P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• GASTRIC CANCER •

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

Feng Zhu, Cai-Xia Jin, Tao Song, Jun Yang, Lei Guo, Ying-Nian Yu

Feng Zhu, Tao Song, Jun Yang, Lei Guo, Ying-Nian Yu, Department of Pathophysiology, Zhejiang University School of Medicine, Hangzhou 310031, Zhejiang Province, China
Cai-Xia Jin, Department of Biology, Ningxia Medical College, Yingchuan 750004, Ningxia Self-governing Region, China
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Correspondence to: Professor Ying-Nian Yu, Department of Pathophysiology, Zhejiang University School of Medicine, Hangzhou 310031, Zhejiang Province, China. ynyu@mail.hz.zj.cn
Telephone: +86-571-87217149 **Fax:** +86-571-87217149
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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 ($P<0.05$) and 5.8 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. The spontaneous *HPRT* mutation was disappeared in *REV3*-disrupted cells, and induced mutation frequency at *HPRT* locus significantly decreased from 8.66×10^{-6} in FL cells to 0.14×10^{-6} in transgenic cells as well ($P<0.01$).

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.

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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[1-5]. Recent studies have also revealed that tobacco smoke significantly increases the risks for oral[6, 7], esophageal[3-5, 8], bladder[9-12], pancreas[11], gastric[13] and colorectal cancers[14]. In addition, men who have a history of chronic indigestion or gastroduodenal ulcer have substantially higher mortality rates associated with concurrent cigarette smoking[13].

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[15]. Epidemiologic studies have suggested an etiological role for N-nitroso compounds from dietary sources in the development of gastric and colorectal cancer in humans[16, 17], and animal experiments have shown that MNNG induces gastric cancer[18-21] and colorectal cancer[22, 23]. 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[24]. 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*[25]. 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, 37-43, 44-47]. The expression of *REV3* appears to be elevated at the transcriptional level in some tumor cell lines[48]. 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 lines 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 *ARF1* (encoding ADP-ribosylation factor 1) as the internal control. RNA from 2×10^6 293 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 ℃, 30 sec denaturation at 94 ℃, 30 sec annealing at 59 ℃, 1 min extension at 72 ℃, and an additional 10 min extension at 72 ℃. PCR primers: *REV3*, 5' -TGT CCA AGG CAC CAT ATC TC-3' (sense), 5' -TGC TAC ACG TGG TAC TAC TG-3' (antisense); *ARF1*, 5' -GAA CAT CTT CGC CAA CCT CTT C-3' (sense), 5' -ACA GCC AGT CCA GTC CTT CAT A-3' (antisense). The sizes of the expected products are 635bp for *REV3* and 515bp for *ARF1*. Ratios of OD_{REV3}./OD_{ARF1} 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 1unit 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' -GCC AAG GAA TAC AGA GGA 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 ℃, 30 cycles of 30 sec denaturation at 94 ℃, 30 sec annealing at 59 ℃, and 1 min extension at 72 ℃. Amplifications were completed by an additional 8 min extension at 72 ℃. 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[51]

2×10^5 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-existed *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 10^6 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, 2×10^5 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 % methyllene blue, and the number counted. The mutation frequency was calculated as following:

Mutation frequency=(number of mutant clones/10^6 cells)×(1/relative cloning efficiency).

Statistical analysis was performed according to the method described by Kastenbaum and Bowman[52].

Detection of untargeted mutation on shuttle plasmid pZ189

The detection of untargeted mutation was performed as described (Figure 1) [33].

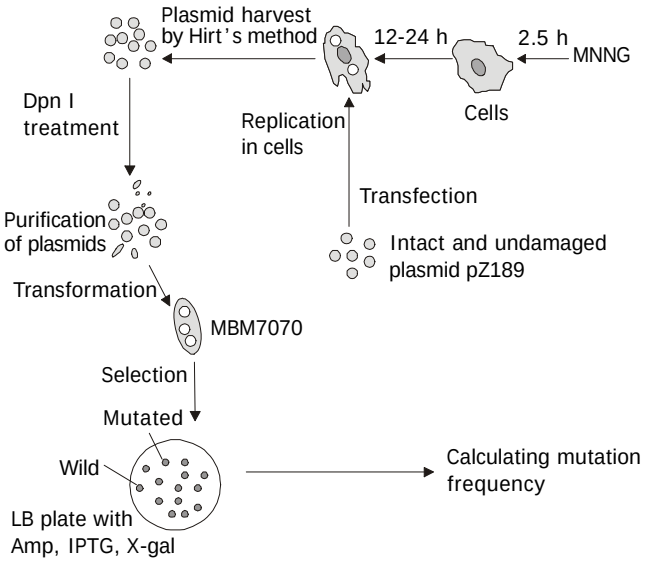

Figure 1 The detection system of untargeted mutation occurring on undamaged DNA template. Cells were pretreated by MNNG, and then intact and undamaged shuttle plasmid pZ189 was transfected into cells after removing MNNG. After replication for 48 h in cells, replicated pZ189 plasmid was rescued and then transformed to host bacterial MBM7070 to screen pZ189 mutants.

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.

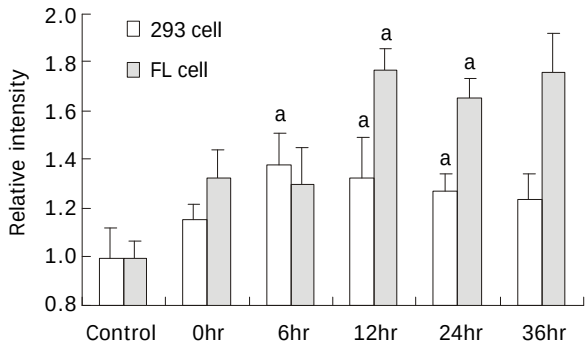

Figure 2 The response of the human *REV3* to MNNG at different time points. The response of *REV3* to MNNG was measured in human 293 cells and FL cells at the transcriptional level by using RT-PCR. $^aP<0.05$, compared with the control.

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

An expected high level of 297bp antisense RNA to C-terminal of *REV3* was detected in transgenic cells by RT-PCR (Figure 3). In addition, the results of Western blotting showed that the REV3 protein level was obviously reduced in transgenic cells (Figure 4). Therefore, it was indicated that the function of REV3 protein was partially blocked by antisense *REV3* fragment in transgenic cells.

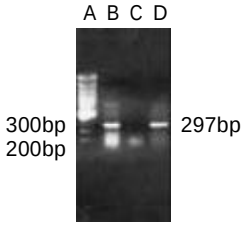

Figure 3 Detection of antisense *REV3* RNA fragment expressed in 293-REV3$^-$ cells using RT-PCR. A, 100bp DNA ladder; B, RT-PCR result in Dex-treated 293-REV3$^-$ cells; C, RT-PCR result in 293-REV3$^-$ cells (Dex-free); D, a positive control.

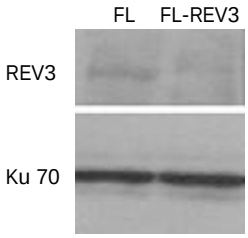

Figure 4 Western blotting showing the loss of REV3 protein in transgenic FL-REV3$^-$ cells. Western analysis showing the level of expression of REV3 protein in nuclear extracts from *REV3* antisense-expressing transfectant FL-REV3$^-$ and its parental strain FL. Ku-70 was used as the loading control.

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.

Previous studies found that MNNG could induce *HPRT* mutation in human cells[53]. 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

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 10^6 cells selected	Mutation frequency (10^{-6})
FL	0	None	2.87	2.87
	0.3		19.25[bc]	8.66
FL-M	0	None	4.00	4
	0.3		18.75[bc]	18.75
FL-REV3$^-$	0	Yes	0[a]	0
	0.3		7[a]	0.14

[a]: mutants screened from 5×10^7 cells; [b]$P<0.01$ compared with spontaneous mutants in FL cell and FL-M cell; [c]$P<0.01$ 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^{-4})	Number of transformant	Number of mutant	Mutation frequency(10^{-4})
293	7954	1	1.26	12205	9	7.37[ab]
293-M	15358	2	1.30	12040	7	5.81[ab]
293-REV3$^-$	39236	9	2.29	19758	3	1.52
FL	13495	7	5.2	13854	38	27.4[cd]
FL-M	13272	7	5.3	10310	28	27.2[cd]
FL-REV3$^-$	10967	3	2.7	12609	5	4.0

a, c χ test $P<0.05$ and 0.01 respectively as compared with spontaneous mutation frequency; b, d χ test $P<0.05$ and 0.01 as compared with induced mutation frequency in 293-REV3$^-$ and FL-REV3$^-$ cells respectively.

induced in MNNG-pretreated 293, 293-M, FL and FL-M cells, with the mutation frequencies occurred in these cells being 4.5-5.8-fold higher than those in control groups. However, the untargeted mutation frequencies significantly decreased by 3.8 fold (from 7.37×10^{-4} to 1.52×10^{-4}, $P<0.05$) and 5.8 fold (from 27.4×10^{-4} to 4.0×10^{-4}, $P<0.01$) respectively in MNNG-pretreated transgenic 293 cells and FL cells in which *REV3* was blocked by antisense RNA, and the mutation frequencies were almost similar to their spontaneous mutation levels.

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 cardia carcinogenesis among Chinese population[54]. Recently, it was found that COX-2 may contribute to progression of tumor in human gastric adenocarcinoma[55]. However, it has become 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*[56-58]. The genetic changes mainly occur in initiation, malignant conversion and progression stages in the development of malignant tumors[59]. 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[40, 42, 60]. On the other hand, UV-light or chemical carcinogen can induce the UTM on undamaged DNA templates[31-33].

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 mammalian cells as early epigenetic events, which indicates that *REV3* could be activated by MNNG via the activation of specific transcriptional factors in advance.

Mutation at *HPRT* locus can be used as an indicator to reflect the degree of genome instability[62]. 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[47]. 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[47], 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 *HRPT* 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,

in the later case no evidence was presented of *REV3* dependent. In this study, however, we proved that MNNG-induced mutation on undamaged DNA templates was *REV3*-depedent (Table 2). To date, we still do not know whether there are other factors involved in untargeted mutation in addition to the human *REV3* gene. Taken together, these data strongly suggest that human *REV3* gene is capable of inducing mammalian genome instability, and this mutator gene could be a potential target for gastric and colorectal cancer prevention and gene therapy.

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