hypermethylation in sputum from heavy smokers has been shown to predict the occurrence of lung cancer (10,12).

In contrast, data available for TSG hypermethylation from non-smokers are much less plentiful. Lower frequencies of hypermethylation have in general been reported in non-smokers’ lung cancer (13–15), but little is known about the role of SHS exposure may play in induction of such epigenetic alterations during the course of lung cancer development in never-smokers. Very few, if any, of the studies published have had access to detailed tobacco smoke exposure data for the cases that would have allowed an investigation of the relation between SHS exposure in the past and TSG promoter methylation in tumour.

We investigated the promoter region of a set of TSGs consistently suggested to be hypermethylated in lung cancer and showing clear association between promoter methylation and protein loss (9,10). The objective was to determine aberrant methylation in tumours from smoking and never-smoking lung cancer cases, in order to characterise prevalence and pattern of promoter hypermethylation in tobacco smoke-related lung carcinogenesis. In particular, our interest was to examine TSG hypermethylation in lifelong never-smokers for whom detailed data on SHS exposure was available. We focused methylation analyses on the following five genes: p16/CDKN2A (cyclin-dependent kinase inhibitor 2A; cell cycle regulation), RARB (retinoic acid receptor β; transcription regulation), RASSF1 (Ras association domain family member 1; cell cycle regulation), MGMT (O6-methylguanine–DNA methyltransferase; DNA repair) and DAPK1 (death-associated protein kinase 1, pro-apoptotic) (9–11).

**Materials and methods**

**Study population and data collection**

Promoter hypermethylation was studied in a total of 212 lung cancer patients, who were smokers or never-smokers. The study comprised two series of lung cancer cases, all of Caucasian origin, collected in Europe. DNAs were included from primary lung tumours of cases collected in a biomarker substudy component of a European multicentre study on non-smokers’ lung cancer (35 smokers—including 27 women and 8 men and 43 never-smokers—33 women and 10 men) (16,17), and of cases from a biomarker study on lung cancer carried out in at the Finnish Institute of Occupational Health (FIOH), Helsinki, Finland (130 smokers—108 men and 22 women and 4 never-smokers—3 men and 1 women) (18,19). Altogether, 103 adenocarcinomas, 79 squamous cell carcinomas and 30 of other histological types (12 large cell carcinomas, 9 carcinoids and 9 tumours with mixed histology) were investigated. Data on disease stage were available for 106 cases. Detailed data on tobacco smoke exposure, including SHS exposure details for never-smokers (n = 42), were obtained at in-person interviews. Smokers included 109 current smokers (those who had smoked at least 1 year before the lung cancer diagnosis) and 56 former smokers (those who had quit smoking >1 year before the clinical diagnosis). In smokers, tobacco smoke exposure was estimated both as cigarettes smoked per day and as pack-years (number of packs smoked per day multiplied by number of years of smoking; 20 cigarettes per pack). For never-smokers, we followed the criteria and classifications as defined earlier (16). Lifelong never-smokers were defined as subjects who had not smoked >400 cigarettes in their lifetime.

**Analysis of promoter methylation**

DNA from paraffin-embedded or frozen lung tumour tissues were assessed for promoter hypermethylation in five TSGs. Also, matching DNA from peripheral blood leukocytes of some of the patients (n = 10), leucocyte DNA from three healthy control subjects as well as DNA from cancer cell lines (T24, H157,
Methylation in smokers and never-smokers

Results

Promoter hypermethylation and clinicopathological features

The most prevalent histology included in the study was adenocarcinoma (49%), 61% of the tumours were from male patients, and the median age of the cases was 63.5 years (Table I). In all, 165 ever-smokers (109 current smokers and 56 former smokers) and 47 never-smokers were investigated.

Promoter hypermethylation was very frequent in the lung tumours, with 71% (150/212) showing methylation in one or more of the genes. The overall mean MI was 0.23 (i.e. on average >1 hypermethylated gene of the five studied per tumour). The prevalence of promoter hypermethylation varied to some extent between the TSGs studied; aberrant methylation was detected in 20–30% of cases for p16, RASSF1 and RARB, whereas MGMT and DAPK1 were methylated in 15–20% of the cases (Table II). All leukocyte DNA samples, whether from healthy controls (n = 3) or cancer patients (n = 10), were negative for methylation in all genes analysed.

Aberrant methylation of p16 occurred significantly more often in males than in females (27.9 versus 9.6%, P = 0.002; Table II), while hypermethylation of RARB was predominant in females (39.8 versus 25.6%, P = 0.044). The DAPK1 gene also tended to be more frequently methylated in tumours from female patients (P = 0.076). After adjustment for age, histology, TP53 and smoking in the multivariate analysis, only p16 retained statistically significant association to sex (P = 0.018; supplementary Table I, available at Mutagenesis Online).

With regard to tumour histology, p16 hypermethylation occurred in 34.2% of squamous cell carcinomas, which was significantly more prevalent than in adenocarcinomas (P = 0.001; Table II). No difference was observed in RARB hypermethylation between these two major tumour histologies, but RARB methylation was significantly less frequent (13.3%) in the mixed group of other histologies (P = 0.012 versus adenocarcinoma; Table II). Both these associations with tumour histology remained statistically significant in multivariate analysis (P = 0.025 for p16; P = 0.006 for RARB; supplementary Table I, available at Mutagenesis Online).

The other patient or tumour characteristics (age at diagnosis and disease stage) were not significantly associated with promoter hypermethylation in any of the genes studied (Table II; data on age at diagnosis not shown).

Aberrant methylation and smoking

Overall, the observed frequencies of hypermethylation of the five TSGs were at higher or comparable level in lung tumours from smoking patients when compared to never-smokers (Table III, Figure 2A), with MI, reflecting the average level of methylation, being 0.23 in the smoker group and 0.22 in never-smokers. Hypermethylation of p16 was more common in ever-smokers than in never-smokers, with borderline significance (OR 2.59; 95% CI 0.93–8.97; P = 0.073). Among ever-smokers, however, former smokers had a statistically significant 2-fold higher risk of p16 hypermethylation as compared to current smokers (OR 2.27; 95% CI 1.02–5.08; P = 0.044; Figure 2B). The risk of former smokers was even more pronounced (OR 4.25; 95% CI 1.36–16.06; P = 0.009) when compared to the never-smoker group. In contrast to p16, RARB hypermethylation was more rarely observed in lung tumours from ever-smokers (27.9%) as compared to never-smokers (42.6%), but this difference was not statistically significant (OR 0.52; 95% CI 0.25–1.09; P = 0.086; Table III, Figure 2A).
the level seen in smokers. However, MI was elevated, although the mean MI in the SHS-exposed never-smokers was close to lung cancers (18). Neither lung tissue fibre counts (tional exposure to asbestos were available for the FIOH series of associated with TSG hypermethylation in smokers (data not available for five never-smokers). Never-smokers, SHS exposure d

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Total n^b</th>
<th>p16 (%)</th>
<th>RARB (%)</th>
<th>RASSF1 (%)^c</th>
<th>MGMT (%)</th>
<th>DAPK1 (%)</th>
</tr>
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<tbody>
<tr>
<td>Never (Ref)</td>
<td>47</td>
<td>5 (10.6)</td>
<td>20 (42.6)</td>
<td>11 (23.9)</td>
<td>5 (10.6)</td>
<td>11 (23.4)</td>
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<tr>
<td>Ever</td>
<td>165</td>
<td>39 (23.6)</td>
<td>46 (27.9)</td>
<td>50 (30.3)</td>
<td>27 (16.4)</td>
<td>31 (18.8)</td>
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<td>OR (95% CI)</td>
<td>2.59 (0.93–8.97)</td>
<td>0.52 (0.25–1.09)</td>
<td>1.38 (0.62–2.37)</td>
<td>1.64 (0.57–5.80)</td>
<td>0.76 (0.33–1.84)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Former smokers (Ref)</td>
<td>56</td>
<td>19 (33.9)</td>
<td>14 (25.0)</td>
<td>15 (26.8)</td>
<td>9 (16.1)</td>
<td>9 (16.1)</td>
</tr>
<tr>
<td>Current smokers</td>
<td>109</td>
<td>20 (18.3)</td>
<td>32 (29.4)</td>
<td>35 (32.1)</td>
<td>18 (16.5)</td>
<td>22 (20.2)</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>0.44 (0.20–0.98)</td>
<td>1.24 (0.57–2.82)</td>
<td>1.29 (0.60–2.86)</td>
<td>1.03 (0.40–2.82)</td>
<td>1.32 (0.53–3.53)</td>
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<tr>
<td>Never-smokers, SHS exposure^d</td>
<td>0 (Ref)</td>
<td>0 (0)</td>
<td>2 (22.2)</td>
<td>2 (22.2)</td>
<td>2 (22.2)</td>
<td>1 (11.1)</td>
</tr>
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<td>Yes</td>
<td>33</td>
<td>4 (12.1)</td>
<td>16 (48.5)</td>
<td>6 (18.8)</td>
<td>3 (9.1)</td>
<td>10 (30.3)</td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>no calculation</td>
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</table>
| TSG methylation and SHS exposure

When the overall effect of SHS was considered in the group of lifelong never-smokers (n = 42 with SHS exposure details), the mean MI in the SHS-exposed never-smokers was close to the level seen in smokers. However, MI was elevated, although non-significantly, in never-smokers with SHS exposure, as compared to never-smokers without such exposure (0.24 versus 0.16; P = 0.19; Figure 2D). Similarly, the number of affected genes was higher in smokers and in the SHS-exposed never-smokers as compared to the non-exposed never-smokers (Figure 2C).

Of the individual genes, hypermethylation of the p16 gene was observed in none of the non-exposed never-smokers (0/9), whereas tumours from smokers (39/165; 23.6%) and those from SHS-exposed never-smokers (4/33; 12.1%) did exhibit p16 methylation (Table III, Figure 2B). Even though RARB hypermethylation was frequent in the overall group of never-smokers as compared to ever-smokers, among the never-smokers, it predominantly occurred in tumours from SHS-exposed cases (48.5 versus 22.2% in SHS non-exposed patients; Table III).
Following a similar pattern, DAPK1 gene hypermethylation also tended to be more prevalent in the SHS-exposed never-smokers as compared to the non-exposed never-smokers (30.3 versus 11.1%; Table III).

**Gene hypermethylation versus TP53 mutation**

The same series of lung cancers were previously analysed for TP53 gene mutations (17,18). In the current study, we compared hypermethylation between lung tumours carrying a TP53 mutation ($n = 79$) and those without ($n = 133$). The mean MI was significantly higher in tumours with TP53 mutation as compared to the cases with a wild-type gene (0.27 versus 0.21; $P = 0.018$). A difference was also seen between the TP53 mutation carrier and wild-type tumours when analysed as hypermethylation present in one or more genes involved in the analysis (OR 1.86; CI 0.94–3.80; $P = 0.078$).

Of the individual genes, hypermethylation of $p16$ and MGMT were more common in TP53-mutated lung tumours than in tumours with wild-type TP53 ($P = 0.034$ and $P = 0.072$, respectively; Table II). After adjustment for age, sex, histology and smoking status, the association between TP53 mutation and hypermethylation in one or more genes remained statistically significant ($P = 0.027$; supplementary Table I, available at *Mutagenesis* Online).

**Discussion**

We investigated promoter hypermethylation in a series of primary lung tumours from smokers and never-smokers, with detailed interview data on SHS exposure available for the never-smokers. Our findings on five lung cancer-related TSGs indicate that promoter hypermethylation in one or more genes was common, affecting two-thirds of all lung cancer tumours studied. Our results further show differences in the frequency of promoter region methylation between the various tobacco smoke exposure groups. Interestingly, we found that the cases that were positive for a TP53 mutation, a hallmark of tobacco-related lung cancer, were at a higher risk of also having aberrant promoter methylation in at least one of the five genes.

Silencing of TSG transcription via promoter hypermethylation is known to be a frequent and early event during lung tumourigenesis (9). Although clinical, pathological and molecular differences between lung tumours from smokers and never-smokers have been described (13,14), understanding of molecular alterations involved in non-smokers’ lung cancer in association to environmental exposure is often difficult due to incomplete or lacking data on exposure history, in particular concerning exposure to SHS. In a set of studies, more frequent hypermethylation in several TSGs and a higher average MI have been reported for lung tumours from smokers as compared to non-smokers (25–27), but in other studies (28–32), no clear differences were observed or even higher rates of hypermethylation in tumours from never-smokers. Such differences may be related to varying daily amounts of smoking among smokers, and, importantly, to lack of systematic and accurate data on SHS exposure among non-smokers.

Our current results demonstrate a higher rate of $p16$ promoter methylation in smokers as compared to never-smokers, but this association was not statistically significant after adjusting for histology, sex and age in multivariate analysis. The promoter methylation pattern seen among never-smokers, however, suggests differences between SHS-exposed and non-exposed never-smokers. In lung cancer cases who were never-smokers not exposed to SHS, we observed a low methylation index, whereas never-smokers with SHS exposure exhibited similar mean MI as smokers. Furthermore, $p16$ hypermethylation was exclusively detected in lung tumours from ever-smokers and never-smokers regularly exposed to SHS. The highest risk of $p16$ hypermethylation was found for former smokers. These data on $p16$ gene, which encodes...
a regulatory protein of the retinoblastoma protein pathway, are well in line with the published findings indicating that hypermethylation of p16 shows association to smoking (9,33,34). In addition to frequent occurrence in lung tumours, hypermethylation of the p16 gene has been detected in sputum, plasma and bronchial brush samples of cancer-free smokers (10,35–38). Moreover, data from several studies suggest that p16 hypermethylation is a biomarker of early stages of malignant transformation and can predict occurrence of lung cancer some years before clinical diagnosis (10,12,35,38). In accordance with the findings in smokers, experimental work has shown occurrence of the p16 gene hypermethylation in rat adenocarcinomas induced by tobacco-specific carcinogen NNK (12).

In addition to smoking, hypermethylation of particular genes was associated with gender and tumour histology in the present study. We found that hypermethylation of p16 was predominant in squamous cell carcinomas and in tumours from men. Conversely, we observed RARB hypermethylation to be more frequent in tumours from females and in adenocarcinomas versus lung tumours of other histologies. Our results are in line with the majority of previous studies on resected tumour tissues, in which p16 hypermethylation was observed in 24–56% of squamous cell carcinomas and to a lesser extent in other histologies (11,25,33,39). Hypermethylated RARB promoter typically predominates in adenocarcinomas from female patients, who are often non-smokers (25,39,40). The current data further suggest that RARB hypermethylation as detected in lung tumours from never-smokers may at least partially be attributed to exposure to SHS.

According to current understanding, lung tumourigenesis likely involves complex interplay between two major mechanisms, the genetic and epigenetic. In our previous study, on the same series of cases, we found that smokers exhibited increased risk of TP53 mutation as compared to non-smokers (17,18) and, further, that exposure to SHS doubled the risk of TP53 mutation in never-smokers (17). The present study revealed association between occurrence of TP53 mutation and overall methylation as well as between TP53 mutation and hypermethylation of the p16 and MGMT genes. Interestingly, recent studies have shown that exposure of cells to DNA damage (such as double-strand breaks induced by low doses of carcinogen also present in tobacco smoke) may directly contribute to gene silencing via increasing promoter hypermethylation in multiple genes (41,42). Our current observation that TP53 mutations in lung cancer are associated with an increased overall level of TSG hypermethylation fits well with these experimental data. Furthermore, there are data indicating that epigenetic inactivation of MGMT, a gene that encodes a DNA repair enzyme responsible for removal of alkyl groups from the O6 position of guanine, increases genetic instability of cells (11). In keeping with such an effect, it has been reported that inactivation of MGMT in lung tumours was associated with increased occurrence of TP53 mutation, especially the G:C to A:T transition (32). Collectively, these data strongly implicate tobacco smoke-related DNA damage as a common origin for the TP53 hallmark mutation as well as TSG promoter hypermethylation in lung carcinogenesis.

In conclusion, our study, although of limited power to provide conclusive evidence on its own, provides an important contribution to the understanding of epigenetic pathways involved in tobacco smoke-induced lung cancer and their relation to mutational pathways. In particular, our data support the role of SHS exposure in lung carcinogenesis in never-smokers and propose that TSG hypermethylation is involved in this process, similar to the findings reported for smokers.

Supplementary data

Supplementary Table I is available at Mutagenesis Online.

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