Original Article

TNF-α-Induced NF-κB Alter the Methylation Status of Some Stemness Genes in HT-29 Human Colon Cancer Cell

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Abstract

Background: Acquisition of stem-like properties requires overcoming the epigenetic barrier of differentiation and re-expression of several genes involved in stemness and the cell cycle. DNA methylation is the classic epigenetic mechanism for de/differentiation. The writers and erasers of DNA methylation are not site-specific enzymes for altering specific gene methylation. Thus, the aim of the present study is investigation of the *in vitro* interaction of ten eleven translocations (TETs) with nuclear factor kappa B (NF-κB) in hypomethylation of stemness genes.

Materials and Methods: This experimental study was performed on HT-29 cells as human colorectal cancer cell lines. The interaction between TETs and DNA-methyltransferases 3 beta (DNMT3s) with p65 was achieved by coimmunoprecipitation. TETs were knocked down using siRNA, and the efficacy was analyzed by reverse-transcriptase polymerase chain reaction. The promoter methylation status of the target genes (NANOG, MYC) was determined by the methylation-sensitive high-resolution melting method.

Results: TET3 and DNMT3b functionally interacted with p65 in samples through 25 ng/ml TNF-α treatment for 48 h in HT-29 cells. Transfection with siRNA significantly decreased the expression of TET enzymes after 72 h. Interestingly, treatment with TET siRNAs enhanced methylation of MYC and NANOG genes in samples with 25 ng/ml TNF-α treatment for 72 h in HT-29 cells. Moreover, methylation effects of TET3 were stronger than those of TET1 and TET2.

Conclusions: These results suggest that inflammation may alter the methylation status of genes required for stemness and predispose the cells to neoplastic alterations.

Keywords: Methylation, NF-kappa B, translocation, tumor necrosis factor-alpha

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Introduction

Colorectal cancer is a leading mortality factor worldwide despite the fact that several studies have been conducted on the molecular mechanism of cancer initiation and new medical treatment strategies.[1] According to World Health Organization, 1,800,000 new cases and 862,000 deaths have been recorded in 2018 due to colorectal cancer. Colon and intestinal epithelia have a highly regenerative capacity, and hence, there is an inherent susceptibility to neoplastic alterations.[2]

Regardless of controversies about the cellular origin of cancer, it has been accepted that differentiation of stem cells

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to progenitors and subsequently to terminally differentiated somatic cells is not a one-way road in physiological and pathological conditions.[3] Dedifferentiation is a process through which differentiated cells return into the cell cycle and hence become susceptible to preneoplastic alterations.[4] The comeback to the stem-like state needs re-expression of some suppressed genes in differentiated cells by epigenetic approaches such as DNA methylation.[5]

Chronic inflammation is a hallmark of cancer, which increases the risk of colorectal tumorigenesis associated with prominent activation of nuclear factor kappa B (NF-κB) signaling.[6,7] There is mounting evidence about the role of NF-κB signaling in cancer initiation[8,9]; however, there are no data on the role of inflammation related to NF-κB activation in transformation of differentiated intestinal epithelial cells.

It has been previously observed that samples derived from the colon of healthy obese men have a decreased *NANOG* promoter methylation; *NANOG* controls stemness in embryonic stem cells. Inflammation-associated NF-κB activation may be involved in hypomethylation of *NANOG* promoter.[10] There is also evidence that some transcription factors influence the methylation/demethylation status of gene promoters via recruiting DNMTs (DNA methyl transferases) or TETs (ten eleven translocations) as coregulators.[11,12] If so, inflammation-induced NF-κB activation may play a role in DNA demethylation of some oncogenic genes (including the stemness genes) to predispose the corresponding cells for re-entering into the cell cycle. Our previous observation suggested that *NANOG*, *POU5F1*, and *MYC* genes have NF-κB consensus binding sites in their promoters, but we could not directly show the interaction of NF-κB and TETs.[10] Other studies also suggest that NF-κB may be involved in dedifferentiation of intestinal epithelial cells.[13,14] NANOG has been considered as a tumor cell stemness marker with its downregulation attenuated colon cancer properties.[15] Also, a recent study revealed that ablation of NANOG impaired stemness of colon cancer cells.[16] Thus, the aim of this work was to look at the role of TET interaction with NF-κB in hypomethylation of stemness genes (NANOG, MYC) *in vitro* using a low-grade human colon cancer cell line. In this regard, human colon adenocarcinoma cell line HT-29 was incubated with TNF-α as a model of obesity-related inflammation for 48 h to analyze the probability of the tumor necrosis factor-alpha (TNF-α)-induced NF-κB activation role in decreasing methylation of MYC promoter genes in addition to NANOG promoter.

Materials and Methods

Cell culture

In this experimental study, HT-29 cells were purchased from the National Cell bank of Iran (Pasteur Institute, Iran) and cultured in Dulbecco's modified Eagle medium (DMEM) (Biosera, Inc., Loire Valley, France) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine under standard conditions. The culture medium was replaced twice a week. When HT-29 cells reached 80% confluency, they were treated with TNF-α (25 ng/ml for 72 hours). The cells were harvested by ethylenediamine tetraacetic acid (EDTA)-trypsin.

Coimmunoprecipitation

To analyze the interaction between p65 and DNMT3s (DNMT3a, DNMT3b) or TETs (TET1, TET2, TET3), TNF-α-treated HT-29 cells (for 48 h) were harvested and lysed with radioimmunoprecipitation assay buffer (RIPA) buffer (Santa Cruz Biotechnology Inc., CA) on ice with occasional vortexing. After centrifuging the lysate (14,000 rpm and 4°C), the lysates containing 700 µg protein were immunoprecipitated using 1.5 µg anti-NF-κB p65 (D14E12) (Cell Signaling MA, USA) overnight at 4°C with rotation. After removing the insoluble material by centrifugation, the lysates were subjected to 50 µL of settled Immobilized Protein A/G agarose (100 µL) (Santa Cruz Biotechnology Inc., CA) overnight at 4°C. The beads were then resuspended in NP-40 buffer, boiled for 10 min, and subjected to immunoblot analysis using anti-TETs and anti-DNMT3b primary antibodies.

Gene silencing using RNA interference (siRNA)

HT-29 cells were transfected with combined siRNA against TET1, 2, and 3 (Santa Cruz Biotechnology Inc., CA). Each siRNA generally contains pools of three to five target-specific 19–25 nucleotides. Briefly, 2×10^5 cells were seeded in a six-well plate with 2 ml antibiotic-free normal growth medium supplemented with FBS. The cells were incubated until they reached 80% confluency. Approximately 1 µg from each siRNA was diluted into 100 µl siRNA transfection medium (solution A). Afterward, 2–8 µl of siRNA transfection reagent was diluted into 100 µl siRNA transfection medium (solution B). Both solutions were mixed and remained at room temperature for 45 min. The cells were harvested and washed using 2 ml transfection medium, and the mixed solution was added to cells. The cells were then put in the incubator for 8 h. Later, the fresh medium supplemented with 20% FBS and antibiotic was added to cells without removing the transfection mixture. After 24 h, the medium containing 10% FBS was refreshed, and transfection analysis was performed after 72 h.

Gene expression analysis by quantitative real-time RT-PCR

To analyze the efficacy of TET knockdown using siRNA, reverse-transcriptase polymerase chain reaction (PCR) was performed on HT-29 cells transfected with a combination of siRNA against TETs. Total RNA was extracted using an RNA extraction kit (Sinaclon Bioscience, Iran) according to the manufacturer's protocol. One µg of total RNA was transcribed to cDNA using a cDNA synthesis kit (Vivantis Technologies, Malaysia). TET genes were amplified with MasterMix plus SYBER green (Sinaclon Bioscience, Iran) according to the manufacturer's procedure. The expression of TET genes was calculated relative to GAPDH as the internal control in each sample, and the data were presented by the ΔΔCt method.

DNA extraction and sodium bisulfite modification

Treated cultured cells were subjected to DNA extraction using an EZ-10 spin column animal DNA mini-preps kit (Bio Basic Inc.). Following this step, an EZ DNA methylation-gold kit (Zymo Research) was used to mediate sodium bisulfite conversion of the total genomic DNA. Hence, the unmethylated cytosines were converted to uracils, while the methylated ones remained intact. The modified samples were subsequently stored at -80°C for further analysis.

Primer design

The primers used in HRM were specifically designed to complement CpG islands in the promoter region of selected genes in accordance with HRM primer design guideline. Specifically, closeness to the NF-kB binding site was considered while designing primers. NF-kB binds to the kB binding site (5-GGGRNYYYCC-3) and exerts its role as a transcriptional activator or repressor. To analyze NF-kB in human NANOG and MYC, Softberry (http://www.softberry.com/berry.phtml?topic = nsite and group = programs and subgroup = promoter) and MatIn-spector programs (http://www.genomatix.de) were used.

Methylation-sensitive high-resolution melting

The methylation status of target genes (NANOG, MYC) was determined by the HRM method. Therefore, 1 ul bisulfite-modified template along with 4 ul of 5x hot FIREPOL Eva Green HRM mix-Rox kit (Solis BioDyne) and primers (0.3 pmol) were mixed, and a final 20 ul volume was obtained using double-distilled water to perform PCR. All test tubes were prepared as duplicates. At first, to perform PCR, the samples were denatured for 15 minutes at 95°C for one cycle, followed by annealing for 15 s at 95°C. Then, the extension step was performed at 72°C for 20 s for 45 cycles. The next step was HRM, which involved treating the samples at 95°C for 1 minute, 40°C for 1 minute, and 74°C for 5 s and continuous acquisition to 90°C at 25 acquisitions per 1°C performed by StepOnePlus, Applied Biosystem. A DNA sample that was not modified by bisulfate served as the negative control. To set the completely methylated (100%) or unmethylated (0%) status, commercial samples were purchased from Zymo Research. To analyze the methylation percentage of unknown samples, a standard curve was generated for NANOG using 0, 50, and 100% methylated samples, while for MYC, the standard curve was drawn using 0 and 100%. To determine the melting temperature of PCR product by HRM version 2.2 software (Thermo Fischer Scientific), the regions before and after major fluorescent decrease were used to create the normalized melting curves.

Statistical analysis

Statistical tests were performed using SPSS software (version 16.0). $P \leq 0.05$ was considered as the significance level, and independent-samples *t*-test was used. Where the distribution of continuous variables was not normal, the Mann–Whitney U test was employed. Gene methylation was assessed as medians based on the distribution among controls. Regression analysis was also performed as needed.

Results

TET3 and DNMT3b functionally interact with p65 in HT-29 cells incubated with TNF-α

To demonstrate physical interactions between TETs and DNMTs with NF-κB/p65, the coimmunoprecipitation method was utilized using p65 antibody. It has been previously shown that NF-κB induction in HT-29 cells decreases the methylation of some embryonic genes in their promoter regions.[10] Therefore, it was hypothesized that NF-κB interacts with TET enzyme family. As shown in Figure 1, coimmunoprecipitation of total HT-29 cell lysate revealed that TET3 interacts with NF-κB/p65 in samples with TNF-α treatment for 48 h. Concomitantly, NF-κB was bound to DNMT3b in HT-29 cells [Figure 1]. The results of the present study did not show the interaction of other TET or DNMT family proteins with NF-κB/p65.

NF-κB-induced hypomethylation of NANOG and MYC modulated by downregulation of TETs

It has recently been shown that the treatment of HT-29 cells with TNF-α to induce NF-κB promotes the hypomethylation of NANOG and cMYC genes in their promoter just close to NF-κB binding sites. Promoter hypomethylation is often associated with gene expression. Since it was hypothesized that TETs mediate hypomethylation of oncogenic and embryonic genes induced by NF-κB, HT-29 cells with transfected siRNA (80 pmol) repress TET1, 2, and 3 genes for 72 h. As shown in Figure 2a, real-time PCR indicated that TET knockdown for 72 h markedly reduced the mRNA expression of all TETs. This reduction was 43% and 39% for TET1 and TET 2, respectively, and it was 51% for TET3 in comparison to control [Figure 2a].

Methylation-sensitive high-resolution melting (MS-HRM) revealed that promoter hypomethylation of MYC gene was induced by TNF-α, 25 ng/ml for 72 h, which also was decreased by knockdown of TET expression in HT-29 cells. TET3 was more effective than TET1 and TET2 in hypomethylation of MYC gene, respectively [Figure 2b].

As shown in Figure 2c, the hypomethylation of NANOG induced by TNFα was markedly decreased in cells transfected

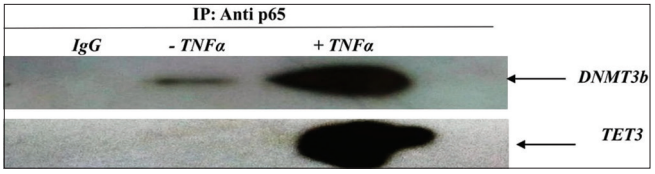

Figure 1: Coimmunoprecipitation of p65 with TET3 and DNMT3b. HT-29 cells were either incubated by TNF-α (+TNF, 10 ng/ml) or without TNF-α (-TNF) for 48 h, and lysates were incubated with p65 antiserum. The anti-p65/NF-κB complex is then pulled out of the sample by protein A/G-coupled agarose beads. The immune complexes were analyzed by immunoblotting with TET1, 2, and 3 or DNMT3b primary antibodies

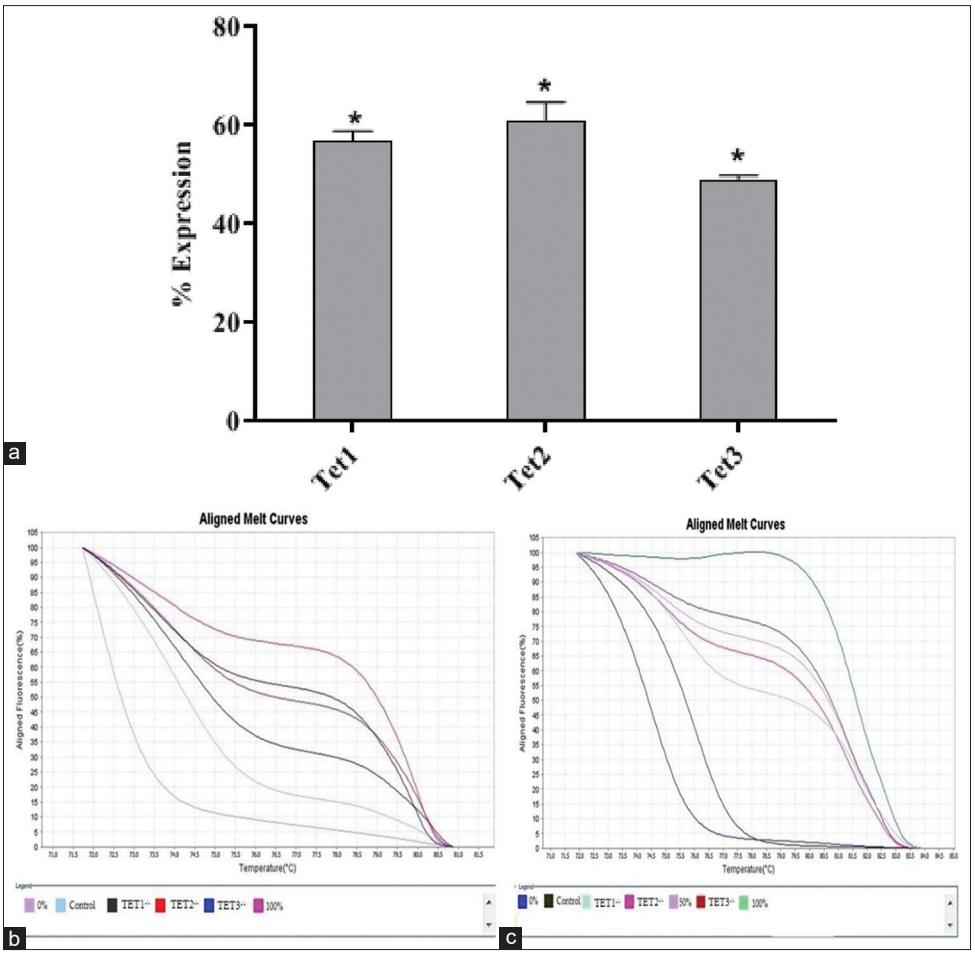

Figure 2: (a) HT-29 cells transfected with a combination of siRNA against TET1, 2, and 3 according to the manufacturer's protocol. After 72 h, the cells were harvested and RNA was extracted. One μg total RNA was subjected to cDNA synthesis, and then quantitative-PCR was performed using TET primers. Gene expression was calculated relative to GAPDH expression using ΔΔct method. * denotes statistically significant difference compared to control cells. (b and c) Methylation-sensitive high-resolution melting curve for *cMYC* (b) and *NANOG* (c) genes. HT-29 cells with downregulation of TET genes were subjected to bisulfite modification and then analyzed with HRM real-time PCR using primers designed for promoters of desired genes near a specific NF-κB binding site

by TETs. A major hypermethylation effect was seen in cells transfected by TET3 siRNA in comparison to negative siRNA control, and a weaker effect was seen for TET1. Knockdown of TET2 mRNA had a moderate effect on modulation of NF-κB-induced hypomethylation of NANOG genes.

Discussion

Our previous study showed that NF-κB activation induced by TNF-α promotes hypomethylation of stemness genes such as NANOG, POU5F1, and MYC in HT-29 colon cancer cells.[10] In the present study, experimental evidence was provided regarding the fact that DNA methylation and demethylation enzymes can bind to NF-κB. Moreover, downregulation of TET enzymes decreased the hypomethylation of NF-κB-mediated stemness genes.

At present, it is widely accepted that chronic inflammation is one of the major risk factors for tumorigenesis. Evidence clearly confirms the role of inflammation in cancer initiation, including Helicobacter pylori infection/stomach cancer, viral hepatitis/hepatocellular carcinoma, and inflammatory bowel disease/colorectal cancer.[17] Also, a major mechanism proposed for obesity-associated cancers is inflammation accompanied by accumulation of adipose tissue.[18,19] After activation of a pattern recognition receptor, innate immune cells recruit NF-κB transcription factor to express proinflammatory cytokines.[20] NF-κB has a crucial role in linking inflammation and cancer. In response to TNF-α treatment, NF-κB translocates to the nucleus and promotes the expression of inflammatory-responsive genes through TNF receptor.[6,21] Our previous data showed that TNF-α treatment of HT-29 cells increases the canonical pathway of NF-κB activation.[22] Many studies revealed that transcription factors recruit histone-modifying enzymes as coactivators to open chromatin structure and promote transcription.[23] P300/CBP transcription factor shows histone acetyl transferase activity during transactivation. Furthermore, corepressors are recruited by transcription factors to repress gene expression.

There is no obvious evidence for DNA methylation enzymes as coregulators of NF-κB. DNMTs and TETs are non-site-specific enzymes, and preferable alteration of DNA methylation of specific genes requires a guide to target genes.[24] The results of coimmunoprecipitations showed that TET3 and DNMT3b bind to NF-κB and that TNF-α treatment potentiates this binding. DNMTs and TETs are writers and erasers of DNA methylation epigenetic mark. Some pioneering studies suggest that transcription factors play a pivotal role in the alteration of DNA methylation by recruiting enzymes of DNMTs and TETs to guide them toward their target genes. A study has shown that re-expression of RE1-Silencing Transcription factor (REST) in REST-knockout cells results in the inhibition of methylation around the binding sites, suggesting that REST can bind to the methylated region and promote DNA demethylation.[25] Other experimental results also revealed that during osteoclastogenesis, PU-1 interacts with DNMT3b and TET2 to perform DNA methylation and demethylation of target genes.[26] This causes hypomethylation of some genes and hypermethylation of others during differentiation of monocytes to osteoclasts.[27] There are NF-κB binding sites in NANOG and cMYC promoter regions (NANOG: −485/−475, MYC: +288/298s from the transcription start site). HRM-specific primers were designed near the NF-κB binding site in the promoter region. Since TNFα-induced NF-κB activation decreased methylation in these regions, it is plausible to propose that NF-κB exerts promoter demethylation via recruitment of TETs. We also showed that knockdown of TET expression using transfection of siRNAs into HT-29 cells attenuates the hypomethylation of TNF-α-induced stemness genes. These results suggest that inflammation-associated NF-κB activation may play a critical role in overcoming epigenetic barriers during dedifferentiation of epithelial cells.

The simultaneous interaction between both DNMT3b and TET3 with NF-κB raises a question: How does NF-κB choose these enzymes each time to exert methylation or demethylation of CpGs in target genes? Although our results cannot answer this question, post-translational modification of transcription factors or their coregulators determines which enzymes/coregulators would be exposed and hence affect the promoter methylation status.

It has been suggested that dedifferentiation of somatic cells and neoplastic nonstem cells can be a source of cancer stem cells with tumor-initiating capacity.[28] Cancer stem cells share some embryonic and adult stem cell genes and the transcriptional network. However, most of these genes are silenced epigenetically during the development of adult tissues. Therefore, reprogramming of terminally differentiated cells should overcome this epigenetic barrier to re-enter the cell cycle to dedifferentiate.[29] Overexpression of these pluripotency genes *in vivo* results in dysplasia and tumorigenesis.[30] The role of inflammation in the generation of tumor-initiating cells was confirmed by Schwitalla *et al*.,[13] who showed that colonic epithelial cells can be reprogrammed into cancer stem cells via activation of Wnt signaling by inflammation-associated NF-κB activation.

Conclusion

The results of the present study provide evidence that TNF-α-induced NF-κB decreases methylation of NANOG and MYC in HT-29 cells. Furthermore, coimmunoprecipitation of p65 showed that DNMT3b and TET3 can bind to NF-κB during activation. Also, downregulation of TETs attenuates stemness gene hypomethylation by TNFα-induced NF-κB activation. These findings suggest that the inhibition of NF-κB may prevent early oncogenic events in an epigenetic manner.

Study limitation

Like other studies, this study had some limitations. Limitations in financial resources and project budget and problems in preparing laboratory consumables are regarded as the limitations of the present study.

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Ethics approval and consent to participate

All procedures performed in this study were in accordance with the 1964 Helsinki declaration. The present study was approved by the Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences (grant no.: CMRC-9425) and Department of Cellular and Molecular Nutrition of Shahid Beheshti University of Medical Sciences.

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Conflicts of interest

There are no conflicts of interest.

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