Demethylation of the hTERT promoter in normal human gastric mucosal epithelial cells following *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine exposure

YONG-BO CHENG[1], DIAN-CHUN FANG[2], PING YAO[1], LI-PING GUO[2], XIAO-YAN NING[2] and LEI WANG[1]

[1]Department of Gastroenterology, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830054; [2]Department of Gastroenterology, Southwest Hospital, Third Military Medical University, Chongqing 400038, P.R. China

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Abstract. *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) is an alkylating agent that can induce gastric carcinoma. As a well-known human carcinogen, MNNG has been universally recognized as a methylating agent and is believed to act through methylation mechanism. In the present study, the epigenetic status of the human telomerase reverse transcriptase (hTERT) promoter was investigated in MNNG-treated normal human gastric mucosal epithelial cells. After 4 h exposure to MNNG at different concentrations, 6.8 and 68 μM, bisulfite sequencing polymerase chain reaction showed that five methylated cytosines outside the CpG dinucleotides in the 290-bp fragment from the hTERT promoter were demethylated and all the methylated cytosines in CpG dinucleotides remained intact. Furthermore, the epigenetic status of the target region following MNNG exposure was extremely similar to those of the BGC-823, SGC-7901 and MKN-28 lines; the three cell lines from human gastric adenocarcinoma. The result indicates that MNNG-induced demethylation in cytosines outside the CpG dinucleotides may be an early molecular lesion with the potential for impacting malignant transformation and a possible underlying carcinogenic mechanism of MNNG. Thus, it may provide another insight into the mechanisms of MNNG carcinogenesis.

Introduction

N-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) is a well-known alkylating agent that can induce gastric carcinoma (1,2). As a carcinogenic chemical, the mechanism of carcinogenesis induced by MNNG is not well understood. Previous studies have focused on the methylation aspect (1,3,4) and presently, MNNG has been recognized as a methylating agent universally (5,6).

Thus far, MNNG-induced DNA epigenetic changes have been mostly reported in rats (1,2,4-6), no relevant studies in humans found. Therefore, to improve the insight into the mechanism of human gastric carcinogenesis induced by MNNG, the epigenetic status of the human telomerase reverse transcriptase (hTERT) promoter, the rate-limiting subunit of telomerase whose activity is considered to be an early step in gastric carcinogenesis (7-9), was investigated in normal human gastric mucosal epithelial cells (nhGMECs) following MNNG exposure.

Materials and methods

Chemical. MNNG was purchased from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan) dissolved in redistilled water at 25˚C and formulated with a concentration of 0.1%. Subsequently, the MNNG-containing water was confected into 6.8 and 68 μM in Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Gibco-BRL, Life Technologies, Carlsbad, CA, USA). The MNNG was freshly prepared for the experiment.

Cell culture. Following the approval by the The Third Military Medical University and the affiliated Southwest Hospital Ethical Committees and informed consent from the patient, nhGMECs were isolated from the specimen obtained by routine surgery with our previously developed method (10). The patient that provided the gastric sample was aged 42 years and underwent surgery for a gastric ulcer complicating perforation. The possibility of gastric cancer was excluded by postoperative pathology. BGC-823, SGC-7901 and MKN-28 cell lines were maintained in the laboratory and routinely cultured. All the cells were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) and without any antibiotics.

MNNG-treated cells. nhGMECs were cultured primarily for 48 h. The supernatant was discarded and the cells were washed three times in warmed phosphate-buffered saline (PBS). Exposure for 4 h to MNNG at different concentrations

Correspondence to: Professor Dian-Chun Fang, Department of Gastroenterology, Southwest Hospital, Third Military Medical University, 30 Gaotanyanzheng Road, Chongqing 400038, P.R. China
E-mail: fangdianchun2012@163.com

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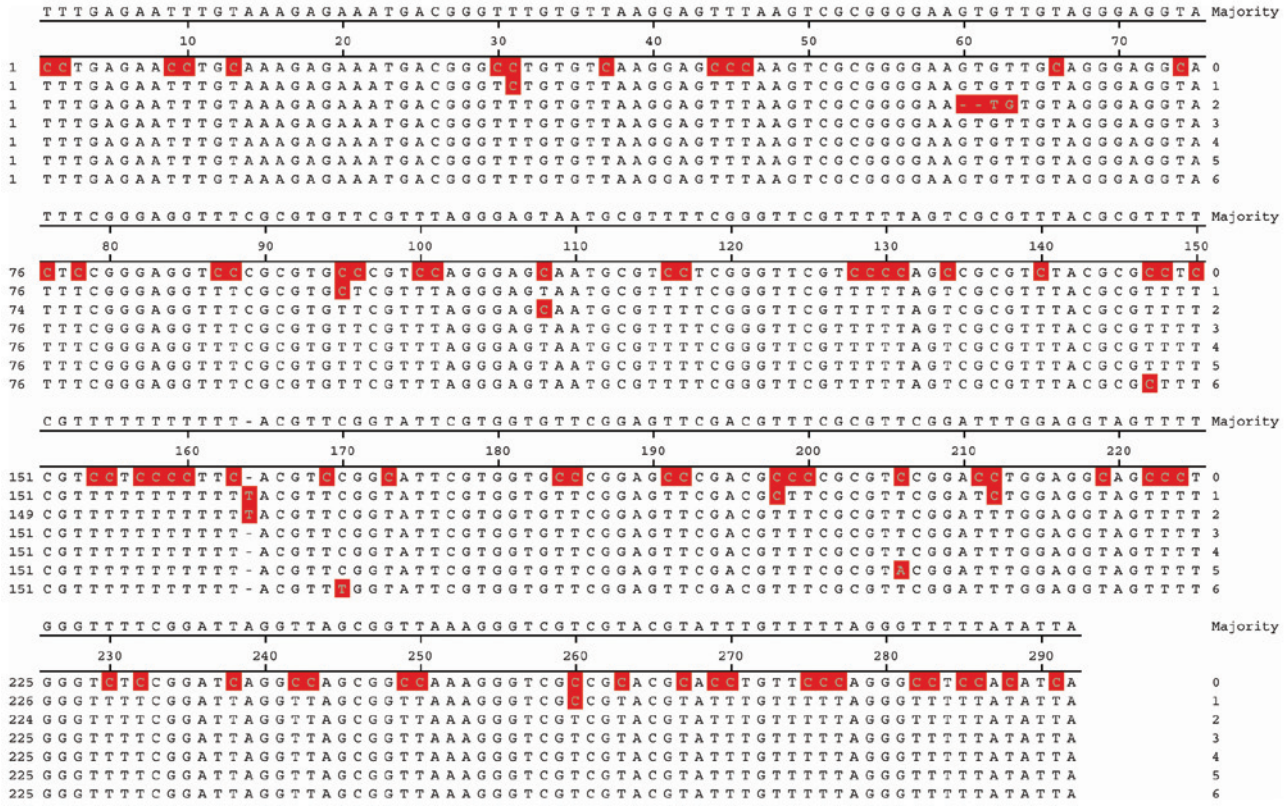

Figure 1. DNA sequencing result of the promoter region of human telomerase reverse transcriptase following bisulfite treatment. 0, Fragment in GenBank accession no. AF098956 sequence; 1, normal human gastric mucosal epithelial cells (nhGMECs) without *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) treatment; 2, nhGMECs treated with 6.8 μM MNNG; 3, nhGMECs treated with 68 μM MNNG; 4, BGC-823 cells; 5, SGC-7901 cells; 6, MKN-28 cells. Sequenator, ABI Prism 3730.

of 6.8 and 68 μM was carried out. The cells were cultured in DMEM-F12 medium without MNNG and FBS as a negative control. Following treatment, the cells were washed five times with warmed PBS to remove any residual MNNG and reincubated in fresh DMEM-F12 medium supplemented with FBS. At intervals of 48 h (≤100 h post-isolation), the cells were harvested and studies were performed as described below.

hTERT promoter methylation assay. DNA were isolated from nhGMECs that were treated with and without MNNG, BGC-823, SGC-7901 and MKN-28 cells using E.Z.N.A.® SQ DNA kit (Omega Bio-Tek Inc., Norcross, GA, USA). The hTERT promoter methylation status was assessed with bisulfite sequencing polymerase chain reaction (PCR) using the EZ DNA methylation kit (Zymo Research, Irvine, CA, USA) as follows: Genomic DNA was modified by sodium bisulfite. A segment of 290 base-pairs (bp) of the hTERT promoter (GeneBank accession no. AF097365) was amplified by PCR with primers: Forward, 5'-TTTGAGAATTTGTAAAGAGAAATG-3' and reverse, 5'-AATATAAAAACCCTAAAAACAAATAC-3'; under 32 cycles of 94˚C for 50 sec, 53˚C for 50 sec and 72˚C for 1 min, followed by 8 min at 72˚C. Sequencing of all the PCR products following cloning was performed by Sangon, Shanghai, China using the ABI Prism 3730 sequenator. All the sequence comparisons were carried out with the DNA Star software (DNASTAR Inc., Madison, WI, USA).

Results

MNNG treatment and the methylation status. The nhGMECs were dissociated and cultured. DNA were isolated from nhGMECs that were treated with and without MNNG, BGC-823, SGC-7901 and MKN-28 cells. Bisulfite treatment of genomic DNA can cause unmethylated cytosines to be completely converted into uracil, which are detected as thymine following PCR amplification, whereas methylated cytosines remain unchanged. The six sequences of the bisulfite-treated DNA were compared to those published in GenBank respectively. All 5'-CpG-3' dinucleotides were found to be methylated, with only five cytosines at positions 31, 95, 198, 212 and 260 outside the CpG dinucleotides methylated in the 290-bp fragment from the hTERT promoter region of the nhGMECs. Following 6.8 and 68 μM MNNG exposure, all the methylated 5'-CpG-3' dinucleotides remained intact, the five cytosines were all demethylated and the methylation status of the target region was extremely similar to those of the BGC-823, SGC-7901 and MKN-28 lines (Fig. 1). The demethylated cytosines were at position 31, 95, 198, 212 and 260, which corresponded respectively to the -716, -652, -550, -536 and -488 positions, relative to the ATG translation initiation site (GeneBank accession no. AF097365).

Discussion

Primary cultured nhGMECs have a clear similarity with their corresponding cells *in vivo* and are taken as the ideal tool for

gastric pathological studies. The methylation status of the hTERT promoter plays a role in hTERT expression and subsequent telomerase activation. The process may be an early step in gastric carcinogenesis (9). The aim of the present study, using nhGMECs, was to elucidate the mechanism of human gastric carcinogenesis induced by MNNG from the point of the epigenetic state of the hTERT promoter. As a carcinogenic agent, MNNG has been reported to produce DNA methylation and is usually recognized as a methylating agent (1,4-6). The present study observed that the epigenetic change of MNNG-treated nhGMECs was in agreement with those in previous studies. By contrast, it showed a selective demethylation in the 290-bp fragment from the hTERT promoter region. Furthermore, the demethylation of cytosine occured at position cytosine outside the CpG dinucleotides, and the methylated cytosine in the CpG dinucleotides remained intact. Notably, such epigenetic status of the target region following MNNG exposure was extremely similar to those of BGC-823, SGC-7901 and MKN-28 lines; the three cell lines from human gastric adenocarcinoma. This experiment could not be repeated due to the limited gastric specimens from similar patients. Therefore, the mechanism of human gastric carcinogenesis induced by MNNG may be correlated with demethylation in the hTERT promoter region or another undetected region. Previous studies on the mechanism of carcinogenesis have focused on more methylation and less demethylation. In neoplasia, demethylation of the genome as a whole occurs *in vivo* (11). Additionally, increasing the incidence of cancer during aging is accompanied by decreasing DNA methylation (12,13), although there is controversy regarding this (8). From another perspective, the promoter of the *hTERT* gene becomes methylated during the development of some but not all tumors (14). All these indicate a possible role for demethylation in carcinogenesis. The epigenetic state of cytosine outside the CpG dinucleotides may be involved in the carcinogenesis. By computational prediction it has been estimated that 29,000 CpG-rich regions are distributed in the human genome (15); therefore, the majority of studies on gene methylation focused on cytosine in the CpG dinucleotides and revealed that the CpG islands within gene promoters generally become methylated during human carcinogenesis. As cytosine in the CpG dinucleotides, cytosine outside the CpG dinucleotides are also distributed in the promoter and first exon of genes and contain putative binding motifs, such as myeloid-specific zinc finger protein 2 (16,17). Their epigenetic state may also affect the binding of transcription factors (16) and possibly result in carcinogenesis. Therefore, the epigenetic status of cytosine outside the CpG dinucleotides requires further study.

In the present study, to prevent interference MNNG was dissolved in redistilled water and not dimethylsulphoxide and was freshly prepared for the experiment. nhGMECs were all primary, not passage cells (8,12) and were cultured in DMEM-F12 medium without any antibiotics. In general, alkylating agents, such as MNNG, produce increased G→A, not C→T, transition mutations. All five C→T outside the CpG dinucleotides are not considered to result from the mutagenic effect of MNNG (5).

In conclusion, a selective demethylation in the hTERT promoter in nhGMECs was observed following exposure to different MNNG doses *in vitro*. Demethylation in cytosine outside the CpG dinucleotides may be an early molecular lesion with the potential for impacting malignant transformation and a possible underlying carcinogenic mechanism of MNNG. Thus, it may provide another insight into the mechanisms of MNNG carcinogenesis.

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