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The tobacco-specific carcinogen NNK induces DNA methyltransferase 1 accumulation in Laryngeal carcinoma

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SUMMARY

Over-expression of DNA methyltransferase 1 (*DNMT1*) correlates with hypermethylation of tumor suppressor genes (TSGs) in tobacco-induced cancers. The tobacco component nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) increases protein levels of the *DNMT1* in human lung cancer. However, the role of *DNMT1* expression induced by NNK is not clear during laryngeal carcinogenesis. We investigated *DNMT1* expression levels in 101 cases of human laryngeal carcinoma specimens and 54 cases clear surgical margin specimens by reverse transcription polymerase chain reaction (RT-PCR), Western blotting and immunohistochemistry (IHC) detection. Then, we analyzed the relationship between the DNMT1 expression and the smoking status of the patients with laryngeal carcinoma. Moreover, we investigated the effects of tobacco carcinogen NNK on *DNMT1* expression in Hep-2 cells. We found that *DNMT1* mRNA and protein expressions were up-regulated in laryngeal cancer tissues ($p < 0.05$ and $p < 0.01$, respectively). Among the 101 cases, DNMT1 protein from patients with heavier smoking habit had a significant trend of an increase with IHC scores ($p < 0.01$). The overall survival rates of patients DNMT1-positive were significantly lower than those of patients DNMT1-negative ($p < 0.05$). We observed that NNK increased DNMT1 protein levels, not mRNA levels, in cultured Hep-2 cells significantly in both dose- and time-dependent manner ($p < 0.05$). These results supported the idea that NNK-induced *DNMT1* expression may result from protein stabilization. Increased DNMT1 protein expression may play a critical role in the malignant progression of larynx.

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common malignancy worldwide, and has a profound impact on quality of life.[1] Tobacco is notably genotoxic and associated with head and neck carcinogenesis. According to the International Union Against Cancer (IUAC), approximately 95% of larynx cancer cases are related to smoking, and the probability increases when associated to alcohol.[2,3] Cigarette carcinogens have the capacity to alter early response gene expression in tobacco-related malignancies.[4]

The exposure of aerodigestive tract epithelium to tobacco carcinogens often leads to histologic changes over large areas of the tissues, resulting in field cancerization with potential multifocal unsynchronized premalignant and primary malignant lesions.[5] 4-(Methylnitro-samino)-1-(3-pyridyl)-1-butanone (NNK) is the key ingredient of tobacco smoke carcinogen. Clinical studies indicated that smoking is associated with promoter hypermethylation at more than 20 tumor suppressor genes (TSGs) in lung tumors.[6,7] Haussmann et al. reported that the larynx was considered the organ most sensitive to histopathological changes after exposure to cigarette smoke. There is dose-dependent diffuse squamous metaplasia of the pseudostratified epithelium and squamous hyperplasia on the base of the epiglottis.[8]

Recently, it was identified that *DNA methyltransferase 1* (*DNMT1*) expression was induced by NNK in human lung cancer samples and cell lines. The NNK-induced *DNMT1* accumulation and subsequent hypermethylation of the promoter of TSGs may lead to tumorigenesis in patients of lung cancer.[9] Increased *DNMT1* always occurs in the process of malignant genesis, especially tobacco-induced cancers, and diminished survival of patients with these neoplasms.[9–14] *DNMT1* over-expression is significantly correlated with poorer differentiation of liver, stomach, lung and pancreatic cancers. Moreover, in these tumors, *DNMT1* overexpression is significantly correlated with accumulation of DNA

Abbreviations: HNSCC, head and neck squamous cell carcinoma; NNK, 4-(methyl-nitro-samino)-1-(3-pyridyl)-1-butanone; TSGs, tumor suppressor genes; IHC, immunohistochemistry; DNMT1, DNA methyltransferase 1; RT-PCR, reverse transcription polymerase chain reaction; HPV, human papillomavirus.

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hypermethylation of multiple tumor-related genes.[9,15–19] With respect to laryngeal carcinogenesis, some studies have revealed accumulation of DNA hypermethylation of tumor-related genes in squamous cell carcinoma.[5,20–23]

In this study, we performed cell and clinical studies to analyze the role of DNMT1 and NNK in the progression of laryngeal carcinoma.

Materials and methods

Patients

One hundred and one cases of laryngeal carcinoma tissues and 54 cases of clear surgical margin were obtained from patients treated at the Ear, Nose and Throat (ENT) Department of Shengjing Hospital of China Medical University after receiving their informed consent and the approval of the hospital authorities. None of the patients received radiotherapy or chemotherapy prior to the genetic analysis. The patients were evaluated according to the 6th AJCC TNM staging system.[24] The age of the laryngeal cancer patients in this study ranged 39–78 years, with an average of 57 years. All the other clinical information of the patients was shown in Table 1. Patients were categorized as those who did not smoke (never smoked and formerly smoked) and those who continuously smoked (both regular and occasional continuous smoking). The definition of a former smoker was an individual who had smoked at least 100 cigarettes in his or her life time and had quit >12 months before the laryngeal cancer diagnosis.[25]All specimens were frozen after collection and stored at −80 °C immediately. Patients were also categorized by smoking history based on pack-years, calculated as the number of packs smoked per day multiplied by the number of years smoked. The two groups in this categorization smoked 20 or more pack-years or fewer than 20. Follow-ups of 101 patients were performed at 2-month intervals in the first year after surgery and at 3-month intervals thereafter at outpatient clinics or by routine phone calls. The end of the followup period was December 2010 for all patients. Overall survival was calculated from the date of surgery to the date of death or of the final followup. Approval for the study was received from the Ethics Committee of China Medical University.

Cell culture and treatment with NNK

Hep-2 (human laryngeal squamous cell carcinoma) cell line was maintained in RPMI-1640 with 10% FBS and antibiotics at 37 °C in 5% CO_2 humidified atmosphere. For the dose-dependent study, cells were treated with NNK at indicated doses for 2 h at 37 °C. For the time-course study, cells were treated with 1.0 μM NNK for the indicated times at 37 °C which was then replaced with fresh medium without NNK for the indicated times.

Table 1
Association analysis between DNMT1 protein expressions and clinical characteristics in all 101 patients of laryngeal cancer.

Characteristics	*DNMT1* Negative *n* = 13		*DNMT1* Positive *n* = 88		*p*-Value
	No.	%	No.	%	
Age[a]	56 ± 10		57 ± 10		0.414
Gender					
Male	11	84.6	67	76.1	
Female	2	15.4	21	23.9	0.727
Site					
Supraglottic	3	23.1	27	30.7	
Glottic	7	53.8	55	62.5	
Infraglottic	1	7.7	6	6.8	0.948
TNM stage					
I + II	11	84.6	44	50.0	
III + IV	2	15.4	44	50.0	0.034

[a] Mean ± SD.

Immunohistochemistry (IHC) analysis

IHC detection was performed as previously described[26] to determine the expression levels of *DNMT1*, the sections were treated over night at 4 °C with a rabbit anti-DNMT1 polyclonal antiserum (1:200, Abcam, UK). The sections were counterstained in hematoxylin and mounted. Immunostaining signals were scored by two independent observers.

The criteria for IHC results were as follows: positive tumor cells were defined as having brown-yellow granules distributed in the nucleus or cytoplasm, with stain intensity higher than the unspecific background. DNMT1 protein expression in the cancer tissue was considered as overexpression if 10% or more of the cancer cells were positive for DNMT1.[27]

RNA extraction and RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction, and reverse transcripted using AMV RNA PCR kit (TaKaRa Bio, Inc., Japan). Expression levels of *DNMT1* were detected by RT-PCR assay using *GAPDH* as an internal control. The primer sequences for *DNMT1* reaction were 5′-GAGGAAGCTGCTAAGGACTAGTTC-3′ (forward) and 5′-ACTGCACAATTTGATCACTAAATC-3′ (reverse). The primer sequences for *GAPDH* reaction were 5′-ATCATCAGCAATGCA ATGCCTCC-3′ (forward) and 5′-CATCACGCCACAGTTTCC-3′ (reverse). The PCR mixture contained 10× PCR buffer (TaKaRa Bio, Inc., Japan), deoxynucleotide triphosphates (2.5 mM), primers (final concentration, 0.5 μM each per reaction), 0.5 U of HotStarTaq (TaKaRa Bio, Inc., Japan). The PCR analyses with annealing temperatures at 58 °C were performed. The PCR products were analyzed on 3% agarose gels and were visualized under ultraviolet illumination. Clear surgical margins were used as negative controls against the tumor cells regarding the intensity of *DNMT1* mRNA expression.

Western blotting

Conventional immunoblotting was performed as previously described,[28] using rabbit anti-DNMT1 polyclonal antibody (1:1000, Abcam, UK). Western blotting was performed using rabbit anti-β-tubulin polyclonal antibody (1:1500, Santa Cruz Biotechnology, USA) as a loading control for its appropriate band size. Detection was carried out using ECL reagents (Beyotime, China) and exposing them to X-ray film. Clear surgical margins were used as negative controls.

Statistics

SPSS17.0 software (SPSS Inc.) was used for all statistical analysis. The comparisons of the mRNA and protein expression levels in tumor and clear surgical margin tissues were performed using the Wilcoxon signed-rank test. Fisher exact χ^2 test was used to compare the frequency of DNMT1 alterations between the patients with different smoking statuses and pack-years. The relationship between DNMT1 expression levels and clinical characteristics was analyzed by Fisher exact χ^2 test (with respect to gender, tumor site and TNM stage) and Mann–Whitney *U*-test (with respect to age). To analyze the difference of DNMT1 protein levels among various treatments in cell model studies, 2-tailed Student's *t* test was used. Type III censoring was performed on subjects who