

DNA methylation at promoter regions of interleukin 1B, interleukin 6, and interleukin 8 in non-small cell lung cancer

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Abstract Epidemiologic and experimental evidences support the concept that inflammation promotes the development and progression of cancers. Interleukins (ILs) regulate the expression of several molecules and signaling pathways involved in inflammation. High expression of some ILs in the tumor microenvironment has been associated with a more virulent tumor phenotype. To examine the role of IL-1 β , IL-6, and IL-8 in non-small cell lung cancer, we measured mRNA levels and promoter DNA methylation in a panel of cultured human lung cells ($n = 23$) and in matched pair lung tumor versus adjacent non-tumorous tissues ($n = 24$). We found that lung cancer cells or tissues had significantly different DNA methylation and mRNA levels than normal human bronchial epithelial cells or adjacent non-tumorous tissues, respectively. High DNA methylation of ILs promoters in lung cancer cells or tissues was associated with low mRNA levels. We found an inverse correlation between DNA methylation of *IL1B*,

IL6, and *IL8* gene promoters and their corresponding mRNA levels, such inverse correlation was more significant for *IL1B* (i.e., all cancer cell lines used in this study had a hypermethylated *IL1B* promoter which was associated with silencing of the gene). Our results underline for the first time the role of epigenetic modifications in the regulation of the expression of key cytokines involved in the inflammatory response during lung cancer development.

Keywords Interleukins · DNA methylation · Inflammation · Lung carcinogenesis · Epigenetic

Introduction

Inflammation has recently been defined as one of the enabling characteristics of cancer [1]. Approximately 25 % of all cancers are somehow associated with chronic inflammation [2]. Chronic inflammation has been implicated in lung carcinogenesis arising as a result of continuous exposure to components in tobacco smoke [3, 4].

Inflammation is a complex process involving different cells and mediators; it can affect tumor progression and development through multiple mechanisms [5]. Interleukins (ILs) regulate the expression of several molecules involved in inflammation. They are in the context of carcinogenesis produced by normal cells, tumor cells, and cellular elements of the tumor microenvironment [6]. IL-1 β is an important pro-inflammatory cytokine that has been shown to be expressed at tumor sites. IL-1 β is involved in various phases of the malignant process, such as initiation, promotion of carcinogenesis, tumor growth, and invasiveness [7]. High levels of IL-1 β in the tumor microenvironment have been associated with a more aggressive tumor phenotype [8, 9].

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The cytokine IL-6 and the chemokine IL-8 have different systemic functions, they are inducible by IL-1 β [10, 11] and elevated serum levels have been found in various cancer types [12, 13], including lung cancer [14].

We previously reported an association between single nucleotide polymorphisms in the regulatory region of *IL1B*, *IL6*, or *IL8* and lung cancer risk [15, 16]. The relationship between genetic variations and *IL1B*, *IL6*, or *IL8* gene expressions in human lung has been extensively investigated [16–18]. Comparatively, the impact of promoter DNA methylation on interleukin mRNA levels has not received much attention. Epigenetic alterations such as DNA methylation are factors not only affecting cancer cells but also cells of the tumor-associated stroma [1]. Changes in DNA methylation patterns have been suggested to be an early event in lung carcinogenesis and may be a possible biomarker for early detection of lung cancer [19]. In the present study, we investigated whether DNA methylation could be involved in the regulation of the mRNA levels of *IL1B*, *IL6*, and *IL8* genes in non-small cell lung cancer (NSCLC).

Materials and methods

Tissues from lung cancer patients

All patients were Caucasians of Norwegian origin diagnosed with early stage lung cancer. Adjacent non-tumorous lung tissues were collected from the resected lung tissue as far as possible from the tumor (>5 cm) at the time of surgery for lung cancer at Haukeland University Hospital in Bergen between 1986 and 2010. Only tumor tissues containing at least 80 % of tumor cells were analyzed in the study. After resection, the lung tissue samples were snap-frozen in liquid nitrogen and kept at -80°C until further processing. Patients were interviewed by trained health personnel using standardized questionnaires and were categorized as never smokers, ex-smokers, or current smokers (Table 1). Never smokers are patients indicating having smoked less than 100 cigarettes in their lifetime. Ex-smokers were defined as patients having quit at least 1 year prior to surgery, and current smokers were those indicating that they were smoking up to the time of surgery. Written informed consent was obtained from each subject and the investigation was performed after approval by the regional ethical committee.

Cell culture conditions and treatments

Primary normal human bronchial epithelial cells (NHBE)

Immediately after surgery for lung cancer from randomly selected patients, bronchial tissues were dissected from

Table 1 24 matched pair characteristics

Age (mean \pm SEM)	60.75 \pm 10.1
Sex (male/female)	16/8
Number of cigarettes per day	$N = 20$
Mean \pm SEM	16.80 \pm 6.1
Median (min–max)	16.0 (6–40)
Smokers/ex-smokers/non-smokers ^a	11/9/4
Total pack years ^b	$N = 20$
Mean \pm SEM	28.22 \pm 13.2
Median (min–max)	24.88 (9.3–76)
Bulky aromatic/hydrophobic DNA adducts ^c	$N = 15$
Mean \pm SEM	14.53 \pm 6.5
Median (min–max)	13.85 (4.31–25.46)
Histology	
Adenocarcinoma	10
Squamous cell carcinoma	5
Large cell carcinoma	9

N number of subjects, *SEM* standard error of the mean

^a For definitions see “Materials and methods”

^b Pack years (number of cigarettes smoked per day \times number of years smoked)/20

^c DNA adducts per 10^8 nucleotides

areas with no visible tumor tissue and were immediately submerged in PBS and transported to our laboratory. Normal human bronchial epithelial cells (NHBE cells) were propagated by the explants outgrowth procedure. Explants (approximately 0.3×0.3 cm) were maintained in serum-free LHC-9 (Invitrogen, Carlsbad, CA) medium on collagen-coated dishes [20]. Before reaching confluence, primary NHBE cells were plated without the presence of the explants.

Immortalized human bronchial epithelial cell lines (HBEC)

Three HBEC immortalized by expression of cyclin-dependent kinase 4 and telomerase [21] were a kind gift from Dr. J. D. Minna. Cells were plated on collagen-coated dishes and cultured under serum-free conditions using LHC-9 medium.

Human lung cancer cell lines (HLCC)

Nine HLCC cell lines were included in this study. The A427, A549, NCI-H838, NCI-H1435, NCI-H1793, NCI-H23, and NCI-H1395 cell lines were from the American Type Culture Collection. Dr A.F. Gazdar provided the human lung tumor cell lines NCI-H2009 and HCC-78 [22]. All cell lines have been recently authenticated by an approved laboratory (German Biological Resource Centre). Cell lines were maintained in RPMI 1640 without phenol red (Invitrogen; San Diego, CA, USA), supplemented with

10 % dialyzed fetal bovine serum (PAA Laboratories GmbH; Pasching, Austria).

Culture conditions and treatments

Culture media were supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) (Invitrogen). Cells were maintained at 37 °C in a humidified 5 % CO₂ atmosphere. Proliferating cell cultures were treated 5 days with 5'-aza-2'-deoxycytidine (AZA; 0.5 µM). The plates were washed with cold phosphate-buffered saline and stored at –80C for preparation of RNA or DNA.

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen, crushed tissues or cultured cells and reverse transcribed using qScript cDNA Synthesis kit for cultured cells (Quanta Biosciences) or Roche cDNA Synthesis kit for tissues (Molecular Biochemicals, Basel, Switzerland). Quantitative analysis of the specific expression of various genes was performed by real-time PCR on an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA), using the PerfeCTa SYBR Green fast mix (Quanta Biosciences). The amount of target cDNA in each sample was established by determining a fractional PCR threshold cycle number (Ct) and estimated by interpolation from a standard curve. The standard curve was made from known amounts of the corresponding product with the same primer sets and was run on each PCR plate. The expression levels of target genes were normalized to the expression of the B-actin gene. The expression of another housekeeping gene 18S was also measured as a second internal control. We found no significant differences in all the results using 18S or B-actin as a housekeeping control gene. Primers sequences can be found in Supplementary Table 1.

DNA extraction, bisulfite conversion, and pyrosequencing analysis

DNA was extracted from biological samples (primary cells, cell lines, and frozen tissues) either with DNA isolation kits (Qiagen, Hilden, Germany) or standard proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation. DNA bisulfite conversion was carried as previously described [23]. 1 µg of genomic DNA in 20 µl TE buffer was fragmented 20 min at 95 °C and denatured by addition of 2 µl of NaOH (6.3 M) and incubation 10 min at 39 °C. For bisulfite DNA conversion, 416 µl of bisulfite (2.5 M) hydroquinone (6.56 M) pH = 5 solution was added to the DNA, the mixture was incubated 4 h at 58 °C, every 15 min a denaturation step was added

(95 °C, 1 min). Converted DNA was desalted using Promega Wizard DNA clean up kit following the instructions of the manufacturer, resuspended in 50 µl of TE buffer, and desulfonated by addition of 22.5 µl of NH₄OAc (10 M; pH = 7; 39 °C). Finally, DNA was precipitated, washed, and resuspended in TE buffer. Regions of interest of the converted DNA were amplified using PyroMark PCR kit (Qiagen, Hilden, Germany). The methylation status of CpGs present in the amplicon was analyzed by pyrosequencing using PyroMark Gold Q24 reagents (Qiagen, Hilden, Germany) and the PyroMark Q24MDx instrument (Qiagen, Hilden, Germany). Primers sequences used are available on demand. We used 100 % methylated DNA and non-methylated DNA to assess the functionality of our assays.

Statistical analyses

Statistical analyses were carried out using the GraphPad InStat software v. 3.05 (San Diego, CA, USA) and SPSS software version 17.0. Correlations between gene expression and DNA methylation levels were determined using Spearman's nonparametric correlation analysis. The Mann–Whitney test or the ANOVA Kruskal–Wallis tests followed by Dunn post-test were used to compare medians. $p < 0.05$ was considered statistically significant: * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. In the box-and-whiskers plots, the line within each box represents the median. Upper and lower edges of each box represent 75th and 25th percentile, respectively. The whiskers represent the lowest datum still within [1.5*(75th – 25th percentile)] of the lower quartile, and the highest datum still within [1.5*(75th – 25th percentile)] of the upper quartile. All results from the in vitro experiments are from at least three independent experiments. The mean level of methylation of two independent bisulfite treatments and pyrosequencing was used for the tissue samples.

Results

Effects of AZA on *IL1B*, *IL6*, and *IL8* mRNA levels in cultured human lung cells

To investigate the relationship between DNA methylation and mRNA levels of *IL1B*, *IL6*, or *IL8*, we exposed 23 cultured human lung cells: 11 primary normal human bronchial epithelial cells (NHBE), 3 immortalized human bronchial epithelial cell lines (HBEC), and 9 human lung cancer cell lines (HLCC) for 5 days during proliferation phase to the demethylating agent 5'-aza-2'-deoxycytidine (AZA; 0.5 µM) and measured the mRNA levels. We found that AZA increased mRNA levels in all cultured cells

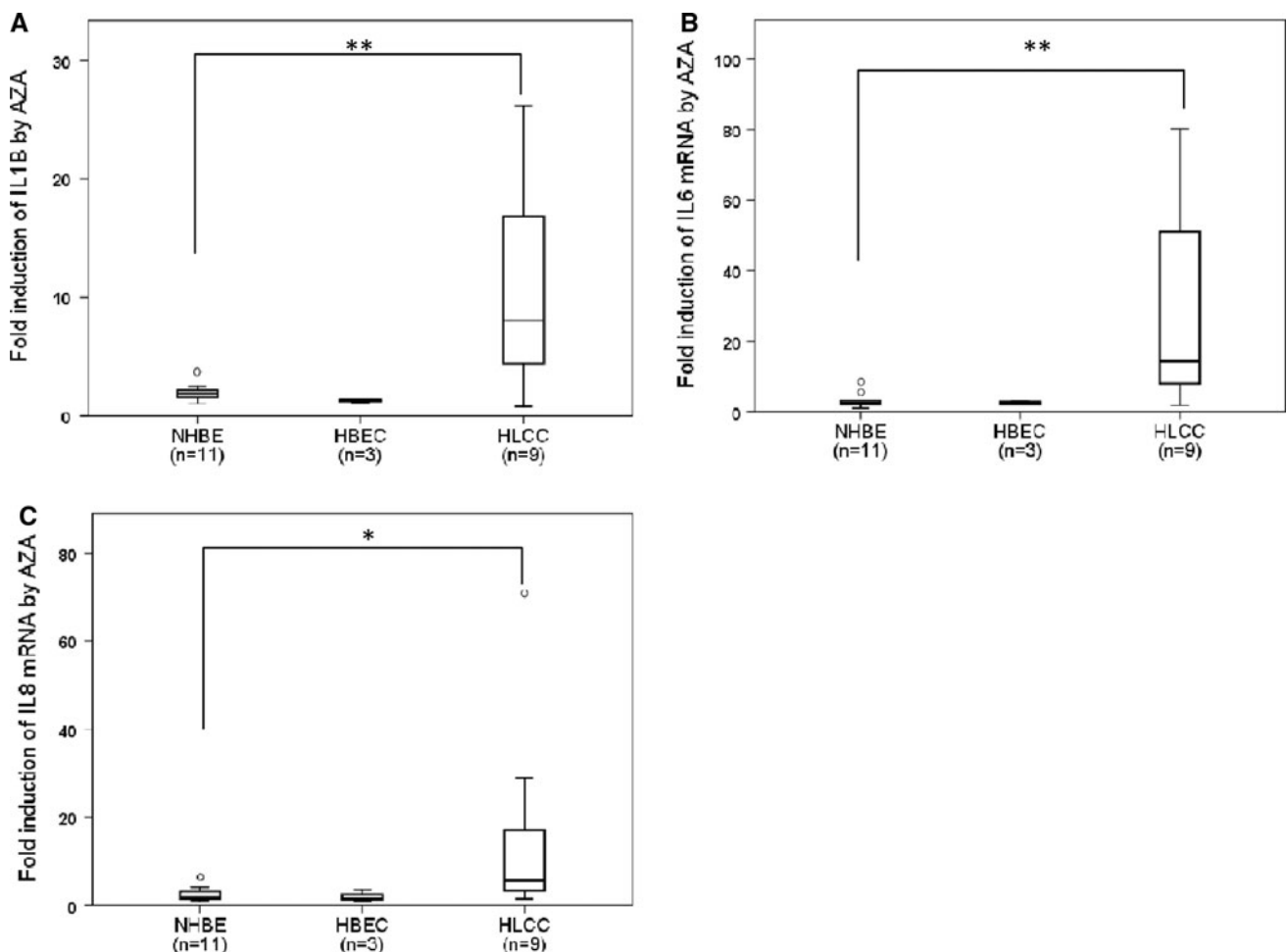


Fig. 1 AZA effects on *IL1B*, *IL6*, and *IL8* mRNA levels. Primary cultured normal human bronchial epithelial cells (NHBE), human bronchial epithelial cells (HBEC), and human lung cancer cell lines (HLCC) were exposed 5 days during proliferation phase to the demethylating agent 5'-aza-2'-deoxycytidine (AZA). mRNA levels of

IL1B, *IL6*, or *IL8* were normalized to the mRNA expression of B-actin ($n = 5$ independent experiments). * $p < 0.05$; ** $p < 0.001$. **a** Fold induction of *IL1B* mRNA levels by AZA, **b** fold induction of *IL6* mRNA levels by AZA, **c** fold induction of *IL8* mRNA levels by AZA

tested, with the most prominent effects in cancer cell lines. Fold inductions of *IL1B* (Fig. 1a), *IL6* (Fig. 1b), or *IL8* (Fig. 1c) mRNA levels by AZA were significantly higher in human lung cancer cell lines (HLCC) compared to NHBE cells.

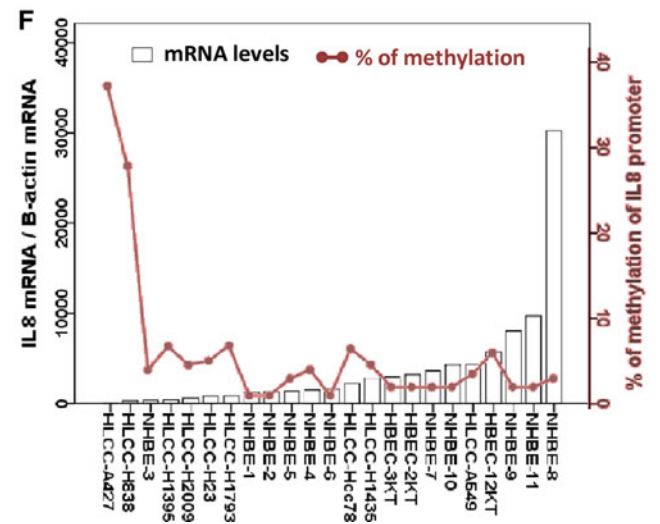
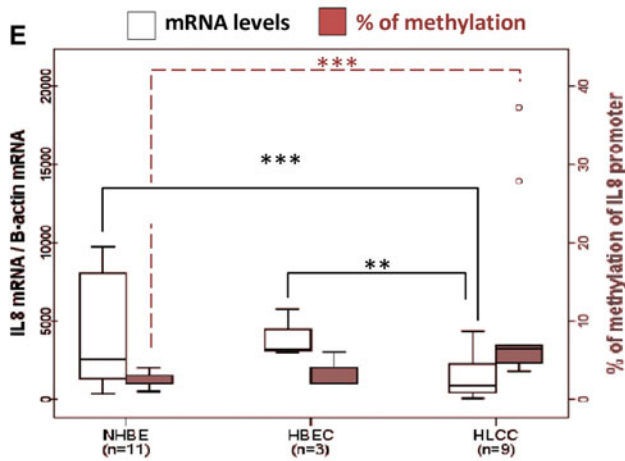
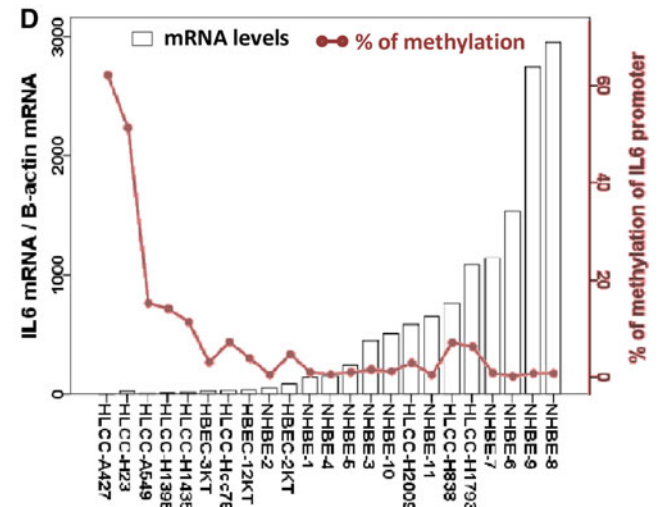
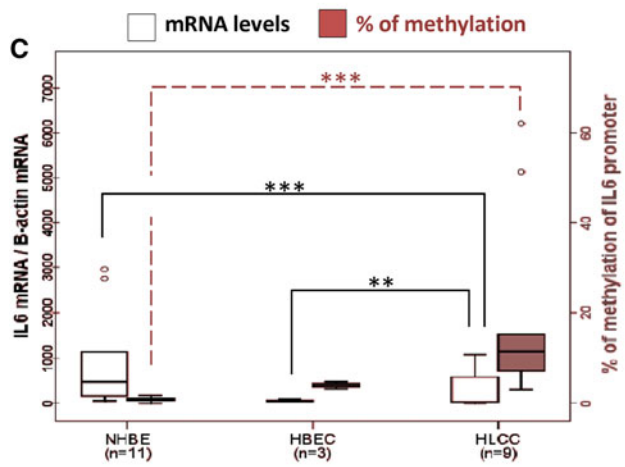
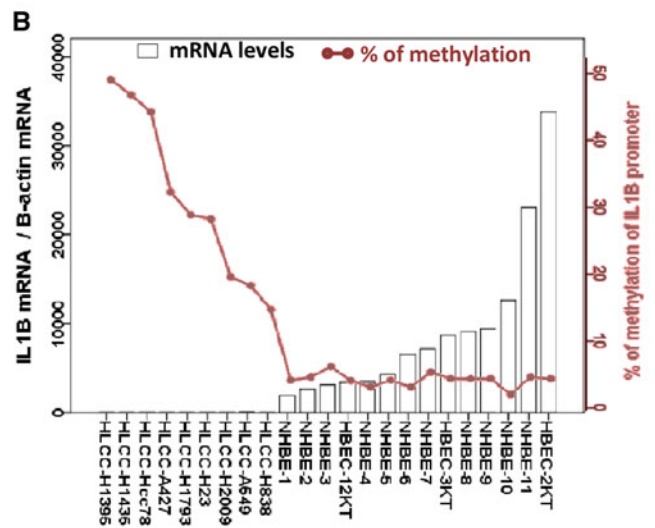
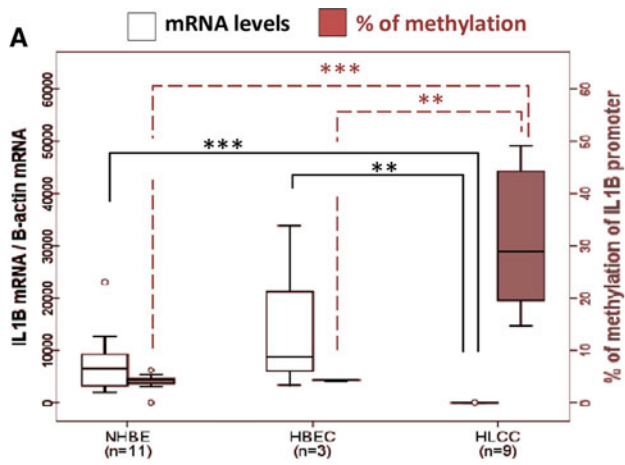
IL1B, *IL6*, and *IL8* mRNA levels and promoter DNA methylation in cultured human lung cells

AZA treatment suggested a relationship between *IL1B*, *IL6*, or *IL8* DNA methylation and their respective mRNA levels. Using DNA bisulfite conversion and pyrosequencing, we investigated for methylation of each cytosine-phosphoguanine (CpG) in the core promoter of each interleukin in our panel of cultured human lung cells.

The region of *IL1B* promoter studied was situated over the transcription start site (−322; +31 nucleotides). The

Fig. 2 mRNA levels and promoter DNA methylation of *IL1B*, *IL6*, and *IL8* in cultured human lung cells. mRNA levels of *IL1B*, *IL6*, or *IL8* were normalized to the mRNA levels of B-actin in primary cultured normal human bronchial epithelial cells (NHBE), human bronchial epithelial cells (HBEC) and human lung cancer cell lines (HLCC). Percentages of DNA methylation were obtained by analyzing the mean methylation of all the CpG sites present in the promoter of each genes ($n = 4$ independent experiments). ** $p < 0.001$; *** $p < 0.0001$. **a–b** mRNA levels and promoter DNA methylation of *IL1B*, **c–d** mRNA levels and promoter DNA methylation of *IL6*, **e–f** mRNA levels and promoter DNA methylation of *IL8*

mean percentage of methylation of the four CpGs of this region was below 5 % in NHBE and HBEC cells (<5 % methylation). In cancer cells (HLCC), the *IL1B* promoter was significantly more methylated and this was associated with lower *IL1B* mRNA levels (Fig. 2a); DNA hypermethylation frequency of the *IL1B* promoter in HLCC was 100 % (all cancer cell lines had higher DNA methylation



of the *IL1B* promoter than the mean plus standard deviation for methylation levels observed in normal cells). DNA methylation of the *IL1B* promoter and mRNA levels were inversely correlated in the panel of cultured human lung cells tested (Spearman $r = -0.74$; $p < 0.0001$; Supplementary Fig. 1a); thus, the cells with the least methylation had the highest mRNA levels and vice versa (Fig. 2b).

The region of *IL6* promoter studied was situated near the transcription start site (-240 ; -82 nucleotides). The percentage of methylation of the four CpGs of this region was below 5 % in NHBE and HBEC cells (<5 % methylation); DNA hypermethylation frequency of the *IL6* promoter in cancer cell lines was 100 %. The occurrence of methylation was significantly higher in cancer cell lines (HLCC) and was associated with lower *IL6* mRNA levels (Fig. 2c). The DNA methylation of the *IL6* promoter and mRNA levels were inversely correlated (Spearman $r = -0.68$; $p = 0.0007$; Fig. 2d; Supplementary Fig. 1b).

The region of *IL8* promoter studied was -205 , -66 nucleotides from the transcription start site and contained four CpGs. The mean methylation of the CpGs was below 7 % in most of the cells tested. However, DNA methylation of the *IL8* promoter was significantly higher in HLCC than in NHBE (Fig. 2e). This was due to higher methylation levels in two (H838 and A427) human lung cancer cell lines (Fig. 2f), the DNA hypermethylation frequency of the *IL8* promoter in HLCC was 89 % since 8 out of 9 cancer cell lines had a percentage of methylation above the mean plus standard deviation for methylation levels seen in normal HBEC and NHBE. In these two cell lines, we also observed lower *IL8* mRNA levels. An inverse correlation between DNA methylation and mRNA levels was found (Spearman $r = -0.536$; $p = 0.0087$; Supplementary Fig. 1c).

IL1B, *IL6*, and *IL8* mRNA levels and promoter DNA methylation in matched pair tissues from lung cancer patients

We analyzed 24 matched pair lung tissues (tumor tissue vs adjacent non-tumorous). In the adjacent non-tumorous human lung tissues, the *IL1B* mRNA and DNA methylation levels were higher than in the corresponding tumor tissues (Fig. 3a); DNA hypermethylation frequency of the *IL1B* promoter in the non-tumorous human lung tissues was 87.5 % (21 out of 24 adjacent non-tumorous tissues had DNA methylation above the mean plus standard deviation for methylation levels seen in tumor tissue). We found in the 24 tumor samples tested that low mRNA levels of *IL1B* were associated with high DNA methylation levels (Fig. 3a, b). An inverse correlation between DNA methylation and mRNA levels of *IL1B* in the 24 tumor tissues was found (Spearman $r = -0.72$, $p < 0.0001$; Supplementary Fig. 2a). However, there was no inverse

correlation between *IL1B* mRNA levels and DNA methylation in the corresponding adjacent non-tumorous tissues (Supplementary Fig. 3a).

We also found significantly higher mRNA levels of *IL6* in the adjacent non-tumorous tissues compared to the tumor tissues (Fig. 3c). Like for *IL1B*, DNA methylation and mRNA levels were inversely correlated in the tumor tissues (Fig. 3d; Spearman $r = -0.49$, $p = 0.0150$; Supplementary Fig. 2b) but not in the non-tumorous tissues (Supplementary Fig. 3b).

Our in vitro results showed little differences in DNA methylation of the *IL8* promoter and a low inverse correlation between DNA methylation and mRNA levels; thus, we did not analyze DNA methylation and mRNA levels of *IL8* promoter in the tissue matched pairs.

Discussion

Pro-inflammatory interleukins play various roles in cancer development and progression [9]. In a panel of cultured human lung cells ($n = 23$) and matched pairs tumor versus adjacent non-tumorous human lung tissues ($n = 24$), we investigated whether DNA methylation was associated with the mRNA levels of *IL1B*, *IL6*, and *IL8* genes. We used powerful and reproducible methods to quantitatively measure DNA methylation and mRNA levels.

We found an inverse correlation in vitro and in tumor tissue samples between mRNA levels and CpG methylation of the *IL1B* promoter (-322 ; $+31$ nucleotides from the transcription start site). It has been shown that demethylation of a single CpG situated -299 bp from the transcription start site of the *IL1B* gene was associated with increased mRNA levels in human chondrocytes [24]. This study and the data presented here suggest that DNA methylation of the CpGs of the *IL1B* promoter close to the transcription start site may influence mRNA levels. However, it must be noted that in the 24 adjacent non-tumorous tissue samples tested, DNA methylation of the *IL1B* promoter was not inversely associated with mRNA levels. This may indicate that other factors may override the effects of DNA methylation on transcription in these tissues; or it is possible that the heterogeneity of the cell types in the adjacent non-tumorous tissues conceals the inverse correlation that we observed in vitro and in tumor samples. It is well known that the use of highly purified cell population is important for the assessment of the impact of DNA methylation on gene expression [25]. As a matter of fact, when we scrutinized the histological slides at distance from the tumor, we found large inter-individual differences in the number of lymphocytes present in the non-tumorous tissues (data not shown). Concerning *IL6*, a previous study suggested that methylation of a single CpG in the promoter

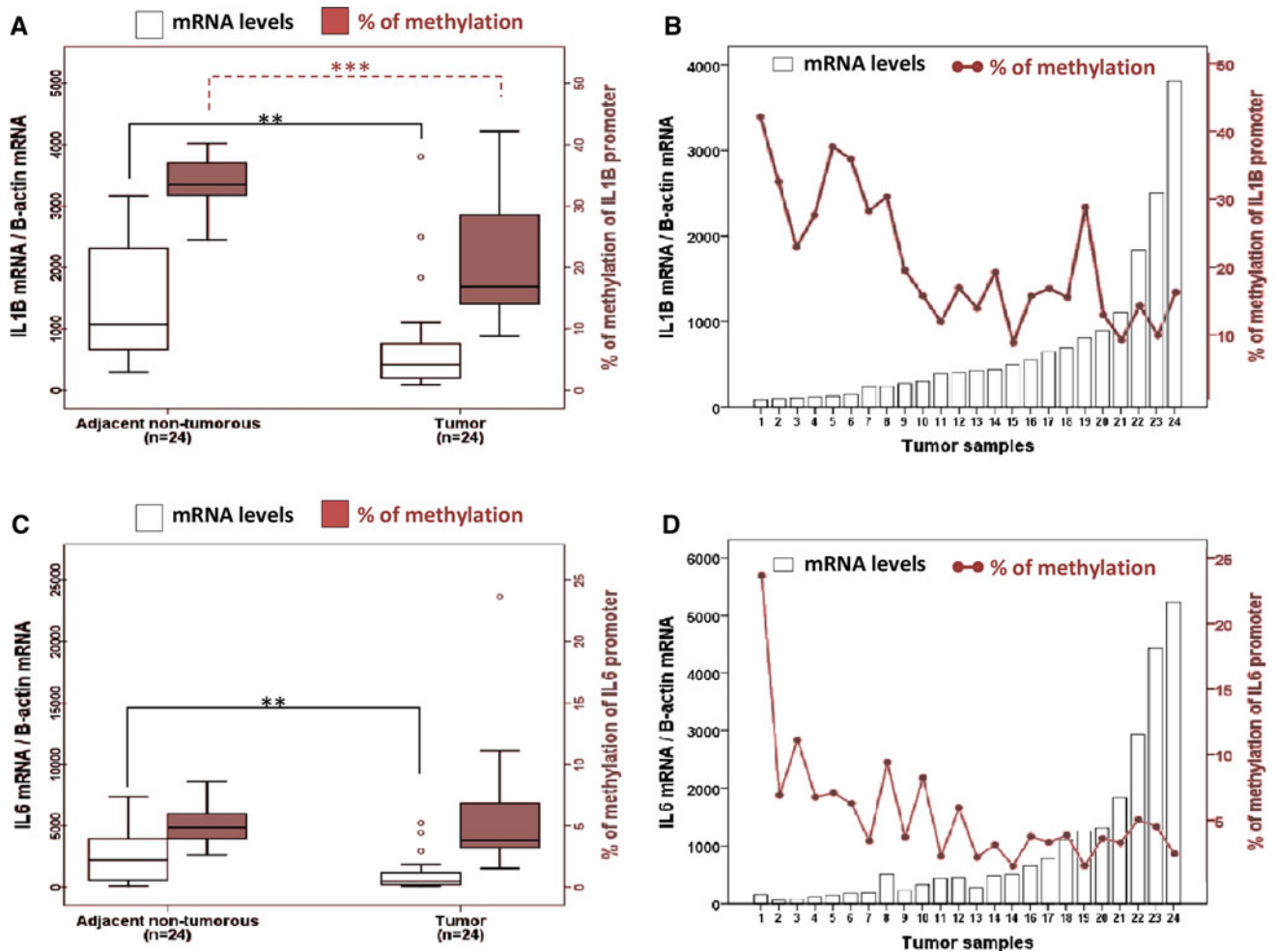


Fig. 3 mRNA levels and promoter DNA methylation of *IL1B*, *IL6*, and *IL8* in matched pair lung tissue. mRNA levels of *IL1B* or *IL6* were normalized to the mRNA levels of B-actin in matched pair series. Percentages of DNA methylation were obtained by analyzing the mean methylation of all the CpG sites present in the promoter of each genes. The mean level of methylation of two independent

bisulfite treatments and pyrosequencing was used for the tissue samples. $**p < 0.001$. **a** mRNA levels and promoter DNA methylation of *IL1B*, **b** mRNA levels and promoter DNA methylation of *IL1B* in individual tumor tissue samples, **c** mRNA levels and promoter DNA methylation of *IL6*, **d** mRNA levels and promoter DNA methylation of *IL6* in individual tumor tissue samples

region (−1,099 from transcription start site) affected mRNA levels in mononuclear cells [26]. We studied the methylation status of CpGs near the transcription start site of *IL6* (−240; −82 nucleotides). Our results suggest an association between the methylation of these CpGs and *IL6* mRNA levels in cultured human lung cells and in lung tumor tissues. Our results and those of Nile et al. [26] imply that different regions of the *IL6* promoter may be methylated and associated with mRNA levels. Here, we also investigated for the first time the DNA methylation of the *IL8* promoter in cultured human lung cells. Our results suggested that the CpGs at the *IL8* promoter were not frequently methylated, but when DNA methylation occurred it was associated with lower mRNA levels.

The relationship between inflammation and epigenetic modifications in cancer is beginning to be recognized

[25, 27]; however, the DNA methylation of the promoter of interleukins has not received much attention. Our study is the first to assess the DNA methylation of *IL1B*, *IL6*, and *IL8* promoters in a cancer context. Several interleukins have been shown to regulate the activity or expression of DNA methyltransferases (DNMTs) and thereby modulate the DNA methylation and the expression of genes involved in carcinogenesis [2, 28]. IL-6 treatment of multiple myeloma cells results in an increase in DNMT1 activity linked with p53 inactivation via promoter hypermethylation [29]. A relationship between DNA methylation of interleukin promoters and DNA methylation of oncogenes or tumor suppressor genes may represent an important step in lung carcinogenesis. Our results suggest that DNA methylation of *IL1B*, *IL6*, and *IL8* promoters occurs in human lung cells and tissues, and this may affect the corresponding mRNA

levels. Since, epigenetic modifications may be involved in interleukins gene regulation, a relationship between *IL1B*, *IL6*, and *IL8* promoters methylation and histone modifications may deserve further focus, especially since DNA methylation is often accompanied with important histone modifications which are acting in concert with the latter to regulate gene expression [30]. Thus, to completely elucidate epigenetic regulation of the expression of the three interleukins studied here, the importance of chromatin condensation and histone modifications should be investigated in the future.

We found higher *IL1B* and *IL6* mRNA levels in normal cultured cells or tissues compared to cancer cell lines or tissues. Similar findings have recently been reported, that is, mRNA levels of *IL6* and *IL1 α* were higher in adjacent tissues from hepatocellular carcinoma compared to the tumor tissues [31]; Jiang et al. further showed that high *IL1 α* mRNA levels in the adjacent non-tumorous tissues were due to high *ER α* expression. However, in our study, we did not find a similar correlation between *IL1B* or *IL6* and *ER α* or *ER β* mRNA levels (data not shown). In adjacent non-tumorous tissue of lung tumor, *IL1B* may be expressed by alveolar macrophages [32]; cancer cells can also express *IL1B* or induce cells within the tumor micro-environment to do so [33]. In vitro data showed that there were significantly higher levels of cytokines TNF α and IL-1 β in the culture supernatant of human monocytes co-cultured with lung cancer cells than in the supernatant of monocytes co-cultured with non-malignant BEAS-2B or HUVACs cells [34]. High *IL1B* or *IL6* mRNA levels in the adjacent tissues of tumor could enhance the tumor progression [35] or participate in the tumor initiation [36]. In fact, it has been shown that IL-1 β stimulates the expression of the pro-metastatic chemokine CX3CL1 in the adjacent normal tissue of prostate tumors [37]; thus, high *IL1B* and *IL6* mRNA levels in adjacent tissues from tumor may stimulate nearby cells to produce angiogenic proteins and growth factors and this may be involved in tumor metastasis and invasiveness [38]. On the other hand, immune cells expressing *IL1B* can release reactive oxygen species that are mutagenic for nearby cancer cells [3, 4]. The inflammation-related reactive species can induce DNA damage, including point mutations in cancer-related genes, and modifications in essential cellular proteins that are involved in DNA repair, apoptosis and cell cycle [2]; thus, high *IL1B* or *IL6* mRNA levels in adjacent tissues from tumor may also promote carcinogenesis. We preliminarily found in 15 non-tumorous tissues of smokers and ex-smokers (where both *IL1B* mRNA and hydrophobic PAH-DNA adduct levels were available) that high *IL1B* mRNA levels were related to high levels of smoking-induced hydrophobic PAH-DNA adduct (data not shown).

In conclusion, our study suggests for the first time a role for epigenetic modifications in the regulation of the expression of *IL1B*, *IL6*, and *IL8*, such a feature is fundamentally important since the expression of these cytokines plays diverse roles in chronic inflammation which might be associated with increased susceptibility to carcinogenesis or tumor development.

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Conflict of interest None declared.

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