IL-6-induced DNMT1 activity mediates SOCS3 promoter hypermethylation in ulcerative colitis-related colorectal cancer

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Ulcerative colitis (UC) is associated with a high risk of developing colorectal cancer (CRC). The mechanisms by which chronic inflammatory responses in the colon may promote CRC remain only partially understood, but may involve reduced negative regulation of interleukin (IL)-6 signaling towards signal transducer and activator of transcription 3 activation through the loss of SOCS3 expression, unleashing the full carcinogenic potential of this transcription factor. Thus, we analyzed SOCS3 expression in the colon of healthy controls, as well as in a cohort of UC patients with varying degrees of dysplasia. We observe that the loss of epithelial SOCS3 expression delimits the areas subject to dysplasia in UC, suggesting an important tumour-suppressive role of SOCS3 downregulation, early in the transformation process. Importantly, methylation of the SOCS3 promoter appears to constitute an important regulatory mechanism for colonic SOCS3 expression as SOCS3 methylation status in CRC cells correlates with a disability to upregulate SOCS3 upon IL-6 stimulation, whereas forced demethylation using 5-aza-2’-deoxycytidine restores SOCS3 expression and inhibits IL-6-induced p-Signaling and activator of transcription 3 activation and proliferation. Expression of the DNA methyltransferase gene DNMT1 is prominent in dysplastic cells and correlates with low or absent SOCS3 expression. Thus, induction of DNMT1 expression in the chronically inflamed colon may release IL-6 signaling towards signal transducer and activator of transcription 3 from inhibition through SOCS3 increasing the propensity to malignant transformation. Hence, DNMT1 emerges as a rational target in preventive strategies aimed at counteracting UC–CRC.

Introduction

Ulcerative colitis (UC) is a major manifestation of inflammatory bowel disease (IBD) and is clinically characterized by chronic recurrent colitis and is histopathologically characterized by the presence of a continuous inflammatory infiltrate in the intestinal mucosa ascending from the rectum into the colon (1). Chronic UC is associated with an increased risk to develop colorectal cancer (CRC) and UC–CRC underlies approximately 10–15% of all mortality in patients with IBD. In these patients, the risk for contracting CRC is approximately 20–100 times higher in the population at large, depending on the absence or presence of Primary sclerosing cholangitis (PSC) as a co-morbidity (2,3). As such, UC–CRC represents a special challenge in the prevention and treatment of gastrointestinal cancer.

The pathophysiological mechanisms explaining the unusual high risk of colon cancer in UC remain only partially understood but may involve the chronic proliferation necessary to repair the damage inflicted on the epithelial monolayer by the constant inflammation (4,5). In this context, high interleukin (IL)-6, detected in colonic biopsies and serum samples of UC patients, is considered an especially deleterious factor with respect to the development of malignant disease. IL-6 is a pro-inflammatory cytokine that is associated with carcinogenesis at multiple places in the human body. Signaling through the canonical cytokine receptor pathway, IL-6 evokes activation of Janus kinase 2, in turn activating the signal transducer and activator of transcription 3 (STAT3), which can mediate evasion of immunosurveillance, promote cell growth and increase survival signaling (6,7). Full manifestation of these pro-oncogenic effects of STAT3, however, requires inactivation of the negative regulators of IL-6 signaling. The most important of the latter mechanisms is expression of the suppressor of cytokine signaling 3 (SOCS3), which is a direct target gene of STAT3 and mediates suppression of IL-6 signaling through the ubiquitination and degradation of signaling intermediates. The question of how during UC–CRC carcinogenesis the negative influence of STAT3-dependent induction of SOCS3 is overcome remains one of the most important questions in the field.

We have previously reported on the silencing of SOCS3 expression as a potential mechanism explaining sustained IL-6-dependent STAT3 phosphorylation and activation in the inflammation–dysplasia–carcinoma sequence in UC (8), but left the underlying molecular mechanism unexplored. Obviously, delineation of these mechanisms might be exceedingly useful for devising rational strategies for the prevention and maybe also for treatment of UC-associated CRC. Interestingly, in cholangiocarcinoma, a disease that shares many characteristics with UC–CRC, IL-6 signaling has been associated with increased expression of DNA methyltransferase-1 (DNMT1) (9), a CpG island methylator, which has thus been proposed to provide a link between IL-6 levels and inflammation-associated cholangiocarcinoma (10) and UC-related carcinoma (11) through DNMT1-mediated tumour-suppressor gene silencing. Causative relationships, however, remain to be established and especially identification of the tumour-suppressor genes involved remains a very open issue.

Here, we explore the possibility that DNMT1 induction by IL-6 in UC mediates silencing of SOCS3 expression and consequently CRC we observe that spatially UC–CRC is restricted to areas with loss of SOCS3 expression, that SOCS3 promoter methylation makes the gene resistant to IL-6-dependent induction of its expression in a DNMT1-dependent fashion and that both in vivo, as well as in patients, IL-6-dependent activation of STAT3 signaling is strictly dependent on DNMT1 enzymatic activity. The dependence of carcinogenic progression of UC–CRC on DNMT1 and the subsequent inhibition of SOCS3 transcription make this methyltransferase a prime candidate target for chemopreventive strategies in this disease.

Materials and methods

Patient biopsies and cell lines

Patients’ characteristics are shown in Table 1. Paraffin-embedded biopsies from 9 normal controls, 27 active UC, 11 UC–CRC and 14 sporadic CRC were included. Colon biopsy specimens were obtained, after informed consent, during colonoscopy performed in the course of routine clinical care and surveillance. The diagnosis of UC was based on conventional clinical, endoscopic and pathohistological criteria as described by Lennard-Jones (12). An independent pathologist reconfirmed carcinoma by hematoxylin and eosin staining.
Table I. Patients' characteristics

<table>
<thead>
<tr>
<th>Gender</th>
<th>Healthy control (N = 9)</th>
<th>Active UC (N = 27)</th>
<th>UC–CRC (N = 11)</th>
<th>Sporadic CRC (N = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>6</td>
<td>15</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>12</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Mean age, year ± standard deviation</td>
<td>39.6 ± 17.8</td>
<td>42.0 ± 15.3</td>
<td>(56.1 ± 16.5)²</td>
<td>(68.3 ± 12.2)²</td>
</tr>
<tr>
<td>Mean duration of disease, year ± standard deviation</td>
<td>–</td>
<td>11.9 ± 9.6</td>
<td>(22.3 ± 5.8)²</td>
<td>NA</td>
</tr>
</tbody>
</table>

Mean age, duration of disease of patients in UC–CRC patients (22.3 ± 5.8) was significantly longer than that of patients in other groups (P < 0.01).

Mean age of patients in sporadic CRC group (68.3 ± 12.2) and UC–CRC group (56.1 ± 16.5) was significantly larger than those of healthy control group and active UC group.

No gender difference in each group.

DNA isolation and bisulphate modification
Different cell-line DNA was isolated using trizol reagent. Cell-line DNA samples were bisulphate modified using the EZ DNA methylation™ kit (ZYMO Research). Methylated DNA and unmethylated DNA (milipore) were acquired commercially and used as a positive and negative control in the enzymatic assay.

Methylation-specific PCR
Methylation-specific PCR (MSP)-specific primers were either designed by Methprimer or as described previously by Weber Hengge (13) and used to amplify methylated and unmethylated DNA sequences as appropriate. A schematic overview of the strategy employed is provided in Supplementary Figure 2, available at *Carcinogenesis* Online. MSP was performed on bisulphite-treated genomic DNA. The PCR was performed in a 25 µl volume containing 40 ng bisulphite-modified DNA, 5x Gold Tag buffer, 0.2 µM deoxynucleoside triphosphates, 10 pmol specific primer mix (forward and reverse primers) and one unit Gold Tag enzyme (Promega). Three regions of SOCS3 promoter were targeted; the PCR conditions and sequences of each primer mix are given in Table 2. The PCR products were visualized on a 2% agarose gel using ethidium bromide and UV illumination.

Normal PCR and pyrosequencing
Bisulphite genomic DNA was amplified region exon 1 to intron before exon 2, on SOCS3 promoter. Product was purified and sequenced by input 15 ng per 100-bp-purified PCR product per 20 µl reaction. SOCS3 promoter region 5'-untranslated region (UTR) before exon 1 was amplified and prepared using PyroMark™ Vacuum Prep Workstation. The methylation status was quantified by pyrosequencing machine PyroMark™MD, BiotaGe. The PCR conditions and sequences of each primer mix were shown in Table 2.

Demethylation treatment
For demethylation, experiments were seeded using 1:10 dilution of a 1 x 10⁶ cells/ml stock cell suspension into 12-well plates. Twenty-four hours later, 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma) was added to a final concentration of 10 µM. Three days (72 h) after 5-Aza-dC treatment, the cells were stimulated by IL-6 (100 ng/ml) and harvested for quantitative real time polymerase chain reaction (QPCR) and western blot. Independent cells (control and demethylated cells) cultured after 3 and 6 days were viewed by microscope.

RNA isolation and real-time PCR
Total RNA was isolated from cell lines using TRIZol (Sigma). About 1 µg of RNA was reversely transcribed using iScript™ cDNA synthesis kit (Bio-rad), quantitative real time polymerase chain reaction (QPCR) reactions were performed using real-time PCR kit sensimix™ SYBR&Fluorescence (Quntace) in a final volume of 25 µl containing 10 pmol primers in IQ5 (Bio-rad). The experimental conditions and primers employed for specific experiment is provided in Table 2. Data obtained are expressed as the ratio between SOCS3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA delta-Ct to yield the fold induction in mRNA expression relative to the appropriate control.

Western blot
Western blotting was performed as described earlier (14). Immobilon IF transfer membranes (Millipore) were probed with primary antibodies specific for SOCS3 (rabbit polyclonal, abcam, ab16030), p-STAT3 (rabbit monoclonal, #9145, cell signal) and DNMT1 (rabbit polyclonal, Abcam, ab19905). α-tubulin (Abcam ab6046) and β-actin (Santa Cruz, E0610) were used to provide the levels of a reference protein to ascertain equal loading. Horseradish peroxidase–labelled goat anti-rabbit and goat anti-mouse were used as secondary antibody (LI-COR Biosciences). Transfer membranes were incubated with antibodies and washed in 50 ml sterile light-protecting centrifuge tubes (Greiner bio-one). Protein expression was visualized using fluorescence Odyssey system (LI-COR Biosciences, Lincoln, NE, USA). Quantitative expression data were determined by Odyssey 3.0 software and normalized using β-actin or α-tubulin for reference gene expression.

Thiazolyl blue tetrazolium bromide assay
Control cells and demethylated cells were cultured in 96-well plates for in total four days. Subsequently 0.5mg/ml thiazolyl blue tetrazolium bromide was added to the cell medium. As described previously (15), the absorbance was measured using a microplate reader Model 680XBR (BIO-RAD) at 490 nm. All experiments were repeated three times. Percentage of cell viability after demethylation was calculated as % Viability = mean OD in sample well/mean OD in control well x 100, where OD is optical density.

Statistical analysis
Statistical analysis was performed using SPSS software version 15.0. The possible statistical differences between were determined using the Mann–Whitney
We reasoned that the mechanisms mediating inhibition of SOCS3 expression in the colonic epithelial compartment are likely to involve SOCS3 promoter methylation as a major regulator of SOCS3 expression in UC. Indeed, it is now well established that SOCS3 promoter methylation is a major regulator of SOCS3 expression in colorectal cancer (CRC) cells (13, 14). Few studies have explored the potential role of SOCS3 methylation in UC. We previously demonstrated that SOCS3 promoter methylation is significantly increased in UC colon compared to normal colon tissue (15, 16). In addition, we reported that SOCS3 mRNA expression is inversely correlated with SOCS3 promoter methylation in UC colon tissue (16). These findings suggest that SOCS3 hypermethylation in UC is related to the downregulation of SOCS3 expression and may contribute to the pathogenesis of UC.

Loss of epithelial SOCS3 expression delimits the neoplastic domain in UC–CRC

We previously proposed that silencing of SOCS3 expression explains sustained IL-6-dependent STAT3 phosphorylation and activation in the inflammation–dysplasia–carcinoma sequence in UC (8). Similarly, downregulation of SOCS3 was not found in CD-dysplasia and CD-CRC patients (Supplementary Figure 1, available at Carcinogenesis Online), which highlights the specificity of SOCS3 downregulation in UC-carcinogenesis. A prediction from the hypothesis that SOCS3 downregulation in UC unleashes the full oncogenic potential of IL-6 to stimulate transcription of SOCS3 is widely divergent, such induction being very strong in caco-2 cultures but absent in DLD1. A preliminary analysis of IL-6 receptor levels revealed that alternative expression of IL-6 signal transduction elements was unlikely to account for these differences (Supplementary Figure 3, available at Carcinogenesis Online). As identification of the mechanisms underlying the different cell lines (Figure 2B), demonstrating that regulation is different in established colon cancer cell cultures, but that especially the capacity of IL-6 to stimulate transcription of SOCS3 is widely divergent, such induction being very strong in caco-2 cultures but absent in DLD1.

Expression of DNMT1 negatively correlates with capacity for SOCS3 induction

Searching for candidate genes possibly involved in the methylation of the SOCS3 promoter in colonic cells, we decided to study the possible involvement of DNMT1 (16), because the UC–CRC highly expressed cytokine IL-6 is able to upregulate the expression of DNMT1 (10). Earlier, we showed that activity of this gene
Fig. 1. Specific SOCS3 down regulation in epithelial cells during progression from low-grade dysplasia (LGD) to UC–CRC in the dysplastic and/or cancer region. (A) Immunohistochemistry (IHC) staining shows SOCS3 expression in dysplastic/cancer and adjacent non-dysplastic/cancer area in the same biopsies. (B) Epithelial SOCS3 expression was compared in the same biopsies between dysplastic/cancerous areas and non-dysplastic areas. SOCS3-positive epithelial cells are significantly more numerous in the non-dysplastic/non-cancer area than in the dysplastic/cancer area from the same biopsies (P < 0.05). To assess SOCS3 expression in different areas of cancer tissues, experienced gastro-pathologist evaluated dysplasia/cancer after hematoxylin and eosin staining and marked dysplastic/cancerous area in every slide. Then two independent trained scientists scored the slides with SOCS3 positive epithelial cells in a blinded manner. The scoring criteria were defined as mild expression <30%, positive expression 30–60%, strong positive expression >60% of SOCS3 positive epithelial cells.
increased chemosensitivity of colorectal cancer cells by inducing epigenetic reprogramming and reducing colorectal cancer-cell stemness (17), thus the activity of this enzyme constitutes an important epigenetic regulatory event. In this study, we established that ideal time frame for this epigenetic experiment at 48 h and thus also in the present study, this temporal window for the epigenetic effects of IL-6 stimulation on nuclear reprogramming was chosen. We observe that IL-6-induced stimulation of colorectal cancer cell cultures provokes DNMT1 expression at both mRNA and protein (Supplementary Figure 4, available at Carcinogenesis Online), at this time point, suggesting that expression of DNMT1 methylating activity under the control of inflammatory mediators in the colon. Furthermore, we found that the IL-6-induced DNMT1 expression correlated with enhanced SOCS3 promoter methylation (Figure 2C). Hence, DNMT1 appears to be one of the enzymes responsible for increased methyltransferase activity leading to methylation of the SOCS3 promoter in turn negatively influencing feedback on IL-6-dependent signal transduction.

**DNMT1 regulates the SOCS3 expression and function in colon cancer cell cultures**

Our data suggest that IL-6-dependent DNMT1 induction mediates SOCS3 downregulation, in turn releasing IL-6 signaling towards STAT3 from its negative regulation. A prediction from this hypothesis is that inhibition of DNMT1 should restore IL-6-dependent induction of SOCS3 and counteract methylation of SOCS3 promoter. Thus, we tested the effect of the methylation inhibitor 5-aza-2′-deoxycytidine (DAC) on DLD1 cells. Indeed, DAC treatment increased SOCS3 mRNA and protein levels in otherwise unchallenged cells (Figure 3A and 3B). Furthermore, following the treatment with the methylation inhibitor, cells became susceptible to IL-6-dependent SOCS3 induction. Finally, concomitantly IL-6-dependent induction of STAT3 was impaired (Figure 3B), as well as IL-6-dependent cell proliferation was impaired (Figure 3C and 3D). To illustrate the effect of methylation inhibitor on cell growth, DLD1 cells were cultured in medium with or without DAC for 3 days, then for an additional 3 days, with or without IL-6. Cells treated with DAC for the entire 6 days showed a significant reduction in cell growth compared with those cultured without DAC (P < 0.05). Whereas IL-6 stimulation further increased the growth of cells in the absence of DAC, DAC treatment prevented enhanced proliferation by IL-6. Thus, these in vitro experiments provide support for the notion that an IL-6-dependent downmodulation of methyltransferases reduces regulation of IL-6 signaling towards STAT3 and is permissive for uncontrolled colonic cell proliferation and subsequently we investigated whether support for this notion could be obtained in patient samples of UC–CRC.

**Fig. 2.** Different SOCS3 promoter methylation in CRC cell lines. (A) The methylation status was determined in five CRC cell lines using MSP at three different regions of SOCS3 promoter. HT29, SW480, SW48 and DLD1 showed more SOCS3 methylation than caco-2 cells. (B) Induction of SOCS3 expression upon IL-6 stimulation in CRC cell lines. (C) IL-6-induced methylation of SOCS3 gene in caco-2 cells after 48 h.

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IL-6 is associated with DNMT1 over-expression in CRC

To investigate the possible importance of DNMT1 expression in the pathogenesis of UC–CRC, we investigated biopsies from controls, UC, UC–CRC and sporadic CRC for the expression of IL-6 (shown previously (8)) and DNMT1 (Figure 4A). As expected, IL-6 expression was significantly higher in UC–CRC (P = 0.0002), sporadic CRC (P = 0.0017) and active UC (P < 0.0001) than in normal controls, in agreement with a role of IL-6 in tumor progression in the colon. Furthermore, biopsies from UC–CRC have higher IL-6 expression compared with sporadic CRC (P = 0.006; Figure 4B), in agreement with an important role of IL-6 expression for specifically IBD-associated cancer. Importantly, also DNMT1 expression is significantly higher in UC–CRC (P = 0.020) and active UC (P = 0.004) compared with controls (Figure 4C). There seems to be some correlation between the expression of IL-6 and DNMT1 in the epithelial cells, irrespective of disease phenotype or activity (Spearman r = 0.371, P = 0.002). Taken together, these data support the notion that induction of DNMT1 is an important factor in permitting UC–CRC to develop.

Discussion

The highly increased incidence of CRC associated with chronic inflammatory disorders lumped together under the common denominator IBD is of complex and poorly understood etiology, but a large body of evidence in the corpus of biomedical literature implicates increased IL-6 signaling through STAT3 in this phenomenon (18–20). We previously reported substantial silencing of SOCS3 expression in the inflammation–dysplasia–carcinoma sequence in UC (8), whereas the same situation was not found in CD-dysplasia and CD-CRC patients (Supplementary Figure 1, available at Carcinogenesis Online). Apart from multi-effective immune inducers on SOCS3 expression, the location difference in large and small intestine of these two disease types would also lead the specificity of SOCS3 downregulation exerting the sustained IL-6-dependent STAT3 activation in UC-carcinogenesis. However, limited data could not lead to more clear conclusions. Earlier, it was shown that SOCS3 is an important negative regulator of STAT3 during intestinal inflammation, suggesting that loss of SOCS3 would provoke activation of this pro-oncogenic transcription factor (21). Indeed, we reported that concomitant with the loss of SOCS3, STAT3 activation increased, providing a rational explanation as to why SOCS3 loss is related to an increased propensity to develop UC–CRC. Thus, we proposed that the loss of SOCS3 expression represents a critical event, permissive for the development of UC–CRC, but the molecular mechanism as to why chronic inflammation would provoke the loss of SOCS3 expression remained obscure at most.

In the present study, we demonstrate that SOCS3 downregulation delimits the extent of the dysplastic and cancerous domain in patients...
with UC-dyspalsia and UC-CRC respectively, further supporting SOCS3 downregulation is a critical event here. Furthermore, we provide evidence that in the colonic compartment, the SOCS3 promoter is subject to regulation through methylation. Finally, we show that IL-6 signaling can provoke an increase in methylation of SOCS3 by stimulating increased DNMT1 expression and that results in increased signaling through STAT3. Finally, we provide evidence that DNMT1 is upregulated in vivo in UC and its expression correlates to both the areas with high IL-6 signaling and those which cancer is developed. Hence we propose that under the influence of the prolonged signaling through IL-6, the SOCS3 promoter is methylated releasing signaling towards STAT3 from inhibition and allowing the cancerous process to progress. Thus, DNMT1 emerges as an attractive target for rational strategies aimed at preventing CRC in patients IBD. From our data, it emerges that SOCS3 methylation involves large areas of its promoter (see also Supplementary Figure 2, available at Carcinogenesis Online), as MSP showed strong methylation in three different regions of this promoter. Importantly, one of areas positively identified as being methylated includes the STAT3 binding element, which is considered essential for induction of SOCS3 in response to IL-6 and seen as the main element responsible for limiting the extent of SOCS3 induction in vivo. The relevance of this finding for the cancerous process is illustrated by our observation that absence of SOCS3 expression delimits the dysplastic domain in UC. Thus, it seems that methylation of this particular element might constitute a rate-limiting step in the generation UC-CRC.

In our patient cohort, no specific significant differences between the genders emerged. It has been reported, though, that DNA methylation patterns change with increasing age and these changed methylation pattern contribute to ageing. Specifically, in a study investigating cytosine methylation in the promoter of 80 genes, the degree of methylation showed significant positive correlation with the male sex and with age (22). Also in different neuroendocrine tumours, gene methylation is positively associated with older age of patients and more aggressive manifestation of cancer (23). In our patients, we did detect more methyltransferase expression and SOCS3 gene methylation in older patients, suggesting that age is a factor for the mechanisms described in the present study as well.

IL-6/p-STAT3 signaling in vitro has been shown to stimulate both colonic epithelial cell proliferation and to induce resistance to apoptosis (24). In apparent agreement, in animals genetically devoid of SOCS3 in the colonic mucosa, enhanced sensitivity to repeated dextran sulfate sodium (DSS) challenge is noted with respect to the occurrence of colon carcinoma (25). The current data add to this concept as they provide a mechanistic link to the previous murine data to actual patients. However, the role of STAT3 in colonic physiology is not clear cut. Recent data indicate that STAT3 expression can also inhibit inflammation in the colon through multiple but mainly tolerogenic mechanisms (26). Hence, strategies at increasing SOCS3 expression may be accompanied exacerbation of disease. Disregarding this complication in the clinical management of UC per se, our data do show that preventing SOCS3 promoter methylation may be the way forward for novel strategies aimed at avoiding the development of UC–CRC. Various DNMT inhibitors are currently in clinical testing and might provide a mechanistic link to the previous murine data to actual patients. However, the role of STAT3 in colonic physiology is not clear cut. Recent data indicate that STAT3 expression can also inhibit inflammation in the colon through multiple but mainly tolerogenic mechanisms (26). Hence, strategies at increasing SOCS3 expression may be accompanied exacerbation of disease. Disregarding this complication in the clinical management of UC per se, our data do show that preventing SOCS3 promoter methylation may be the way forward for novel strategies aimed at avoiding the development of UC–CRC. Various DNMT inhibitors are currently in clinical testing and might be interesting to study the effect of these in, for instance, UC patients with PSC co-morbidity as such this way would be a novel rational strategy for dealing with this complication of UC.

Fig. 4. IL-6 and DNMT1 expression and their correlation in patient biopsies. (A) Immunohistochemistry (IHC) staining was used to visualize DNMT1 expression in the biopsies from controls, UC–CRC and active UC. (B) Analysis of the number of IL-6 positive epithelial cells. IL-6 expression is significantly higher in UC–CRC (P = 0.0002), sporadic CRC (P = 0.0017) and active UC (P < 0.0001) than in normal controls. Biopsies from UC–CRC had even higher IL-6 expression than sporadic CRC (P = 0.006). (C) Analysis of DNMT1 positive epithelial cells. DNMT1 expression is significantly higher in UC–CRC (P = 0.020) and active UC (P = 0.004) compared with controls. (D) Overall the expression levels of IL-6 and DNMT1 in all the biopsies show positive correlation (Spearman’s correlation coefficient r = 0.371, P = 0.002).
**Supplementary material**

Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

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**References**


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