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Lineage-specific silencing of human IL-10 gene expression by promoter methylation in cervical cancer cells

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ABSTRACT

Epigenetic analysis was performed to demonstrate that the normal and neoplastic epithelial cells do not serve as the source of the locally elevated IL-10 production during cervical carcinogenesis. Bisulfite sequencing was used to correlate promoter CpG methylation with the transcription of the gene. Lack of IL-10 transcription in HeLa, SiHa, Caski, HT-3, C33-A, HaCaT cell lines and in primary human keratinocytes correlated consistently with the methylated state of the proximal CpG residues, particularly with the two most proximal CpGs at positions –185 and –110. These two sites were also highly methylated in normal and malignant cervical cells directly isolated from patient material. On the other hand, IL-10 producing peripheral blood mononuclear cells had unmethylated CpG residues in the proximal promoter associated with acetylated H3 and H4 histones as determined by chromatin immunoprecipitation. In HeLa carrying epigenetically silenced endogeneous IL-10 promoter, the transfected non-CpG methylated 1 kb and 0.6 kb proximal promoter fragments could drive reporter gene expression, which was reversed by cassette methylation of these promoter fragments. In conclusion, the CpG methylation pattern of the proximal promoter is implicated as a major determinant of transcriptional silencing of human IL-10 expression in cells of cervical epithelial origin.

1. Introduction

IL-10 plays a critical role in limiting the intensity and duration of immune and inflammatory responses. IL-10 production is tightly regulated and seems to be restricted to certain cell types:[1,2] monocytes, macrophages, B cells, dendritic cells and T lymphocytes are the major sources of human IL-10.[3] The immunoregulatory role of IL-10 has been extensively studied. IL-10 cytokine inhibits the effector function of Th1 cells, activated macrophages and monocytes but enhances the growth and differentiation of B cells, granulocytes, dendritic cells, mast cells and endothelial cells. Locally elevated IL-10 production and the consequent down-regulation of cell-mediated defences is associated with the progression of certain malignancies.[4] During carcinogenesis at the uterine cervix, increased IL-10 expression and decreased IFN-γ production is associated with progression.[5,6] Carcinomas of the uterine cervix arise from the epithelium of the transformation zone constitutively expressing IL-10 in physiological conditions,[7] while in other areas of multilayered squamous epithelium, IL-10 is produced in response to injury, i.e. infiltrating inflammatory cells are the source of local IL-10 production.[8,9] On the other hand, IL-10 transcripts are absent from keratinocytes, the dominant cell type of the squamous

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epithelium.[10] Cervical cancer cell lines, which are also of epithelial origin, do not produce IL-10 either,[4,11] suggesting the existence of a lineage specific silencing mechanism.

Jacobs et al. suggested that an immunological disbalance, created by infiltrating inflammatory cells, contributes to cervical carcinogenesis.[12] The inducibility of IL-10 production in inflammatory cells[13–15] and the tissue specific silencing in other cell types[10] raises the possibility of a complex transcriptional regulation. The activity of the IL-10 promoter is regulated by binding of a series of transcription factors including STAT3,[14] SV40 promoter factor 1 (Sp1),[15,16] CCAAT/enhancer binding protein β,[17] and Ets-1.[18] These transcription factors are active in squamous epithelial cells.[19–22] The access of these transcription factors to the IL-10 promoter region can be influenced, however, by the local chromatin status, which in turn is subjected to epigenetic regulation.

In vertebrates, DNA methylation is a key epigenetic mechanism controlling transcription.[23] Downregulation of gene expression and complete silencing of genes often results from CpG methylation in their promoter regions. CpG methylation could either directly block the binding of transcription factors to their recognition sequences, or attract methyl-CpG binding domain (MBD) containing family of proteins that recruit histone deacetylases, thereby inducing a repressed chromatin state.[24]

In this study, an epigenetic analysis was performed to demonstrate that the normal and neoplastic epithelial cells do not serve as the source of the locally elevated IL-10 production during the cervical carcinogenesis. We analysed the CpG methylation pattern of the IL-10 promoter in peripheral blood mononuclear cells (PBMC), primary human keratinocytes and established cell lines of epithelial origin. We found that the IL-10 promoter was transcriptionally active and hypomethylated in PBMC but silent and highly methylated in epithelial cells. Methylation of IL-10 promoter regulatory sequences *in vitro* inhibited promoter activity. We also found that the active IL-10 promoter was enriched in acetylated histones H3 and H4. The methylation state of the two most proximal CpG sites in the IL-10 promoter differed most markedly between IL-10 producing and non-producing cell lines. These two sites were also highly methylated in normal and malignant cervical cells directly isolated from patient material. Our data suggest that methylation of specific CpG sites in the proximal IL-10 promoter can mediate a stable transcriptional repression in both normal epithelial and cervical cancer cells in a lineage specific manner.

2. Materials and methods

2.1. Cell lines and clinical samples

Peripheral blood mononuclear cells (PBMC) were isolated by Hypaque-Ficoll density gradient centrifugation from buffy coat preparations of healthy blood donors. The human keratinocyte cell line HaCaT and the human cervical tumour cell lines HeLa, SiHa, CaSki and C-33A were grown in DMEM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Karlsruhe, Germany) and 10% FCS. Primary human foreskin keratinocytes (Gibco Cat No: 12332-011) were cultured in Defined Keratinocyte serum-free Medium (DK-SFM, Gibco, Karlsruhe, Germany). Archived DNA samples of primary cervical tumours ($n = 10$) and exfoliated normal cervical epithelial cells ($n = 3$) were obtained from previous studies.[25,26]

2.2. RNA extraction, reverse transcription and PCR for IL-10 mRNA

Total cellular RNA was isolated using Tri Reagent (Sigma Aldrich, Deisenhofen, Germany) according to the manufacturer's protocol. RT-PCR was performed by using the Enhanced Avian HS RT-PCR kit (Sigma Aldrich, Deisenhofen, Germany) following the one-step RT-PCR procedure to produce cDNA. IL-10 cDNA was amplified by PCR using the following primers: 5′- TCAAGGCGCATGTGAACTCC -3′ (forward) and 5′- GTCGCCACCCTGATGTCTCA -3′ (reverse). Human cellular control oligonucleotide primers targeting human ribosomal RNA (h36B4) were 5′- AGATGCAGCAGATCCGCAT -3′ (forward) and 5′- ATATGAGGCAGGAGTTTCTCCAG -3′ (reverse).

2.3. Automated genomic sequencing of sodium bisulfite-treated DNA

We used the method of Frommer et al.[27] adapted for automated DNA sequencer.[28] Bisulfite conversion and sequencing of modified genomic DNA was carried out as described previously.[29] The IL-10 proximal promoter region was amplified from bisulfite-modified DNA by nested PCR. An initial PCR was performed with the following outer primers, specific for bisulfite-converted DNA: 5′-GGTAGGGGTTATGGTGAGTATTATTTGA –3′ (forward primer) and 5′- CCTAAACTAACCCTCACCCCAATC –3′ (reverse primer). The reactions included 20 pmol of each primer, 0.2 μM dNTPs, 2× Eppendorf HotMasterMix (Merck, Darmstadt, Germany) and 2 μl of bisulfite-treated genomic DNA. The product of the initial PCR was used as a template for a second PCR, using nested primers. The reverse nested primer corresponding to nucleotides –39 to –14 bp (5′- CCCTAATATATAAACCTTCACCTCTC –3′) was biotin labelled at the 5′ end and the forward primer carried 15 bases of M13 universal primer at its 5′ end. These primers corresponded to nucleotides –349 to –324 bp (5′- GTAAAACGACGGCCA-GAATGAGAATTTATAGTTGAGGGTTT-3′) and –681 to –657 bp (5′- GTAAAACGACGGCCA-GGATAGTTGAAGAGGTGGAAATATG-3′) of the bisulfite converted sequence, respectively. For these nested amplifications, 2 μl from the first PCR reaction product was used as template. Reactions included 20 pmol of each primer, 0.2 μM dNTPs and 2× Eppendorf HotMasterMix (Merck, Darmstadt, Germany). The product of the second PCR reaction was bound to streptavidin-coated magnetic beads (Dynal, Oslo, Norway) and the purified, biotin-labelled DNA was sequenced using the Alfexpress Autoread Sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) The reaction products were separated on acrylamid gel using an automated DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.4. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a modified protocol.[30] Briefly, cultured PBMC, HaCaT, HeLa and pHKC cells were fixed with formaldehyde at a final concentration of 1% at 25 °C for 10 min followed by addition of glycine to stop cross-linking.

Cells were lysed in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.0) and DNA was sheared by sonication to lengths between 200 and 1000 bp. Cell lysate was diluted with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, and 167 mM NaCl) and precleared by adding salmon sperm DNA/protein G agarose beads (Upstate Biochem., New York, NY) for 2 h. 10 μl precleared chromatin was kept as an input sample and the remainder was divided into four parts. Samples were incubated with 5 μg anti-acetyl-histone H3 (06-599, Upstate Biochem, New York, NY) and anti-acetyl-histone H4 antibodies (06-866, Upstate Biochem, New York, NY) and normal mouse IgG (sc-2025, Santa Cruz Biotech, Santa Cruz, CA) overnight at 4 °C. DNA-protein complexes were collected with salmon sperm DNA/protein G agarose (16-201, Upstate Biochem, New York, NY) for 3 h at 4 °C. Samples were washed once with 1 ml of low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl), once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate and 1 mM EDTA), and twice with TE buffer (pH 8.0) The washed beads were diluted in 100 μl TE buffer and incubated with 1 μg RNAse overnight at 65 °C. SDS at a total concentration of 0.5% and 20 μg Proteinase K were added to the samples followed by incubation at 45 °C for 3 h. The DNA was purified by using PCR purification kit (QIAGEN, Hilden, Germany). The recovered DNA fragments were resuspended in TE buffer (pH 8.0) and used for PCR amplifications.

2.5. Quantitative PCR analysis

Quantitations of immune precipitates were performed by real-time PCR using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers were designed to amplify 150–250 bp amplicons from IL-10 promoter. Two primer pairs were used to amplify the −531 to −669 (10CH1: forward, 5′- GGGACAGCTGAAGAGGTGGA -3′ and reverse, 5′- CCTCAAAGTTCCCAAGCAGC -3′) and −233 to −70 (10CH7: forward, 5′- GCTGTAATGCAGAAGTTCATGTTC-3′ and reverse, 5′-AGGGAGGCCTCTTCATTCA -3′) fragments of proximal promoter. PCR analyses were performed in 20 μl volume using Power SYBRGreen PCR Master Mix (Applied Biosystems, Foster City, CA). Standard curves were prepared for each primer set by tenfold serial dilution of input DNA.

2.6. Plasmid constructs

In the first step, 1089 bp and 618 bp fragments of the IL-10 proximal promoter were amplified from PBMC genomic DNA by using PCR. The first primer pair (forward, 5′- CTACTAAGGCTTCTTTGGGAG -3′; reverse 5′-CCCTGATGTGTAGACCTTCACC-3′) amplifies the area between −1102 and −14 and the second primer pair (forward, 5′- GGTAAAGGAGCCTGGAACACA -3′; reverse 5′-CCCTGATGTGTAGACCTTCACC-3′) amplifies the area between −631 and −14 relative to the IL-10 gene transcription start site.

These PCR fragments were ligated to linearised Xa-2 vector DNA (PinPoint^{TM} Xa-2 Vector, Promega, Madison, WI) that was cleaved at an *Eco*RV site and had a single 3′-terminal thymidine added to both ends. Then, the promoter fragments were excised from Xa-2 vector using the restriction enzyme sites for *Kpn*I and *Bgl*II (NEB, Beverly, MA) that allowed insertion into the pGL2 vector (pGL2-Basic, Promega, Madison, WI), which is a luciferase reporter vector. The digested gel purified promoter fragments were ligated into similarly treated pGL2 vector by using T4 DNA ligase (Promega, Madison, WI) and expanded in competent XL-1 *Escherichia coli* strain. All plasmid constructs were confirmed by nucleotide sequencing.

2.7. Methylation cassette assay

The methylation cassette assay was performed as described previously with modifications.[31] Briefly, pGL2 plasmid constructs containing IL-10 promoter fragments were digested with *Kpn*I and *Bgl*II restriction endonucleases (Promega, Madison, WI). One aliquot was methylated for 4 h at 37 °C in a 30-μl reaction mixture with 10 U of *Sss*I CpG methylase (NEB, Beverly, MA), 10× buffer and 160 μM *S*-adenosyl-methionine. The other aliquot (mock-methylated control) was treated in the same way except that the CpG methylase enzyme was omitted. The methylated and unmethylated cassette fragments were gel purified using the Qiaquick gel extraction kit (QIAGEN, Hilden, Germany) and eluted in 30 μl of sterile deionised water. Full methylation of promoter cassettes in each construct was confirmed by digestion with Fast Digest^{TM} *Hpa*II and Fast Digest^{TM} *Msp*I (both from Fermentas GmbH, St. Leon-Rot Germany). The percentage of cassettes left uncut after digestion was quantified by real-time PCR amplification using standard curves determined by serial dilution of the uncut cassette. The effectiveness of restriction cleavage was expressed as a percentage of cleaved cassettes. 2.5 ng of gel purified DNA in a total volume of 20 μl of reaction solution containing 10 U of *Hpa*II or 10 U of *Msp*I was digested for 5 min at 37 °C. 2 μl of hundred-fold dilutions of the reaction mixtures were used for real-time PCR amplification by using the 10CH7 primer pair as described above. The methylated or mock-methylated cassette fragments were then ligated back into unmethylated pGL2-Basic vector in a 1:1 molar ratio at 16 °C for 12 h with 10 U of T4 DNA ligase (NEB, Beverly, MA) The efficiency of the ligation reaction was assessed by analysis of 1 μl of the ligation reaction mixture on a 1.5% agarose gel. The ligation mixtures were transfected using Lipofectamine^{TM} 2000 reagent (Invitrogen, Carlsbad, CA) into HeLa cell line according to the manufacturer's protocol. Cells were plated in 6-well plates and transfected 24 h later at 80% confluency. Transiently transfected cells were incubated at normal growth conditions for 24 h and assayed for transgene expression by using Luciferase Reporter Assay (Promega, Madison, WI). Briefly, the cells were rinsed with PBS and lysed in the plate by using Reporter Lysis Buffer (Promega, Madison, WI).

3. Results

3.1. Activity and methylation status of the human IL-10 promoter

Using RT-PCR, we detected IL-10 transcripts in peripheral blood mononuclear cells but not in primary keratinocytes or HaCat or cervical cancer cell lines (HaCaT, HT-3, C33-A, HeLa, CaSki, SiHa) (Fig. 1). The results of CpG methylation analysis

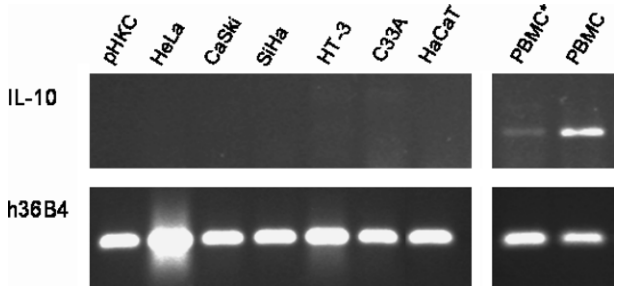

Fig. 1 – Detection of IL-10 mRNA in primary human keratinocytes (pHKC), HaCaT, C-33A, HT-3, CaSki, HeLa, SiHa cell lines and in peripheral blood mononuclear cells from adult (PBMC) and umbilical cord vein (PBMC*).

of the cell lines are presented in Fig. 2. There are eight CpG sites in the analysed region (comprising cytosines located at positions −634, −599, −373, −352, −350, −320, −185 and −110, relative to the transcriptional start site). The cytosines at positions −373, −352, −350, −320, −185 and −110 were highly methylated both in primary keratinocytes and immortalised epithelial cell lines. Cytosine −110 was completely methylated in all of the epithelial cells studied. In contrast, cytosine −110 was unmethylated in IL-10 expressing PBMCs regardless of immunological maturation. The methylation of the two distal CpG sites (comprising cytosines −634 and −599) varied in the analysed cell lines. A high level of cytosine methylation in these positions did not prevent the activity of IL-10 promoter in PBMCs.

The methylation state of the two most proximal CpG sites in the IL-10 promoter differed most markedly between IL-10 producing and non-producing cell lines. To prove that promoter methylation is not induced during extensive culturing of the epithelial cell lines, we determined the methylation status of the two CpG sites in normal exfoliated cervical cells ($n = 3$) and in cervical cancer tissue specimens ($n = 10$). CpG site −110 was uniformly methylated in cervical cancer biopsies and

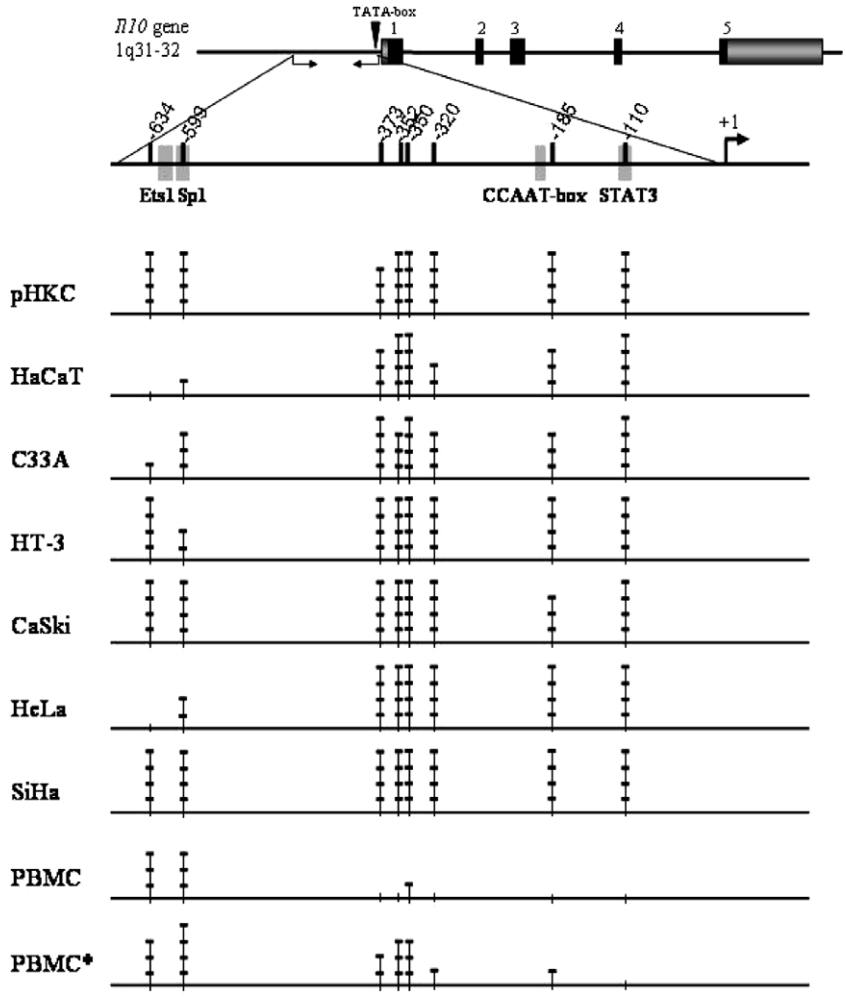

Fig. 2 – Methylation patterns in the proximal region of the human IL-10 promoter. On the schematic map of IL-10 gene (top), bent arrows indicate the studied promoter region and the transcriptional start; numbered short vertical lines across the horizontal bar indicate CpG sites; faint bars represent relevant transcription factor binding sites in the human IL-10 promoter. The degree of cytosine methylation is indicated by the height of the lollipops as follows: spot only, 0%; one lollipop unit, 0 to 25%; two units, 25 to 50%; three units, 50 to 75%, four units, 75 to 100%. (pHKC: primary human keratinocytes, PBMC*: PBMC isolated from umbilical cord vein).

in normal cervical epithelial cells. CpG site –185 also tended to be highly methylated in the clinical samples, although partial demethylation at this site was found in two cervical cancer biopsies.

3.2. *In vitro CpG methylation of IL-10 promoter regulatory sequences inhibits promoter activity*

The proximal promoter fragments –1102 to –14 and –613 to –14 were cloned into PGL2-Basic reporter plasmid. The recombinant plasmids designated as pGL2-1102 and pGL2-613, respectively, were used to study the effect of CpG methylation of IL-10 promoter regulatory sequences on promoter activity. The IL-10 promoter inserts were excised from the constructs and methylated *in vitro* with *Sss*I methylase. The effectiveness of *Sss*I methylation was tested by *Hpa*II and *Msp*I restriction enzymes, respectively. The recognition sequence of these enzymes contains the most proximal CpG at position –110. *Hpa*II, an isoschizomer of *Msp*I, is sensitive to methylation of either cytosine in the recognition site 5′ CCGG 3′. Using real-time PCR quantification, we confirmed complete methylation (98–99%) at this site by its resistance to *Hpa*II. Methylated or mock-methylated casettes were ligated back into the *Kpn*I-*Bgl*II sites of the unmethylated pGL2-Basic vector.

The pGL2-1102 and pGL2-613 constructs carrying CpG methylated or mock-methylated IL10 promoter regulatory sequences used for transient transfections of HeLa cells and tested for their ability to drive luciferase expression. Luciferase activity was normalised to total cellular protein content of transfected HeLa cell cultures. We detected similar levels of inhibition in promoter activity after CpG methylation of the 1 kb and the 0.6 kb cassettes (Fig. 3 a,b). We note that the activity of the unmethylated constructs in an epithelial cell line suggests that the lack of activity of the endogenous IL-10 promoter in HeLa cells is not due to the absence of cognate transcription factors activating IL-10 promoter.

3.3. *Differences in the histone acetylation status of IL-10 promoter in IL-10 expressing and non-expressing cells*

To determine whether histone acetylation is associated with IL-10 expression, we compared the acetylation status of histones H3 and H4 bound to the IL-10 proximal promoter region in the IL-10 expressing PBMCs and three IL-10 non-expressing epithelial cell lines (pHKC, HaCaT, HeLa). In the immunoprecipitated chromatin, we quantified the relative amount of two IL-10 promoter regions, one (–669 to –531) carrying Ets-1 and Sp1 binding sites and two CpGs, and the other (–233 to –70) carrying CCAAT-box and STAT-3 binding sites and the two most proximal CpGs. Acetylated histones were bound to the IL-10 promoter only in the IL-10 expressing PBMCs and in none of the IL-10 non-expressing epithelial cell lines

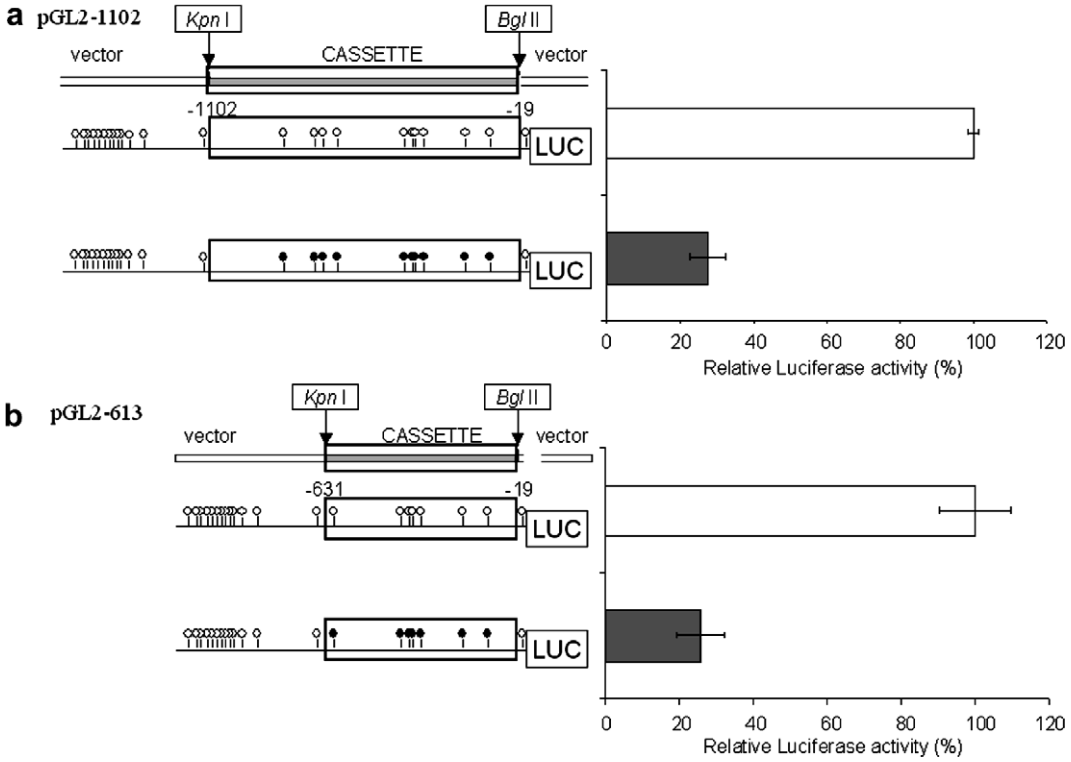

Fig. 3 – Effect of IL-10 promoter methylation on the transcriptional activity of pGL2 reporter plasmid constructs by *methylation cassette assay*. (a) CpG methylation of the entire proximal IL-10 promoter. (b) CpG methylation of the most proximal 600 bp segment of the IL-10 promoter. In the vector and the insert sequences, filled lollipops indicate *in vitro Sss*I methylated CpGs and empty lollipops indicate the unmethylated ones. The relative luciferase activity of methylated contructs (filled bar) is expressed as a percentage of the unmethylated ones (empty bar). Error bars represent standard deviation in a representative experiment.

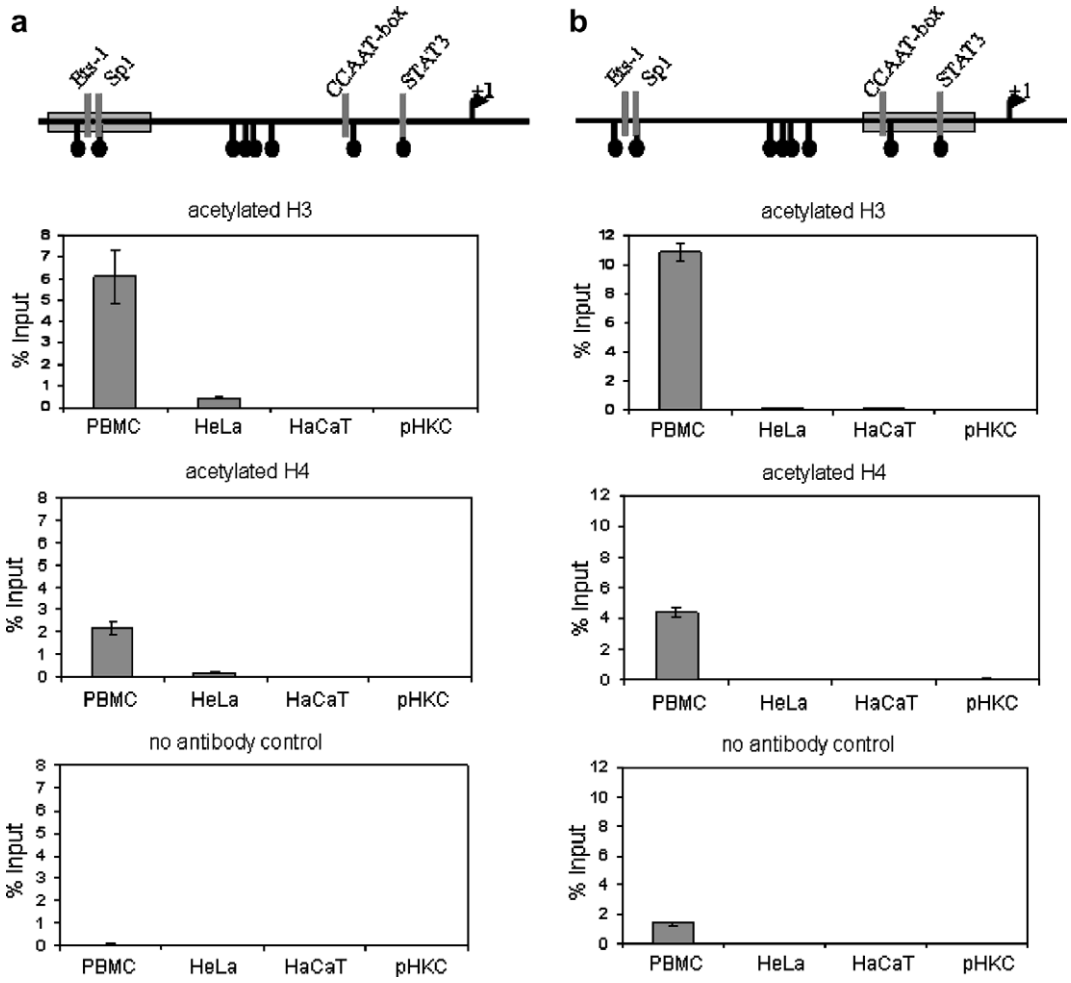

Fig. 4 – Histone acetylation status of IL-10 promoter regions involved in transcriptional regulation. Cross-linked chromatin derived from peripheral blood mononuclear cells (PBMC), HaCaT, HeLa and primary human keratinocyte (pHKC) cells was immunoprecipitated with antibodies against acetylated H3 or acetylated H4. The relative amount of two promoter regions was quantified by real time PCR amplification. The amplified sequences (a,b) are indicated with a horizontal shadowed box on the genomic representation (black lines on the top) also showing CpG sites (black lollipops) and the transcription factor binding sites (vertical grey bars).

(Fig. 4). In the chromatin associated with the actively transcribed promoter, acetylation of H3 histones was more prominent than acetylation of H4 histones. We found uniform histone acetylation status along the examined promoter region suggesting that the enrichment of acetylated histones H3 and H4 in the IL-10 proximal promoter region is associated with open chromatin and transcriptional activation in IL-10 expressing cells.

4. Discussion

Local IL-10 production contributes to the progression of certain neoplasms by inhibiting cell mediated immune defenses. The malignant cells of colorectal carcinoma or thyroid carcinoma also secrete IL-10,[4] and IL-10 serves as an autocrine growth factor for the latter.[32] In addition, infiltrating inflammatory cells are also a potential source of IL-10 in solid tumours.[33] Thus, dissecting the different cellular types can help to identify the IL-10 producing cells in tumours. The presence or absence of IL-10 in established cell lines may reflect their *in vivo* cytokine synthesising capacity. It is noteworthy that cell lines derived from breast, lung, prostate and cervical cancers consistently lack IL-10 expression, which suggests a lineage specific silencing of the IL-10 gene in these tumours.[4,11] In the normal transformation zone of the uterine cervix, where most cervical neoplasias arise from, there is an increased expression of TGF-β1 and IL-10, which can locally suppress the cell mediated immunity.[7] In premalignant cervical lesions, IFN-γ expression is decreased and IL-10 expression is increased.[5,7] In contrast to the established cervical cell lines[11], transcription of IL-10 gene is detected generally in cervical cancer biopsies[34] containing cell types other than cervical tumour cells, suggesting that the source of IL-10 production is different from the neoplastic cell. A uniform lack of IL-10 transcription in cells of cervical epithelial origin suggests that IL-10 promoter is permanently repressed in a lineage specific manner. One could speculate, however, that this repression is not due to the absence of transcription

factors, because major transcription factors involved in the regulation of IL-10 promoter (STAT3, Sp1, Ets-1 and CCAAT-box-binding factor) are expressed in squamous epithelial cells.[19–22]

CpG methylation in promoter regions is a major determinant of epigenetic transcriptional silencing, acting as a common regulatory mechanism for lineage specific gene expression. Tissue specific genes tend to harbour low number CpG sites in their promoters[35] including critical CpG sites prominently involved in epigenetic transcriptional regulation. This regulatory mechanism controls the expression of certain human cytokine genes, such as the IFN-gamma and IL-2, where site-specific methylation of promoter proximal CpG dinucleotides strongly correlates with transcriptional activity.[36–38] The human IL-10 promoter also has a low CpG content: there are eight CpG sites in the 700 bp long proximal promoter region that contains binding sites for critical transcription factors. In the present study we investigated the methylation state of this proximal IL-10 promoter region in established cervical cancer cell lines and cervical cancer biopsies. The concordant DNA methylation pattern suggested that the established cell lines served as reliable models of transcriptional regulation in cervical cancer cells. Primary human foreskin keratinocytes and HaCaT keratinocyte cell line were used to model the CpG methylation pattern in normal epithelial cells. Since these cells are not of cervical origin, we also determined the methylation status of the promoter proximal CpG sites in exfoliated normal cervical epithelial cells directly isolated from patient material. We found that similarly to the promoters of other cytokine genes, the methylation state of the most proximal CpG site in IL-10 promoter regulatory sequences correlated best with IL-10 transcription. This cytosine (position –110) is located within the binding site of STAT3, a transcription factor essential for human IL-10 expression.[4,14,39] It is of note that STAT3 binding is also inhibited by CpG methylation of STAT3 consensus sequence in the promoter of the human glial fibrillary acidic protein (GFAP) gene.[40]

In general, demethylated state of critical CpG sites provides a basis for further fine regulation of chromatin structure through histone modifications.[24] We showed histone H3 acetylation along the regulatory region of human IL-10 promoter in mononuclear blood cells. In a mouse model, a common DNAse I hypersensitivity site (HSS) was found at 0.12 kilobase from the transcriptional start in high IL-10 producing macrophages, Th2, IL-10-TReg cells,[41] but not in Th1 cells.[42] Although this HSS corresponds to proximal CpG sites of the mouse IL-10 promoter, there is no information on the methylation status of these CpG sites. Assuming common epigenetic regulation of IL-10 expression is shared by humans and mice, the results of this and the mouse studies suggest that potential IL-10 producing cells have demethylated promoter proximal CpG sites, and the commitment to high IL-10 production is mediated by loosening the chromatin structure, making the promoter accessible for transcriptional activators.

The methylation state of the next five CpG sites located upstream from cytosine –110 correlated well with the methylation pattern of the promoter proximal (position –110) CpG site, whereas the CpG sites at position –599 and –634 were methylated regardless of the origin and IL-10 expressing ability of the examined cells. In addition, we found that the methylation pattern of IL-10 promoter in human fibroblasts (an IL-10 non-producing cell type) was identical to that of primary keratinocytes (data not shown). This raised the possibility that the CpG methylation mediated silencing of IL-10 expression can be a general mechanism in different cell lineages. The histone acetylation results showed that in IL-10 producing cells, the demethylated state of the six CpG sites proximal to the transcriptional start site also seems to ensure the euchromatic structure along adjacent upsteram promoter sequences (from –669 to –531) in spite of the presence of two methylated CpG sites. One could speculate that the binding of MBD proteins to sequences of low methyl-CpG density is probably insufficient to attract histone deacetylases.

Although there is a marked IL-10 production in cervical cancer, the results of this and other studies suggest that the cancerous cells themselves do not serve as the source of the local IL-10 production. Thus, the stromal compartment of the tumour or the infiltrating lymphocytes are plausible sources of IL-10. These IL-10 producing components of the tumour certainly have a different methylation pattern than the squamous carcinoma cells. In cervical cancer biopsies tested in this study, the overall volume of the IL-10 producing component was obviously too low to influence the semiquantitative determination of CpG methylation with bisulfite sequencing.

The functional tests in this study were based on transient reporter expression assays. We observed that the exogenous IL-10 promoter constructs were actively transcribed in HeLa indicating that an epithelial cell line can express all transcription factors necessary to activate the IL-10 promoter. Using methylation casette assay, we observed that CpG methylation of IL-10 promoter regulatory sequences inhibited the expression of the reporter gene. From the shorter plasmid construct containing the proximal 0.6 kb promoter sequence (pGL2-613), transcription was initiated at a similar level as from the 1 kb plasmid construct (pGL2-1102) spanning the entire proximal IL-10 promoter, suggesting that the sequences adjacent to the transcriptional start site play an essential role in IL-10 transcription by binding crucial transcription factors.[13–17]

In conclusion, we found that the CpG methylation pattern of the promoter proximal regulatory region is a major determinant of transcriptional silencing of human IL-10 expression in normal and neoplastic epithelial cells of the uterine cervix. Our results regarding CpG methylation and histone acetylation data in IL-10 negative epithelial cells and IL-10 producing PBMCs suggest that in addition to the potential influence of genetic polymorphisms,[26,43] epigenetic mechanisms also affect the activity of the human IL-10 promoter.

Conflict of interest statement

None declared.

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