Response of human REV3 gene to gastric cancer inducing carcinogen N-methyl-N’-nitro-N-nitrosoguanidine and its role in mutagenesis

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Abstract

AIM: To understand the response of human REV3 gene to gastric cancer inducing carcinogen N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) and its role in human mutagenesis.

METHODS: The response of the human REV3 gene to MNNG was measured in human 293 cells and FL cells by RT-PCR. By using antisense technology, mutation analysis at HPRT locus (on which lesion-targeted mutation usually occurs) was conducted in human transgenic cell line FL-REV3 by 8-azaguanine screening, and mutation occurred on undamaged DNA template was detected by using a shuttle plasmid pZ189 as the probe in human transgenic cell lines 293-REV3 and FL-REV3. The blockage effect of REV3 was measured by combination of reverse transcription-polymerase chain reaction to detect the expression of antisense REV3 RNA and Western blotting to detect the REV3 protein level.

RESULTS: The human REV3 gene was significantly activated by MNNG treatment, as indicated by the upregulation of REV3 gene expression at the transcriptional level in MNNG-treated human cells, with significant increase of REV3 expression level by 0.38 fold, 0.33 fold and 0.27 fold respectively at 6 h, 12 h and 24 h in MNNG-treated 293 cells (P<0.05); and to 0.77 fold and 0.65 fold at 12 h and 24 h respectively in MNNG-treated FL cells (P<0.05). In transgenic cell line (in which REV3 was blocked by antisense REV3 RNA), high level of antisense REV3 RNA was detected, with a decreased level of REV3 protein. MNNG treatment significantly increased the mutation frequencies on undamaged DNA template (untargeted mutation), and also at HPRT locus (lesion-targeted mutation). However, when REV3 gene was blocked by antisense REV3 RNA, the MNNG-induced mutation frequency on undamaged DNA templates was significantly decreased by 3.8 fold (P<0.01) respectively both in MNNG-pretreated transgenic 293 cells and FL cells in which REV3 was blocked by antisense RNA, and almost recovered to their spontaneous mutation levels.

CONCLUSION: The expression of the human REV3 can be upregulated at the transcriptional level in response to MNNG. The human REV3 gene plays a role not only in lesion-targeted DNA mutagenesis, but also in mutagenesis on undamaged DNA templates that is called untargeted mutation.

INTRODUCTION

It has long been known that exposure to certain chemicals is associated with the development of specific human cancers, which is largely the outcome of interaction between environmental agents and genetic susceptibility. Examples include the associations between amine dyes and bladder cancer, benzene and leukemia, aflatoxin and hepatocellular carcinoma, and tobacco smoke and lung cancer. Recent studies have also revealed that tobacco smoke significantly increases the risks for oral, esophageal, pancreatic, gastric and colorectal cancers. In addition, men who have a history of chronic indigestion or gastroduodenal ulcer have substantially higher mortality rates associated with concurrent cigarette smoking.

Tobacco smoke consists of many chemicals. One important substance found in tobacco smoke is chemical carcinogen N-methyl-N’-nitro-N-nitrosoguanidine (MNNG), a direct acting carcinogen, that targets the cellular DNA and induces severe genotoxic stress to the cell that can result in various DNA damages. Epidemiologic studies have suggested an etiologic role for N-nitroso compounds from dietary sources in the development of gastric and colorectal cancer in humans and animal experiments have shown that MNNG induces gastric cancer and colorectal cancer. Obviously, the link between DNA damages and MNNG induced cancers is closely related to mutagenesis. To ensure normal growth control and accuracy in DNA replication, cells have developed a variety of responses to stress, such as DNA repair, cell cycle checkpoints, DNA damage avoidance, or in extreme cases, apoptosis. In addition, cells have also evolved a sophisticated lesion bypass system (also called translation synthesis, or TLS) to repair the damaged DNA, resulting in DNA damage lesion-targeted mutation. However, mutation can also occur on undamaged DNA template, which is designated untargeted mutation (UTM), which has been described in SOS-induced mutagenesis in E. coli. It has been known that untargeted and targeted mutations caused by SOS response in E. coli both are resulted from the inhibition of DNA polymerase functions.
that normally maintain fidelity and the involvement of DNA polymerases with low fidelity, which include DNA pol IV (dinB), pol V (UmuD’2C) and other factors[26-30]. In eukaryote, it has been found that up to 40 % of cycl-91 revertants induced by ultraviolet (UV) is untargeted using mating experiments with excision deficient strains of Saccharomyces cerevisiae[31], and that stress response induced by DNA damaging agents (8-methoxy-psoralen or UV) leads to specific and delayed UTM in mouse T-lymphoma cells[32]. Previous studies in our laboratory also shown that low concentration MNNG induces UTM in mammalian cells[33]. Currently, it has been known that specialized DNA polymerases are responsible for DNA damage lesion-targeted mutation in eukaryote. However, it is not clear which factor can be activated and involved in UTM on undamaged DNA templates.

The human REV3 gene, encoding the catalytic subunit REV3 of human pol ζ, has been received intensive attention in recent years[34]. REV3 gene is thought to be the major component of error-prone TLS pathway[34, 35], although a number of other polymerases might also be involved in this process[36]. It is responsible for most of spontaneous and UV-induced mutation in yeast and humans, as well as somatic hypermutation in humans[34, 35, 37-39, 44-47]. The expression of REV3 appears to be elevated at the transcriptional level in some tumor cell lines[49]. However, the response of REV3 gene to gastric cancer inducing carcinogen MNNG and its role in MNNG-induced mutagenesis are still not clear. In order to understand the relationship between the human REV3 gene and the etiology of gastric cancer and colorectal cancer in humans, the response of REV3 to MNNG and its role in MNNG-induced mutagenesis, including both lesion-targeted and untargeted mutation, were explored.

**MATERIALS AND METHODS**

**Cell culture and treatment**

Human 293 cells were grown in DMEM (Dulbecco’s Modified Eagle Medium, Gibco) containing 10 % fetal bovine serum (Gibco), 200 units/ml penicillin, 100 µg/ml streptomycin and 200 µg/ml kanamycin. Human FL cells were grown in MEM (Minimum Essential Medium, Gibco), containing 10 % newborn calf serum (Gibco), 200 units/ml penicillin, 100 µg/ml streptomycin and 200 µg/ml kanamycin. Transgenic cell line 293-REV3[49] and FL-REV3 (unpublished data) were established in this laboratory by transfecting 293 cells and FL cells with pM-RS plasmid[50] that can express anti REV3 RNA when induced by dexamethasone (dex). 293-M and FL-M cell line were established by transfecting 293 cells and FL cells with the control vector pMAM neo-amp alone. These transgenic cell line were grown in MEM containing 200 mg/ml of G418 (geneticin, Gibco). For MNNG treatment, cells were exposed to 0.2 µM of MNNG (Sigma, dimethyl sulfoxide (DMSO) as solvent) in serum-free DMEM (for 293 cells) or MEM (for FL cells) for 2.5 h, and then MNNG was removed and replaced with fresh medium. DMSO treated cells were used as control.

**Response of human REV3 to MNNG**

The response of the human REV3 gene to MNNG was measured at the transcriptional level by using reverse transcription-polymerase chain reaction (RT-PCR) with ARFI (encoding ADP-ribosylation factor 1) as the internal control. RNA from 2×10⁶ 293 or FL cells was extracted at different time point using TRIzol agent (Gibco) after 0.2 µM MNNG treatment, followed by the first-strand cDNAs synthesis with 3 µg of RNA using M-MuLV reverse transcriptase (MBI fermentas) and random hexamer primer. After exponential phase selection, PCR was performed with the appropriate cycles; 5 min pre-denaturation at 95 °C, 30 sec denaturation at 94 °C, 30 sec annealing at 59 °C, 1 min extension at 72 °C, and an additional 10 min extension at 72 °C. PCR primers: REV3, 5’-TGCT AGC AAC CAT ATC TC-3’ (sense), 5’-TG CTG ACG TAT TCG TAC TG-3’ (antisense); ARFI, 5’-GAA CAT CTG CGC CAA CTT CTT-3’ (sense), 5’-ACA GCT AGT CCA GTC TCT CAT A-3’ (antisense). The sizes of the expected products are 635bp for REV3 and 515bp for ARFI. Ratios of ODREV3/ODARFI representing REV3 transcript level were calculated.

**Identification of the antisense blocking effect on REV3 function in transgenic cells**

The antisense blocking effect on REV3 function was analyzed by detecting the expression of antisense REV3 fragment with RT-PCR and the REV3 protein level with Western blotting. RNA was extracted from transgenic cells, which could express antisense REV3 fragment after 10 µM dex treatment for 3 days. 0.1 µg RNA from 1 µg RNA sample digested by lunt DNaseI (Gibco) was reverse transcribed using the REV3 specific sense primer (5’-AAG GCC AGC ATA CAA GAC-3’). For the positive control (with no dex treatment), a random hexamer primer was used as the reverse transcription. Each cDNAs sample was amplified with the specific primers: 5’-GCA AAG GAA TAC AGA AGA AGT-3’ (sense), 5’-CCA GCT GAA GAC ATC AAT ACC-3’ (antisense). The PCR cycling parameter is as following: 5 min pre-denaturation at 94 °C, 30 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 59 °C, and 1 min extension at 72 °C. Amplifications were completed by an additional 8 min extension at 72 °C. For Western blotting, the nuclear protein were extracted from the cell strains as described before[24]. Each nuclear extract (30 µg) was used for Western blotting, and the Ku70 protein was used as the loading control.

**Detection of mutation at HPRT locus**

2×10⁶ cells of the FL, FL-M or FL-REV3 were seeded in 100 ml culture flasks, respectively. After 1 day incubation, the media were replaced with HAT medium (Gibco) for 24 h and HT medium (Gibco) for the next 48 h to remove the pre-existing HPRT cells in the population, then the cells were induced with 10 µM dex for another 48 h. After treatment with 0.2 µM MNNG or DMSO for 2.5 h, the medium was removed and replaced with a fresh medium containing 10 µM dex for an additional 24 h incubation. Cells reaching approximately 80 % confluent were subcultured three or four times, with a consistent density at 10⁶ cells/flask. Then 200 cells were transferred to a 9-cm plate (5 plates total) for 15 days to count the relative cloning efficiency. In the meantime, 2×10⁶ cells were seeded in 100 ml culture flask (5 flasks total). After 24 h, the medium was replaced with fresh one containing 5 µg/ml 8-azaguanine (Gibco). Cells were then maintained for 30 days, with the medium changed every 3 days. After washing with 0.9 % NaCl, the clones were fixed with ethanol: acetic acid (3:1), stained with 1 % methylene blue, and the number counted. The mutation frequency was calculated as following:

\[
\text{Mutation frequency} = \frac{\text{number of mutant clones}}{10^6 \times \text{cells}} \times \frac{1}{\text{relative cloning efficiency}}.
\]

Statistical analysis was performed according to the method described by Kastenbaum and Bowman[51].

**Detection of untargeted mutation on shuttle plasmid pZ189**

The detection of untargeted mutation was performed as described (Figure 1)[30].
RESULTS

Response of human mutator REV3 to MNNG

It was found that PCR with 31 cycles for 293 cells and 28 cycles for FL cells ensured the exponential amplification of REV3 and ARF1 within the same tube (data not shown). The expression of REV3 was upregulated at the transcriptional level in both 293 cells and FL cells after MNNG treatment. In MNNG-treated 293 cells, the level of REV3 expression was significantly increased by 0.38 fold at 6 h, 0.33 fold at 12 h and 0.27 fold at 24 h, when compared with the control (P<0.05, Figure 2). Similarly, the transcriptional level of REV3 was also significantly increased by 0.77 fold at 12 h and 0.65 fold at 24 h in MNNG-treated FL cells, when compared with the control (all P<0.05, Figure 2). The data suggest that the human mutator REV3 gene was activated by low concentration MNNG treatment and could be regulated at the transcriptional level.

Decreased formation of MNNG induced HPRT mutants in transgenic cells

HPRT locus is traditionally used as a genetic marker for genome instability. Normally the spontaneous mutation frequency at HPRT locus was quite low. In the present study, we observed that the spontaneous mutation frequency was 2.87×10^{-6} in FL cells, and 4×10^{-6} in FL-M cells. Interestingly, in FL-REV3 cells, no spontaneous mutants were observed. This observation led to the speculation that REV3 may be involved in the process of spontaneous mutagenesis.

Previously studies found that MNNG could induce HPRT mutation in human cells[1]. It would be of interest to know if MNNG had the same effect on FL and the derived FL-M and FL-REV3 cells. As shown in Table 1, we observed that MNNG treatment significantly elevated the mutation frequency from 2.87×10^{-6} to 8.66×10^{-6} at HPRT locus in FL cells. Similarly, the mutation frequency was also increased in FL-M cells by MNNG treatment from 4×10^{-6} to 18.75×10^{-6}. On the other hand, the induced mutation frequency was only 0.14×10^{-6} cells in FL-REV3 cells, which was significantly lower than that of the spontaneous mutation frequency in FL cells (Table 1).

Decreased untargeted mutation frequency on undamaged plasmid transfected into MNNG pretreated transgenic cells

Intact and undamaged shuttle plasmid pZ189 DNA was introduced into MNNG pretreated human cells. Progeny plasmids were harvested 48 h after transfection, and used to transform MBM7070. White and light blue colonies were picked and the frequency of supF tRNA mutants was scored. As shown in Table 2, the spontaneous mutation frequencies were at comparable level between each cell lines. Untargeted mutation on undamaged DNA templates was increasingly
It was interesting to find that human light or chemical carcinogen can induce the UTM on undamaged DNA templates in lesion-targeted mutation replacing the normal replication polymerases and finally result the activation of pol ζ. Several TLS DNA polymerases, especially REV3, are activated in the repair of DNA damages. This process mainly occurs in initiation, malignant conversion and progression involving a series of genetic and epigenetic changes. For example, the induction of colon cancer requires alterations in the expression of hairy cell leukemia virus (HCV) gene, which is a risk factor for the carcinoma among the Chinese population. Recently, it was found that COX-2 may contribute to progress of tumor in human gastric adenocarcinoma. However, it has been clear that the induction of carcinogenesis is a complex multi-step process involving a series of genetic and epigenetic changes. For example, the induction of colon cancer requires alterations in at least three tumor-suppressor genes (MCC, DCC, and p53) and activation of the oncogene K-ras. The genetic changes mainly occur in initiation, malignant conversion and progression stages in the development of malignant tumors. DNA damaging agents can induce lesions in DNA template, causing the block on DNA replication fork. However, it also leads to the activation of several TLS DNA polymerases, especially the activation of pol ζ, to restart the replication process by replacing the normal replication polymerases and finally result in lesion-targeted mutation. On the other hand, UV-light or chemical carcinogen can induce the UTM on undamaged DNA templates.

It was interesting to find that human REV3 gene, which encodes the catalytic subunit of TLS polymerase ζ, was activated by the carcinogen MNNG that can induce gastric and colorectal cancer. Our computational analysis indicated that transcriptional factor binding sites for CREB, AP-1 and NF-κB were found in the promoter region of REV3 (data not shown). Previous studies in our laboratory have shown that MNNG treatment activates CREB[61], AP-1 and NF-κB (unpublished data) in mammal cells as early epigenetic events, which indicates that REV3 could be activated by MNNG via the activation of specific transcriptional factors in advance.

**DISCUSSION**

The interconnections between environment and human health have been increasingly recognized. With the increasing cases of environmental cancer in the world range, especially in developing countries, investigation on the potential biomarkers for environmental risk assay or new targets for gene therapy is an emergent task to prevent and control the carcinogenesis. In China, the incidence of gastric cardia cancer has greatly increased in the past 2-3 decades, and dietary habits might be one of the risk factors for the carcinoma among the Chinese population. Recently, it was found that COX-2 may contribute to progress of tumor in human gastric adenocarcinoma. However, it has been clear that the induction of carcinogenesis is a complex multi-step process involving a series of genetic and epigenetic changes. For example, the induction of colon cancer requires alterations in at least three tumor-suppressor genes (MCC, DCC, and p53) and activation of the oncogene K-ras. The genetic changes mainly occur in initiation, malignant conversion and progression stages in the development of malignant tumors. DNA damaging agents can induce lesions in DNA template, causing the block on DNA replication fork. However, it also leads to the activation of several TLS DNA polymerases, especially the activation of pol ζ, to restart the replication process by replacing the normal replication polymerases and finally result in lesion-targeted mutation. On the other hand, UV-light or chemical carcinogen can induce the UTM on undamaged DNA templates.

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Mutation at HPRT locus can be used as an indicator to reflect the degree of genome instability. It has been recognized that HPRT mutants are generated directly by DNA damage[62, 63], i.e., the mutation spectrum belongs to lesion-targeted mutation. In human fibroblasts, the number of UV-induced HPRT mutants is significantly increased, whereas, the mutation is remarkably depressed in the human cells that express high levels of REV3 antisense RNA.[64]. In this study, our data showed that the spontaneous mutation of HPRT locus in human cells was dependent on the function of REV3, since mutation at HPRT locus was eliminated in cells expressing antisense REV3 (Table 1). On the other hand, REV3 gene was also involved in MNNG-induced HPRT mutation, like in UV-induced mutation[63], as the antisense block of REV3 function significantly decreased the MNNG-induced mutation frequency. It is also possible that other factors might be involved in MNNG-induced HPRT mutagenesis, for example, the function of human REV1 gene is required for mutagenesis at HPRT locus induced by UV light[64].

Interestingly, our data further indicated that human REV3 gene also played a role in mutation genesis occurred on undamaged DNA templates. Unlike the role of REV3 in lesion-targeted mutation, the spontaneous mutagenesis in SupF tRNA gene in pZ189 replicated in human cells was REV3-independent, i.e., the antisense block of REV3 has no effect on the spontaneous mutations (Table 2). It was suggested that most of the spontaneous mutation occurring in such an experimental system are due to the deletion damage induced by the shear force during transfection. Different mechanisms are involved in repairing the base damage and deletion damage.

**Table 1** Detection of the spontaneous and induced mutation frequency at HPRT locus in FL, FL-M and FL-REV3 cells

| Cell line | MNNG (0.3 μM) | Antisense block of REV3 | No. of mutants per 10⁶ cells selected | Mutation frequency (10⁻⁶) |
|-----------|---------------|------------------------|--------------------------------------|--------------------------|
| FL        | 0             | None                   | 2.87                                 | 2.87                     |
|           | 0.3           | 19.29                 | 8.66                                 |
| FL-M      | 0             | None                   | 4.00                                 | 4                        |
|           | 0.3           | 18.75                 | 18.75                                |
| FL-REV3   | 0             | 0                      | 0                                    | 0                        |
|           | 0.3           | 7                      | 0.14                                 |

*α: mutants screened from 5×10⁶ cells; β: P<0.01 compared with spontaneous mutants in FL cell and FL-M cell;  β: P<0.01 compared with FL-REV3 cells.

**Table 2** Mutation frequency of supF tRNA gene in intact plasmid pZ189 after replicated in cultured human cells

| Cell line | DMSO | MNNG |
|-----------|------|------|
|           | Number of transformant | Number of mutant | Mutation frequency (10⁻⁶) | Number of transformant | Number of mutant | Mutation frequency (10⁻⁶) |
| 293       | 7954 | 1    | 1.26                                 | 12205 | 9    | 7.37<sup>a</sup> |
| 293-M     | 15358 | 2    | 1.30                                 | 12040 | 7    | 5.81<sup>a</sup> |
| 293-REV3  | 39236 | 9    | 2.29                                 | 19750 | 3    | 1.52         |
| FL        | 13495 | 7    | 5.2                                  | 13854 | 38   | 27.4<sup>a</sup> |
| FL-M      | 13272 | 7    | 5.3                                  | 10310 | 28   | 27.2<sup>a</sup> |
| FL-REV3   | 10967 | 3    | 2.7                                  | 12609 | 5    | 4.0          |

*α, β test P<0.05 and 0.01 respectively as compared with spontaneous mutation frequency; β,  β test P<0.05 and 0.01 as compared with induced mutation frequency in 293-REV3 and FL-REV3 cells respectively.
mammalian genome instability, and this mutator gene could suggest that human mutation on undamaged DNA templates was (Table 2). To date, we still do not know whether there are references to be a potential target for gastric and colorectal cancer prevention and gene therapy.

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