IL-8 suppresses E-cadherin expression in nasopharyngeal carcinoma cells by enhancing E-cadherin promoter DNA methylation

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Abstract. Nasopharyngeal carcinoma (NPC) has the highest metastasis potential among head and neck cancers. Distant metastasis is the major cause of treatment failure. Recent studies from our laboratory have revealed that IL-8 promotes NPC metastasis via activation of AKT signaling and induction of epithelial-mesenchymal transition (EMT) in the cells. In the present study, we found that IL-8 treatment for NPC cells resulted in an accumulation of DNMT1 protein through activating AKT1 pathway and consequent DNMT1 protein stabilization. Then DNMT1 suppressed E-cadherin expression by increasing the methylation of its promoter region. LY-294002 blocked IL-8-induced p-AKT1 activation resulting in reduction of DNMT1 and increase of E-cadherin expression, whereas forced demethylation using 5-aza-2'-deoxycytidine restored E-cadherin expression. In conclusion, our study, for the first time, shows that the IL-8/AKT1 signaling pathway stabilizes DNMT1 protein, consequently enhancing hypermethylation of E-cadherin promoter regions and downregulating E-cadherin protein level in NPC cells. Upon blockage of the IL-8/AKT pathway and inhibition of DNMT1, E-cadherin expression can be reversed. These data suggest that targeting the IL-8/AKT1 signaling pathway and DNMT1 may provide a potential therapeutic approach for blocking NPC metastasis.

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Abbreviations: NPC, nasopharyngeal carcinoma; DNMT1, DNA methyltransferase-1 interleukin; 5-aza, 5-aza-2'-deoxycytidine; EMT, epithelial-mesenchymal transition

Key words: interleukin-8, IL-8/AKT1 pathway, DNMT1, E-cadherin, nasopharyngeal carcinoma, hypermethylation

Introduction

Nasopharyngeal carcinoma (NPC) has a high incidence rate in southern China and Southeast Asia, and its metastasis rate is also the highest among head and neck cancers (1-3). The close relationship of Epstein-Barr virus infection suggests NPC as an inflammation-associated cancer (4,5).

Interleukin 8 (IL-8; alternatively known as CXCL8) is a proinflammatory cysteine-X-cysteine (CXC) chemokine, plays multiple roles by mediating the activation and chemotaxis of various immune cell types, to promote immune infiltration and angiogenesis, which in turn establishes a venue for cancer cell local invasion, migration, and metastasis. Studies have shown that IL-8 promotes tumor growth and metastasis in melanoma (6-9), bladder cancer (10), and ovarian cancer (11). We have also previously demonstrated that the overexpression of IL-8 in nasopharyngeal carcinoma cell line S26 cells and HONE-1 cells activated AKT1 signaling and induced EMT (12). In recent years, it has also been demonstrated that a link exists between IL-8 and tumor EMT, which involve decreased expression of epithelial markers such as E-cadherin in lung cancer (13), hepatocellular carcinoma (14,15) and thyroid cancer (16).

E-cadherin is a key mediator of cell-cell adhesion in epithelial tissues, and loss of E-cadherin can promote invasive and metastatic behavior in many epithelial tumors (17). In head and neck cancers, loss of cell-cell adhesion resulting in stromal and vascular invasion as a consequence of E-cadherin dysregulation is well documented (18,19).

It has been suggested that DNA methylation plays a major role in enhancing transcriptional silence, especially in tumor suppressor genes (20). E-cadherin also could be suppressed by DNA hypermethylation and has a close relationship with tumor prognosis in head and neck squamous cell carcinoma (21), breast cancer (22), lung cancer (23), bladder cancer (24).

DNA methyltransferases (DNMTs) are responsible for the transfer of a methyl group from the universal methyl donor, S-adenosyl-L-methionine, to the 5-position of the cytosine residue in DNA. There are four members of the DNMT family: DNMT1, DNMT3A, DNMT3B and DNMT3L. DNMT3A and DNMT3B encode *de novo* methyltransferases, while DNMT1 encodes a maintenance methyltransferases, which are essential for mammalian development and reported to be associated with human tumorigenesis (25).

DNMT1 stability is regulated via various post-translational modifications, previous studies showed that AKT1 can stabilizes DNMT1 and affects genome methylation (26,27). IL-8 can activate AKT1 pathway to promote tumor invasion in breast cancer (28), kidney cancer (29), and breast cancer (30). Our previous study also showed that in NPC, the overexpression of IL-8 can induce EMT through activating the AKT signaling pathway (12). However, to the best of our knowledge, there are no studies regarding the relationship between IL-8 and DNMT1 expression, and though our previous study showed IL-8 can reduce E-cadherin expression in NPC cell lines (12), the underlying molecular mechanisms remain unclear. In this study, we explore the possibility that in NPC cell lines, IL-8 can induce DNMT1 expression through AKT1 signaling then mediates silencing of E-cadherin expression which play an important role in EMT.

Materials and methods

Cell lines and cell culture. The human low-metastasis, low endogenous IL-8 secreating level NPC cell lines CNE-2 and the CNE-2 subclones S22 and S26 have previously been established and reported (12,31), the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum purchased from Gibco/BRL (Grand Island, NY, USA), 100 U/ml penicillin G, 100 U of streptomycin (all from Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Based on our previous study (12), recombinant human IL-8 (PeproTech) at a concentration of 8 ng/ml was added into cell culture medium in experimental groups. For vehicle controls, the cells were treated with equivalent amounts of BSA.

Immunoblotting. Immunoblotting was performed as described previously (12). The sources of the primary antibodies (and their concentrations) were as follows: anti-E-cadherin (1:1,000), anti-AKT (1:1,000), anti-phospho-AKT (Ser473) (1:1,000), and anti-β-actin (1:1,000) these antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA); anti-DNMT1 (1:1,000) was purchased from Santa Cruz Biotechnology (Santa Cruz). Anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Promega. For western blot analysis of AKT1 signaling inhibition studies, LY-294002 (the AKT1 inhibitor) (Cell Signaling Technology) (20 μM) was used one hour prior to IL-8 stimulation in an attempt to inhibit the conventional AKT1 pathway, for western blot analysis of DNMT1 inhibition studies, 5-aza-2'-deoxycytidine (the inhibitor of DNA methylation) (Sigma) (5 μM) was used in an attempt to inhibit DNMT1 function. Cells were pretreated with 5-aza-2'-deoxycytidine for 48 h then were treated with IL-8 for 24 h. Experiments were repeated in triplicate.

Quantitative real-time polymerase chain reaction. Total cellular RNA was extracted using the High Pure RNA kit (Roche Applied Science, Penzberg, Germany). For RT-PCR, the total RNA was quantified spectrophotometrically, and equal amounts (1 μg) were transcribed into cDNA according to the manufacturer's protocol (Roche Applied Science). The sequences of the PCR primers used for amplifications of β-actin, IL-8, DNMT1, E-cadherin were as follows: β-actin forward: 5'-CACGATGGAGGGGCCGGACTCATC-3'; β-actin reverse, 5'-TAAAGACCTCTATGCCAACACAGT-3'. IL-8 forward, 5'-CTCCAAACCTTTCCACCCC-3'; IL-8 reverse, 5'-GATTCTTGGATACCACAGAGAATG-3'. DNMT1 forward, 5'-AGCCAAATCGGATGAGTCCATC-3'; DNMT1 reverse, 5'-CCTCCTTCAGTTTCTGTTTGGG-3'. E-cadherin forward, 5'-AACAGGATGGCTGAAGGTGA-3'; E-cadherin reverse, 5'-CCTTCCATGACAGACCCCTT-3'. Fast SYBR Green Master Mix was used to determine the threshold cycle (Ct) value of each sample in the CFX96 real-time PCR detection system (Bio-Rad, CA, USA). β-actin served as the normalization gene in these studies. The relative expression levels of the target genes were given by 2ΔCt (Ct of β-actin minus the Ct of the target gene). PCR amplifications of β-actin, IL-8, E-cadherin, DNMT1 were performed under the following conditions: denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec, and extension at 72˚C for 30 sec; reactions were carried out for 30 cycles. The experiments were performed in triplicate.

Transient transfection of IL-8. For IL-8 overexpression studies, the IL-8 overexpressing vector (cat no. CH832510) and control pENTER vector were purchased from ViGene Biosciences Inc. (Rockville, MD, USA). These plasmids were transfected into S22, S26, CNE2 cells with X-tremeGENE HP transfection reagent according to the manufacturer's instructions (Roche Life Science). The cells were harvest 48 h after transfection for immunoblot and real time-PCR analyses.

ELISA. Twenty-four hours after IL-8 overexpressing plasmids or control pENTER vector were transfected into S22, S26, CNE2 cells with X-tremeGENE HP transfection reagent, $2x10^6$ of the cells were plated into 100-mm culture plates and incubated for another 24 h in the regular medium. The medium was then replaced with a serum-free medium (10 ml) and the cells were incubated for an additional 12 h. Conditioned medium was collected and subjected to centrifugation, followed by filtration through a 0.45 μm membrane filter to remove the debris. Secreted human IL-8 concentration in the conditioned medium was then measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D, MN, USA) following the manufacturer's instructions. The experiments were performed in triplicate.

BSP (bisulfite sequencing PCR). Bisulfite sequencing PCR was used to examine the methylation status of E-cadherin gene promoter. Genomic DNA from cultured cells were extracted according to the manufacturer's protocol (Qiagen 51304), followed by bisulfite conversion using EpiTect Bisulfite kit (Qiagen 59104). DNA amplification was performed via PCR using primers as follows: E-cadherin-BSP-F: 5'-TGTAGGT TTTATAATTTATTTAGAT-3'; E-cadherin-BSP-R: 5'-CTCA

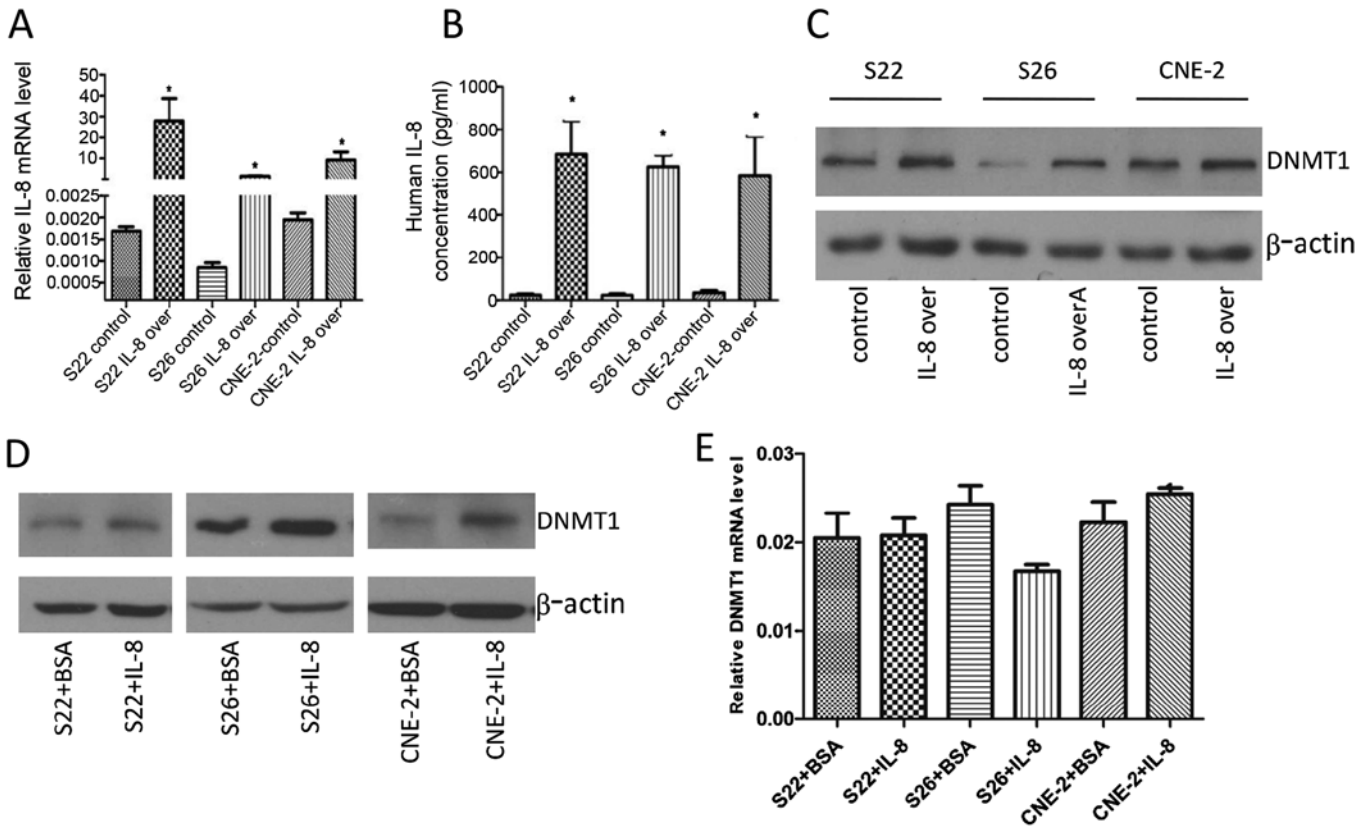

Figure 1. Treatment with IL-8 induces DNMT1 expression in nasopharyngeal cancer cells. (A) Transient transfections of IL-8 in S22, S26, CNE-2 cells. IL-8 mRNA levels were assessed by reverse transcription-PCR. (B) Concentrations of secreted IL-8 in the conditioned media measured by ELISA. (C) Transient transfections of IL-8 in S22, S26, CNE-2 cells, DNMT1 levels were assessed by western blotting 48 h after transfections. (D) S22, S26, CNE-2 cells were treated with 8 ng/ml of IL-8 for 24 h and DNMT1 levels were assessed by western blotting. (E) S22, S26, and CNE-2 cells were treated with 8 ng/ml of IL-8 for 24 h and DNMT1 mRNA levels were assessed by reverse transcription-PCR.

CAAATACTTTACAATT-3'. PCR products were cloned to pTopo TA vector (Invitrogen), and then transfected to Top10 cells for sequencing, at least ten clones from each sample were selected.

Statistics. Student's t-test was used to compare two independent groups of data. A P-value <0.05 was considered statistically significant.

Results

IL-8 treatment induces DNMT1 expression in nasopharyngeal cancer cells. Our previous study showed that IL-8 is lowly expressed in low-metastasis S26 cells (12). In the present study, we investigated whether IL-8 could enhance the expression of DNMT1 by transient transfection of IL-8 plasmid. After transient transfection of IL-8 plasmid in S22, S26, and CNE-2 cells, real-time PCR analysis showed that IL-8 mRNA levels were increased greatly 48 h later (Fig. 1A), and ELISA analysis showed high levels of IL-8 were secreted into the culture medium by the cells (Fig. 1B). Then immunoblotting of the nuclear extracts showed an increase in DNMT1 protein expression 48 h after transient transfections (Fig. 1C). Next, we used exogenous recombinant human IL-8 to verify this finding. Consistently, DNMT1 protein level was increased in the S22, S26, CNE-2 cells following IL-8 treatment with 8 ng/ml for 24 h (Fig. 1D). We next analyzed DNMT1 mRNA expression using real-time PCR, and found that DNMT1 mRNA levels were not significantly altered in these cells (Fig. 1E), suggesting that IL-8-induced accumulation of DNMT1 protein was not due to mRNA overexpression.

IL-8 enhances DNMT1 stabilization through AKT1 signaling. DNMT1 protein stability can be regulated via various post-translational modifications. It has been reported that AKT1 activity can stabilize DNMT1 protein (26,27). Our previous study showed that IL-8 can stimulate AKT1 pathway in NPC cells (12), therefore, we wanted to know whether the IL-8-induced accumulation of DNMT1 protein is partly due to AKT1 signaling. After transient transfection of IL-8, immunoblotting of nuclear extracts showed an increase in p-AKT1 protein expression in S22, S26, and CNE-2 cells (Fig. 2A). Then S22, S26, CNE-2 cells were treated with IL-8 at 8 ng/ml, and total AKT1 and phosphorylated AKT1 levels were tested by immunoblotting. The results showed that treatment of cells with IL-8 lead to activation of the AKT1 pathway as expected. To confirm that the increase of DNMT1 is AKT1-dependent, we treated cultured S22, S26, CNE-2 cells with the AKT1 inhibitor LY294002 and then measured DNMT1 protein levels. LY294002 blocked pAKT1 activation triggered by IL-8 and resulted in reduction of total DNMT1 (Fig. 2B). To confirm DNMT1 protein stabilization, we treated S26 cells

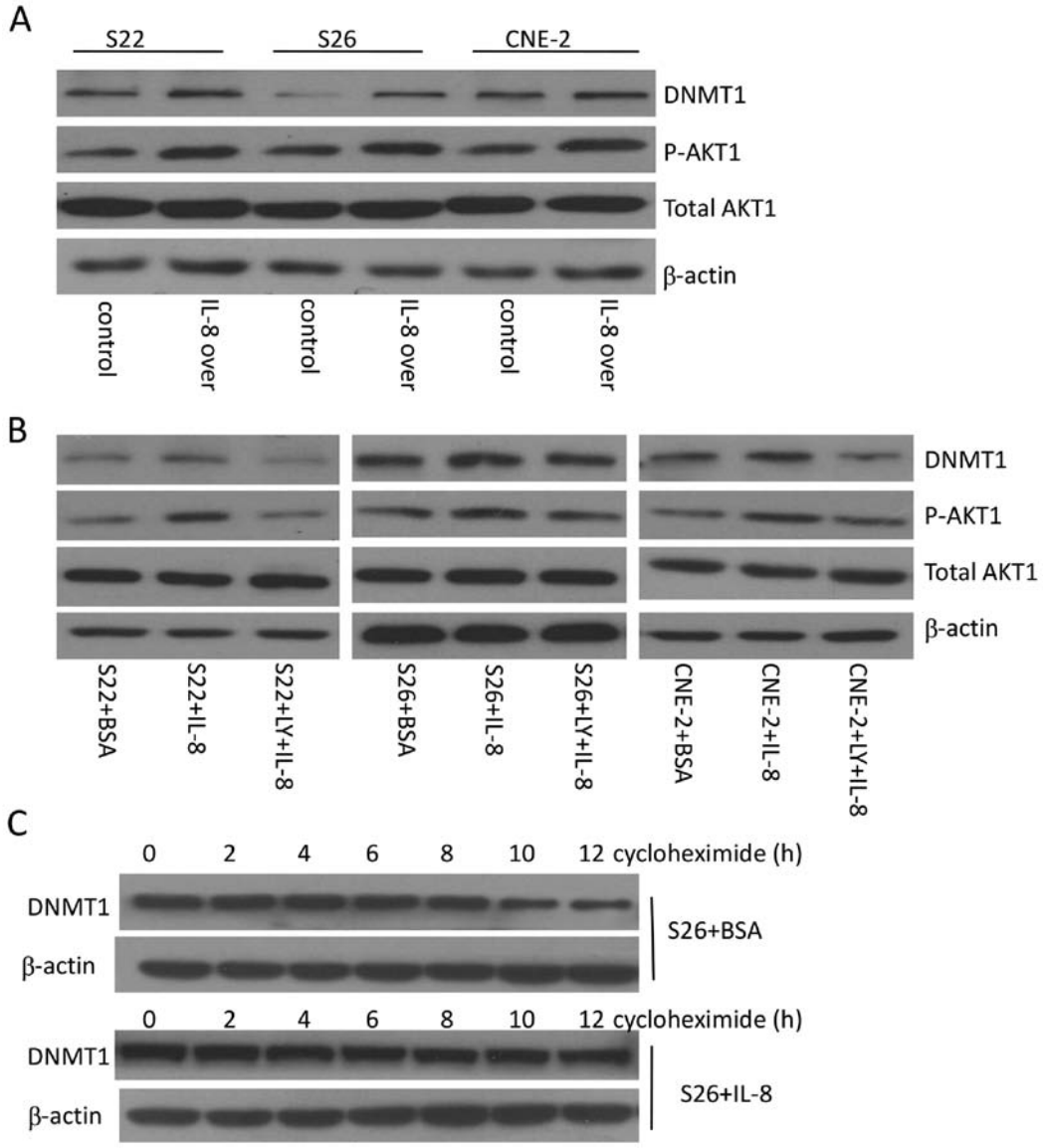

Figure 2. IL-8 increases DNMT1 expression due to stabilization of DNMT1 protein via AKT pathway. (A) S22, S26, CNE-2 cells were treated with 8 ng/ml of IL-8 for 24 h, p-AKT1 levels were assessed by western blotting. (B) S22, S26, and CNE-2 cells were treated with 8 ng/ml of IL-8 for 24 h after pretreatment with 20 μM LY294002 for 1 h. DNMT1, p-AKT1, total AKT1 levels were detected by immunoblotting. (C) Cycloheximide (20 μg/ml) was added to the medium in the presence or absence of IL-8 (8 ng/ml). After the addition of cycloheximide, cells were harvested at the time-points indicated and subjected to western blotting for DNMT1.

with the protein synthesis inhibitor cycloheximide to block the synthesis of DNMT1. Immunoblot analysis showed that DNMT1 levels gradually decreased in 10 h following the addition of cycloheximide to culture media without IL-8. However, the degradation of DNMT1 was slowed down by the addition of IL-8 to the medium, indicating that IL-8 stabilizes DNMT1 protein, an effect persisting for ≥12 h (Fig. 2C). Therefore, we conclude that IL-8 upregulates DNMT1 protein level through activating AKT1 pathway and enhancing DNMT1 stabilization.

IL-8 treatment of S26 cells increases E-cadherin level via DNMT1. Our previous study shows that overexpression of IL-8 in S26 cells can induce EMT with downregulation of E-cadherin (12). In the present study, we continued to explore whether this downregulation of E-cadherin level is partly due to upregulation of DNMT1. Immunoblot analysis showed that IL-8 inhibited the expression of E-cadherin in S26 cell lines and also decreased the transcription of E-cadherin (Fig. 3A and B). As inhibiting AKT1 reduced the overall DNMT1 protein levels, we expected that treating the cells with LY294002 would result in upregulated E-cadherin expression. Then, S26 cells were treated with IL-8 for 24 h, while in the presence of LY-294002, an inhibitor of the AKT pathway, which was added one hour before the addition of IL-8, immunoblot analysis showed that LY-294002 blocked IL-8-induced pAKT1 activation and resulted in reduction of DNMT1 as well as an increase of E-cadherin protein level. Many studies have demonstrated that promoter methylation of E-cadherin is an important mechanism contributing to its downregulation (33). We speculate that methylation of the E-cadherin promoter may play an important role in IL-8 mediated decrease of this gene.

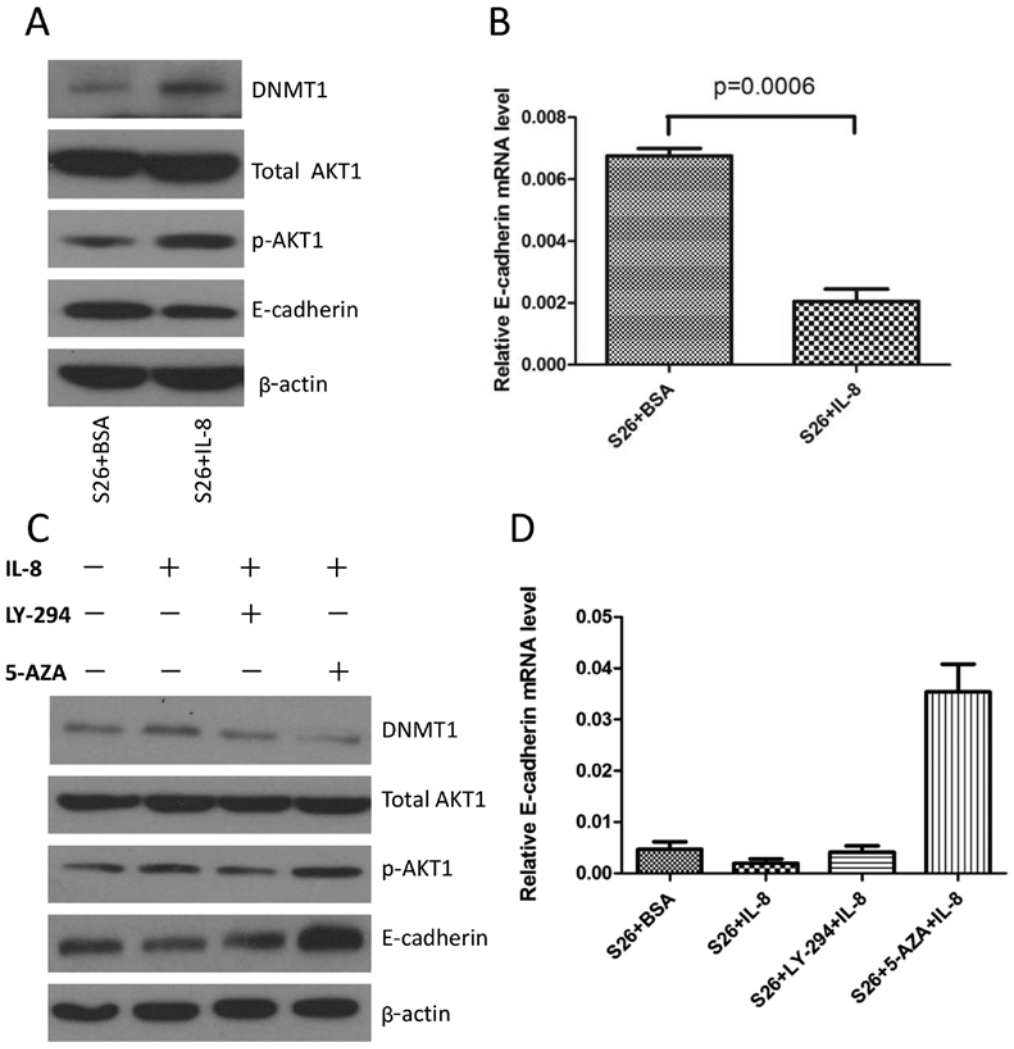

Figure 3. IL-8 downregulates E-cadherin protein level by upregulating DNMT1. (A) S26 cells were treated with 8 ng/ml of IL-8 for 24 h and E-cadherin levels were assessed by western blotting. (B) S26 cells were treated with 8 ng/ml of IL-8 for 24 h and E-cadherin mRNA levels were assessed by RT-PCR. (C) S26 cells were pretreated with 20 μM LY294002 for 30 min or control, then stimulated with 8 ng/ml of IL-8 or control, 5-aza were added 48 h before IL-8 stimulated, DNMT1, total AKT1, p-AKT1, E-cadherin level were tested by western blotting. (D) S26 cells were pretreated with 20 μM LY294002 for 30 min or control, then stimulated with 8 ng/ml of IL-8 or control, 5-aza was added 48 h before IL-8 stimulation. E-cadherin mRNA levels were assessed by RT-PCR.

In addition, while in the presence of 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation, which was added 48 h before IL-8, and IL-8-induced downregulation of E-cadherin was reversed (Fig. 3C). The RNA level of E-cadherin also showed consistent changes (Fig. 3D), suggesting that the effect of IL-8 on E-cadherin expression is transcriptionally regulated via DNMT1.

IL-8 downregulates E-cadherin expression by promoting its promoter methylation via DNMT1. Next, in order to verify that the downregulation of E-cadherin was partly due to its promoter methylation, the bisulfite sequencing PCR of E-cadherin promoter analysis was performed, the methylated cytosine residues (ranging from -280 to +40 of E-cadherin promoter) in each clone were represented by a solid spot as depicted (Fig. 4). In total, 17 CpG methylation sites were detected. Bisulfite sequencing PCR assay of the E-cadherin promoter showed that the promoter CpG island methylation status of E-cadherin in IL-8 treated cells was markedly higher than control cells (Fig. 4). In addition, when LY-294002 or 5-aza-2'-deoxycytidine was added for 48 h together with IL-8 administration, the methylation status of E-cadherin reversed to the control level. These data demonstrated that IL-8 could induce hypermethylation on the specific CpG sites of E-cadherin promoter.

Discussion

The influence of inflammation on tumorigenesis has been intensively investigated for centuries (34). In addition, the expression of IL-8 in the tumor microenvironment is reported to be associated with tumor progression and patient survival (35). We have previously reported that IL-8 promotes metastasis of NPC cells in autocrine and paracrine manner, involving activation of AKT signaling and inducing EMT in NPC cells (12). In the present study, we further demonstrated for the first time that IL-8 inhibited the expression of E-cadherin by inducing its promoter DNA hypermethylation via upregulating

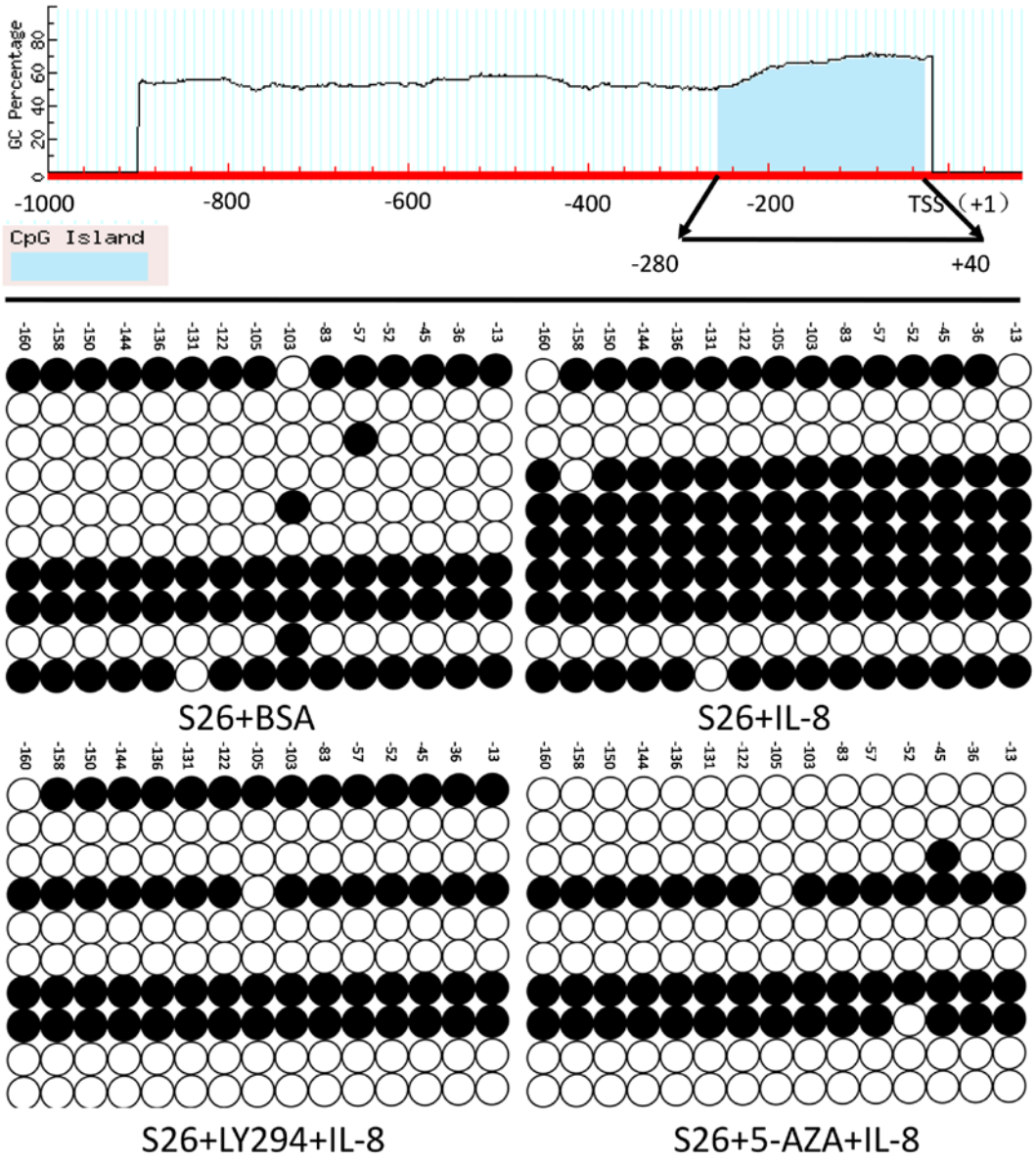

Figure 4. IL-8 downregulates E-cadherin protein level via methylation of E-cadherin promoter region. Bisulfite sequencing PCR was used to examine the methylation status of E-cadherin. The methylated cytosine residues are represented by a solid spot. Upper left, S26 cells; upper right, S26 cells stimulated with 8 ng/ml of IL-8 for 48 h; lower left, S26 cells stimulated with 8 ng/ml of IL-8 and 20 μM LY294002 for 48 h; lower right, S26 cells stimulated with 8 ng/ml of IL-8 and 5 μM of 5-aza for 48 h.

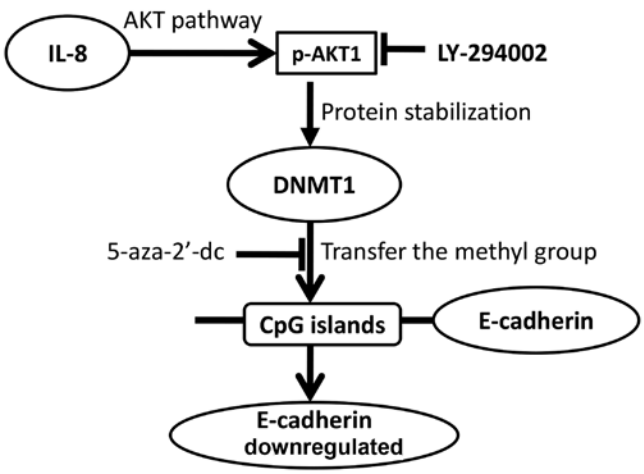

Figure 5. The model of IL-8-p-AKT1-DNMT1 signaling pathway.

DNMT1 protein level. Blockage of the IL-8/AKT pathway and inhibition of DNMT1 reversed the expression of E-cadherin. These data demonstrate a link between inflammation and NPC progression involving epigenetic regulation of E-cadherin.

Dysregulation of DNMT1 activity causes many human diseases, including cancer (36). Post-translational modifications of DNMT1 play a crucial role in how and when it is activated. Other studies have reported that AKT activity can inhibit DNMT1 degradation in multiple cell lines (26,27). IL-8 signals through CXCR1 and CXCR2, and phosphatidylinositol-3 (PI3) is a component in CXCR1/2-signaling. The enzyme PI3-kinase (PI3K) is a principal effector of CXCL8-mediated chemotaxis in neutrophils, and its triggering phosphorylation results in the activation and increased expression of AKT (28,32,37,38). However, to our knowledge, our study is the first one to clarify the relationship between

IL-8 and DNMT1. Our findings showed that IL-8 upregulated DNMT1 though AKT1 pathway by increasing its stability in NPC cells. This increment of DNMT1 by IL-8 stimulation can be eliminated by blocking AKT signaling, confirming that AKT is a key signaling pathway by which IL-8 regulates DNMT1. Upon DNMT1 activation, both transcriptional and translational levels of E-cadherin are reduced. We therefore confirmed E-cadherin as one of the target genes of DNMT1 is regulated by IL-8/AKT signaling. The important roles of E-cadherin in NPC progression relies on its functions as an adhesion molecule regulating cell-cell contact for tissue morphogenesis, cellular polarity and tumor invasiveness (17). It has been reported that loss of membranous E-cadherin expression results in enhanced cell migration activity (39), which significantly correlates with tumor invasion, advanced disease stage, and tumor metastasis (40).

A model for this IL-8-p-AKT1-DNMT1 signaling is then proposed based on our findings (Fig. 5).our data clearly show that the IL-8-activated AKT signaling results in stabilization of DNMT1 and E-cadherin epigenetic regulation. This is the first report to reveal the IL-8-mediated E-cadherin silencing mechanism in NPC. These data suggest that targeting IL-8 signaling is a promising approach for prevention and treatment of NPC metastasis.

Acknowledgements

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