RESEARCH COMMUNICATION

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Induces Retinoic Acid Receptor β Hypermethylation through DNA Methyltransferase 1 Accumulation in Esophageal Squamous Epithelial Cells

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Abstract

Overexpression of DNA methyltransferase 1 (DNMT1) has been detected in many cancers. Tobacco exposure is known to induce genetic and epigenetic changes in the pathogenesis of malignancy. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is an important carcinogen present in tobacco smoke; however the detailed molecular mechanism of how NNK induces esophageal carcinogenesis is still unclear. We found that DNMT1 was overexpressed in ESCC tissues compared with paired non-cancerous tissues, the overexpression being correlated with smoking status and low expression of RARβ. The latter could be upregulated by NNK treatment in Het-1A cells, and the increased DNMT1 expression level reflected promoter hypermethylation and downregulation of retinoic acid receptor β(RARβ). RNA interference mediated knockdown of DNMT1 resulted in promoter demethylation and upregulation of RARβ in KYSE30 and TE-1 cells. 3-(4,5-Dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) and flow cytometric analysis demonstrated that NNK treatment in Het-1A cells could enhance cell proliferation and inhibit cell apoptosis in a dose-dependent manner. In conclusion, DNMT1 overexpression is correlated with smoking status and low expression of RARβ in esophageal SCC patients. NNK could induce RARβ promoter hypermethylation through upregulation of DNMT1 in esophageal squamous epithelial cells, finally leading to enhancement of cell proliferation and inhibition of apoptosis.

Keywords: NNK - retinoic acid receptor β - DNA methyltransferase 1 - esophageal SCC

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Introduction

Human esophageal cancer is the sixth most common cancer in the world and the third most common cancer of the gastrointestinal tract, accounting for more than 335,000 deaths each year (Stewart et al., 2003). In China, as opposed to western countries, the incidence of esophageal squamous carcinoma is higher than that of adenocarcinoma of the esophagus (Gamliel et al., 2000). In order to find new molecular targets for therapeutic means, we need to have a more thorough understanding of the mechanisms responsible for esophageal carcinogenesis.

Genetic, epigenetic and environmental factors are all involved in the pathogenesis of ESCC (Zhao et al., 2011). Previous studies have shown that aberrant expression of cyclooxygenase2 (COX2) or CyclinD1 and tobacco exposure are all involved in the occurrence of ESCC (Zhou et al., 2007; Hu et al., 2009; Li et al., 2009; Zong et al., 2009). Aberrant DNA methylation of tumor suppressor genes, mediated by the upregulation of DNA methyltransferase 1, is one mechanism of carcinogenesis. DNMT1 has been reported to be expressed at higher levels in lung and liver cancer patients who smoked compared to non-smoking patients (Hammons et al., 1999; Lin et al., 2007). Tobacco contains more than 60 carcinogens (Hoffmann et al., 1990; Hecht et al, 2006) and tobacco exposure is known to induce genetic and epigenetic changes in esophageal squamous and lung cancer cells (Hecht et al., 1999; Kim et al., 2004; Soma et al., 2006; Liu et al., 2007; Lin et al., 2010; Huang et al., 2011). NNK is a key ingredient of tobacco smoke carcinogens. It is reported that NNK exposure can induce promoter hypermethylation of several tumor suppressor genes (TSGs) in lung and liver carcinomas, such as cyclin-dependent kinase inhibitor 2A and RARβ (Pulling et al., 2001; Hutt et al., 2005). Lin et al. (2010) reported that NNK can induce TSGs promoter hypermethylation through the accumulation of DNMT1 in mice and human lung cancer. So we reasoned that NNK is likely to play a role in the carcinogenesis of ESCC.

RARβ is a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators. It is considered to be a tumor suppressor gene due to its frequent loss of expression in various types of human cancer, such as esophageal, gastric, colonic and breast

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cancer (Widschwendter et al., 1997; Wang et al., 2003; Kim et al., 2004; Sun et al., 2004). Wang et al. (2003) found that hypermethylation of the RARβ promoter region is an important mechanism for RARβ gene silencing in esophageal squamous cell carcinoma.

In the present study, we aimed to explore the possible role of NNK in causing the downregulation of RARβ in esophageal epithelial cells and to elucidate the molecular mechanisms involved.

Materials and Methods

Cell culture and treatments

Human esophageal squamous carcinoma cell line KYSE30 and TE-1 was obtained from Chinese Academy of Medical Science, and cultured in RPMI1640 supplemented with 10% FBS. An immortalized esophageal epithelial squamous cell line Het-1A was purchased from ATCC, and cultured in BEBM. The Het-1A cells were treated with NNK (0.1 μM, 1 μM, 10 μM, Sigma) for three consecutive days, and KYSE30 and TE-1 cells were treated with 5-Aza-dC (10 μM, Sigma) for three consecutive days.

Clinical samples

Ninety two pairs of paraffin-embedded ESCC samples and paired noncancerous esophageal tissues were retrieved from the Department of Pathology, Provincial Hospital Affiliated to Shandong University of China. The study was approved by the Ethical Review Board. All samples were histologically reviewed by one pathologist to confirm diagnosis. All patients were classified into smoking group and non-smoking group. Tobacco smoking was defined as having smoked at least one cigarette per day for more than 1 year (Gu et al., 2009).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen). For semi-quantitative RT-PCR, 2 μg of RNA was reversely transcribed using Superscript II reverse transcriptase according to the manufacturer's protocol (Invitrogen). cDNA was amplified by using rTaq DNA polymerase (Takara) and gene-specific primers. Primer sequences and specific annealing temperatures are listed in Table 1. Cycling conditions were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, a specific annealing temperature for each gene for 30 s, and 72 °C for 45 s, followed by a final extension of 10 min at 72 °C.

Immunohistochemistry (IHC)

Five micrometer thick sections of formalin-fixed, paraffin-embedded tissues were deparaffinized and dehydrated. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2. Antigen retrieval was conducted by heating in a pressure cooker filled with 10 mM citrate buffer (pH 6.0) for 10 min. After treatment with 10% normal goat serum for 1 hour to block any non-specific reaction, sections were incubated with a mouse anti-human monoclonal antibody against DNMT1 (dilution 1:50; ab13537, Abcam, UK) or RARβ (dilution 1:100; ab15515, Abcam, UK) at 37 °C for 1 hour. Sections were then incubated with a biotinylated secondary antibody (anti-goat or anti-rabbit immunoglobulin) at room temperature for 30 min and then with an avidin-biotin-peroxidase complex at room temperature for 30 min.

3, 3`-diaminobenzidine-hydrogen was used as chromogen, followed by light hematoxylin counterstaining. Slides omitting primary antibody were used as negative controls. For each sample, at least 3 high power fields and 500 cells were randomly counted. If the lesion was small, with less than 500 cells, all of the cells were counted. The DNMT1 or RARβ immunoreactivity rate in each sample was expressed as a percentage of all the cells counted. The average expression rate of DNMT1 or RARβ was chosen as the cut-off value for each patient (n = 92). It was considered to be low if the level of DNMT1 or RARβ protein expression rate in the cancer was below the average, and high if above the average.

Methylation-specific PCR (MSP)

Genomic DNA was isolated using a DNA extraction Kit (Tiangen). 500 ng genomic DNA was bisulfite modified using genomic DNA modification Kit (Zymo research). MSP was performed using HS Taq DNA polymerase with primers specific for methylated and unmethylated sequences of the genes. MSP primers and specific annealing temperatures were listed in Table 1. Cycling conditions were as follows: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, a specific annealing temperature for each gene for 30 s, and 72 °C for 30 s, followed by a final extension of 5 min at 72 °C.

Western Blot

Cells were lysed and centrifuged. Samples containing equal amount of protein (20 μg) were separated by 6% SDS-PAGE and electroblotted onto PVDF membranes(Millipore CO.). The blots were probed with mouse antibodies against DNMT1 (dilution 1:200; ab13537, Abcam, UK) or RARβ(dilution 1:200; ab15515, Abcam, UK), and a rabbit anti-β-actin antibody (1:6000,

Table 1. Primer Sequences for RT-PCR and Methylation-specific PCR Analysis

Primer	Forward primer (5`-3`)	Reverse primer (5`-3`)	Annealing (°C)
RT-PCR			
RAR β	GACTGTATGGATGTTCTGTCAG	ATTTGTCCTGGCAGACGAAGCA	60
DNMT1	ACGACCCTGACCTCAAATAT	CCATTAACACCACCTTCAAGA	56
GAPDH	CGGAGTCAACGGATTGGTCGTAT	AGCCTTCTCCATGGTGGTGAAGAC	56
MSP			
RAR β	M: TCGAGAACGCGAGCGATTCG	M: GACCAATCCAACCGAAACGA	55
	U: TTGAGAATGTGAGTGATTTGA	U: AACCAATCCAACCAAAACAA	55

Santa Cruz) as a loading control. β-actin protein levels were used as a control for equal protein loading.

RNA interference

Specific small interfering RNA (siRNA) molecules targeted DNMT1 mRNA were obtained from GenePharma, and KYSE30 and TE-1 cells were transfected with siRNA molecules using Lipofectamine 2000 according to manufacture's protocol. Cells were harvested for RNA, DNA and protein preparation three days after transfection as described. The siRNA sequences against DNMT1 were 5`-GCACCUCAUUUGCCGAAUATT-3`(sense); 5`-UAUUCGGCAAAUGAGGUGCTG-3` (antisense). The nonspecific control siRNA sequences were 5`-UUCUCCGAACGUGUCACGUTT-3`(sense); 5`-ACGUGACACGUUCGGAGAATT-3` (antisense).

Cell proliferation assay

For cell proliferation measurement, 5×10^3 cells were reseeded into 96-well culture plates, and treated with 0, 1, 10 μM NNK for three days. After incubation, the medium was removed and 20 μl MTT (5 mg/mL, Gibco) was added to each well. Then DMSO was added, the optical density of each well was read on the plate reader at 490 nm. Independent experiments were performed in triplicate.

Cell apoptosis detection

For cell apoptosis detection, cells were harvested by rapid trypsinization to minimize potentially high Annexin V background levels in adherent cells, then washed and stained with FITC-Annexin V and propidium iodide (PI), according to the instructions on the Annexin V-FLUOS (Roche, Switzerland). Independent experiments were performed in triplicate.

Statistical analysis

SPSS 11.5 software was used for all statistical analysis. Data were expressed as the mean ± SD. Correlation between the incidence of DNMT1 and RARβ immunoreactivity and smoking status of ESCC patients were analyzed using the chi-square test. Two-tailed Student's t test was used to determine the difference among various treatments. Difference at P value less than 0.05 was considered significant.

Results

The correlation between DNMT1 and RARβ expression and smoking status in ESCC patients

IHC for DNMT1 protein was performed on 92 ESCC samples and paired noncancerous tissues, and RARβ protein was performed on 92 ESCC samples. Both DNMT1 and RARβ protein were localized in nuclei in tissues. The proportion of DNMT1 positive cells was significantly higher in ESCC samples compared with paired noncancerous tissues (68.36% ± 24.09% vs 25.99% ± 21.47%, P<0.01; Figure 1). Its nuclear expression level was relatively higher in tumor samples of patients who smoked compared with nonsmoking patients, on the contrary, the expression of RARβ and smoking status has significant negative correlation (Figure 2, Table 2). Meanwhile, the high expression of DNMT1 was interrelated with the low expression of RARβ (Table 3).

Table 2. Correlation Between Smoking Status and DNMT1 or RARβ Expression and in ESCC Patients

	Smoker [Number of cases(%)]	Non-smoker [Number of cases(%)]	P value
DNMT1 expression			
High	38(82.6)	26(56.5)	0.012
Low	8(17.4)	20(43.5)	
RARβ expression			
High	11(23.9)	21(45.7)	0.048
Low	35(76.1)	25(54.3)	

Table 3. Correlation Between DNMT1 and RARβ Expression in ESCC Patients

	DNMT1 expression, No. High	Low	P value
High	17	15	0.017
Low	47	13	

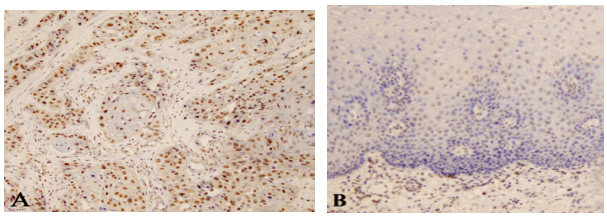

Figure 1. Representative IHC Analysis of DNMT1 Expression in Paraffin Sections from Histologically ESCC Tissues (A) and Paired Noncancerous Tissues (B). Original magnifications: ×100

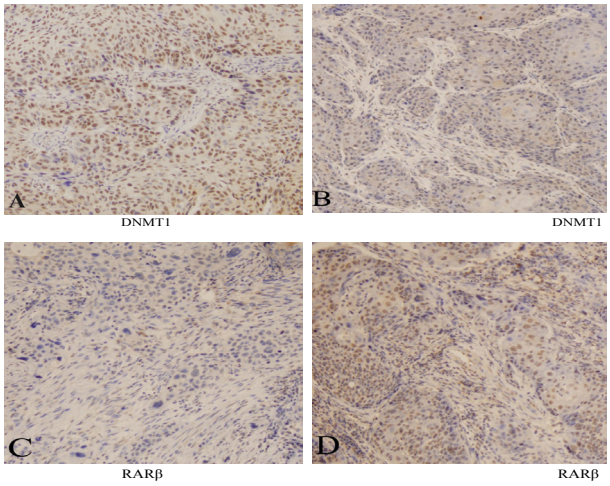

Figure 2. Representative IHC Analysis of DNMT1 and RARβ Expression in Paraffin Sections from ESCC Patients Who were Smokers (A and C) and Those who did not Smoke(B and D). Original Magnifications: ×100

The expression and promoter methylation status of RARβ in KYSE30, TE-1 and Het-1A cells

RARβ were completely silenced in ESCC cells (KYSE30 and TE-1), but was strongly expressed in esophageal squamous epithelial cells (Het-1A). MSP indicated that methylated RARβ alleles were predominant in KYSE30 and TE-1 cells, whereas unmethylated RARβ alleles were common in Het-1A cells (Figure 3A). Treatment of KYSE30 and TE-1 cells with 5-Aza-dC lead

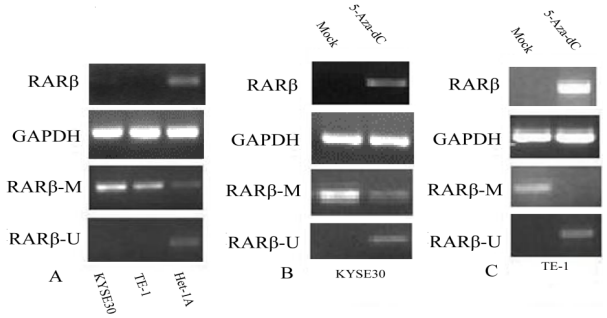

Figure 3. RARβ Expression and Methylation Status in KYSE30, TE-1 and Het-1A Cell Lines. A. mRNA level and promoter methylation status of RARβ detected by RT-PCR and MSP. U represents amplification of unmethylated alleles, and M indicates amplification of methylated alleles; B. RARβ expression and methylation status in KYSE30 cells before and after 5-Aza-dC treatment; C. RARβ expression and methylation status in TE-1 cells before and after 5-Aza-dC treatment

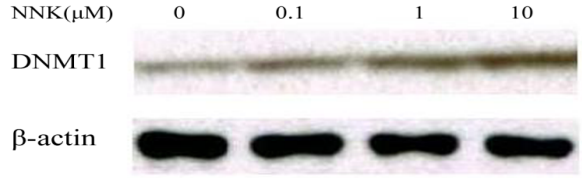

Figure 4. Effect of NNK Treatment of Different Concentrations on DNMT1 Protein Expression in Het-1A Cells

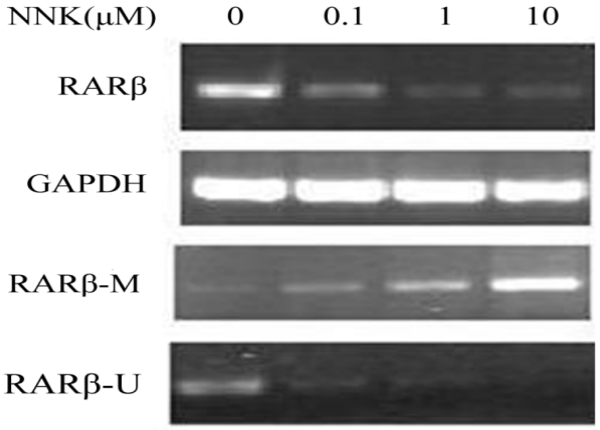

Figure 5. Effect of NNK Treatment of Different Concentrations on RARβ mRNA Levels and Promoter Methylation in Het-1A Cells

to promoter demethylation and upregulation of RARβ (Figure 3B and 3C).

NNK stimulates DNMT1 protein expression, downregulates mRNA levels and induces promoter hypermethylation of RARβ in Het-1A cells

We evaluated the effect of different concentrations (0.1 μM, 1 μM and 10 μM) of NNK treatment on DNMT1 expression in Het-1A cells, and found that NNK effectively induced the upregulation of DNMT1 expression in dose dependent manner (Figure 4). RT-PCR revealed that exposure to NNK of different concentrations caused downregulation of RARβ mRNA levels, and MSP showed the conversion of RARβ CpG islands from a predominantly unmethylated status to a heavily

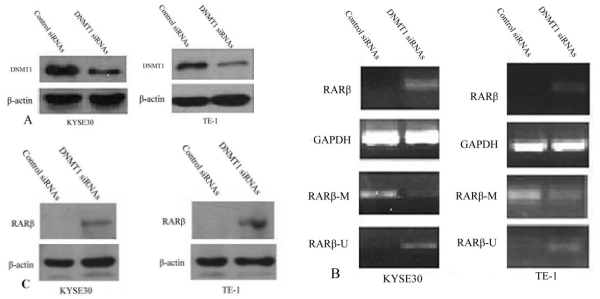

Figure 6. Blocking DNMT1 Expression in KYSE30 and TE-1 Cells with siRNAs Resulted in RARβ Reexpression and Promoter Demethylation. A.siRNAs designed to target DNMT1 mRNA were transfected into KYSE30 and TE-1 cells. B.mRNA reexpression and promoter demethylation of RARβ in KYSE30 and TE-1 cells treated with DNMT siRNAs. C.Protein expression of RARβ in KYSE30 and TE-1 cells treated with DNMT siRNAs compared with nonspecific control siRNAs treated group

methylated status in dose dependent manners (Figure 5). We further examined the effect of DNMT1 siRNAs on RARβ expression and promoter methylation in ESCC cell lines. Western blot analysis demonstrated that DNMT1 targeted siRNAs resulted in a significant reduction of DNMT1 protein levels in KYSE30 and TE-1 cells (Figure 6A). RNA interference mediated knockdown of DNMT1 reduced promoter methylation and induced reexpression of RARβ, as detected by MSP, RT-PCR and western blot analysis (Figure 6B and 6C).

Effects of NNK on cell proliferation and cell apoptosis

An MTT assay showed that the proliferation rate in the NNK treated groups were significantly raised with the increasing NNK concentrations compared with that of the mock group ($P<0.01$). Flow cytometric analysis revealed a dose dependent decrease in the proportion of Annexin-V positive cells in the NNK treated groups, indicating a decrease of early apoptotic cells in these groups, compared with the mock group.

Discussion

Epigenetic disorders can cause a lot of significant human diseases, many of which are mediated by altered DNMT1 expression. DNMT1 plays an important role in the maintenance of DNA methylation status. Overexpression of DNMT1 has been detected in many cancers, such as gastric cancer (Etoh et al., 2004), hepatic cancer (Saito et al., 2003), endometrial cancer (Liao et al., 2008), prostate cancer (Morey Kinney et al., 2008) and pancreatic cancer (Peng et al., 2005), suggesting that DNMT1 overexpression has some significance during tumor carcinogenesis. Previously, our data demonstrated that the expression of DNMT1 was significantly higher both at the protein and mRNA levels in most ESCC tissues compared to paired noncancerous tissues (Zhao et al., 2011). This suggests that DNMT1 overexpression may play an important role in the pathogenesis of ESCC.

In the present study, we have demonstrated that RARβ was completely silenced in ESCC cells, but strongly expressed in esophageal squamous epithelial

cells. MSP results indicated methylated RARβ alleles were predominant in KYSE30 and TE-1 cells, whereas unmethylated RARβ alleles were common in Het-1A cells. We found that RARβ could be reactivated in KYSE30 and TE-1 cells after treatment with 5-Aza-2-dC. MSP results correlated well with the RARβ expression status. This supports promoter hypermethylation being involved in the silencing mechanism of RARβ expression in ESCC.

Gu et al. (2009) reported that smoking was a major risk factor in China for mortality from cancer, including lung cancer, liver cancer, gastric cancer and esophageal cancer, and the multivariate relative risks were 2.44, 1.36, 1.52 and 1.34, respectively. Tobacco smoking can cause genetic and epigenetic changes that can eventually lead to cancer pathogenesis. But the detailed molecular mechanism is not yet fully understood. Liu et al. (2007) showed that cigarette smoke extract (CSE) had a strong inducing effect on Synuclein-γ (SNCG) demethylation through the downregulation of DNMT3B in lung cancer cells. Zong et al. (2009) treated ESCC cells with different concentrations of nicotine, and found cell migration and invasion could be enhanced, accompanied by increasing levels of COX2 protein and matrix metalloproteinase2 (MMP2) activity. Lin et al. (2010) verified that NNK-induced DNMT1 accumulation and subsequent hypermethylation of the promoter of TSGs may lead to tumorigenesis and poor prognosis in lung cancer patients.

Our IHC analysis demonstrated that smoking was correlated with the overexpression of DNMT1 and low expression of RARβ in ESCC, and the high expression of DNMT1 was interrelated with the low expression of RARβ, so we speculated that smoking could cause TSGs promoter hypermethylation through the increase of DNMT1 gene expression and result in the genesis of ESCC. In order to verify this theory, we treated esophageal squamous epithelial cells-Het-1A with NNK of different concentrations. Our study showed that DNMT1 expression in Het-1A cells was significantly upregulated by NNK treatment in dose dependent manner. The increased DNMT1 expression level was reflected by promoter hypermethylation and downregulation of RARβ. We next used siRNAs targeted against DNMT1 to inhibit DNMT1 expression in KYSE30 and TE-1 cells, in which the CpG inlands of RARβ were completely methylated. The protein level of DNMT1 was downregulated in NNK treated cells compared with control cells. MSP showed that downregulation of DNMT1 resulted in the conversion of the completely unmethylated RARβ CpG islands into a partially methylated status. This data demonstrates that RARβ CpG island methylation was controlled by DNMT1.

RARβ is a tumor suppressor gene, and our data have shown that NNK treatment caused promoter hypermethylation and downregulation of RARβ. Therefore, we hypothesized that treatment with NNK might affect the biological behavior of esophageal epithelial cells. MTT and flow cytometric analysis demonstrated that NNK treatment caused an enhancement of cell proliferation and an inhibition of cell apoptosis in Het-1A cells, which was consistent with our hypothesis.

In conclusion, DNMT1 overexpression is correlated with the smoking status and the low expression of RARβ in ESCC patients. NNK can induce RARβ promoter hypermethylation and downregulation through the upregulation of DNMT1 in esophageal squamous epithelial cells, leading to the enhancement of cell proliferation and the inhibition of cell apoptosis.

Acknowledgements

The author(s) declare that they have no competing interests.

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