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N-Methyl-N'-nitro-N-nitrosoguanidine-induced senescence-like growth arrest in colon cancer cells is associated with loss of adenomatous polyposis coli protein, microtubule organization, and telomeric DNA

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Abstract

Background: Cellular senescence is a state in which mammalian cells enter into an irreversible growth arrest and altered biological functions. The senescence response in mammalian cells can be elicited by DNA-damaging agents. In the present study we report that the DNA-damaging agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is able to induce senescence in the HCT-116 colon cancer cell line.

Results: Cells treated with lower concentrations of MNNG (0–25 microM) for 50 h showed a dose-dependent increase in G_2/M phase arrest and apoptosis; however, cells treated with higher concentrations of MNNG (50–100 microM) showed a senescence-like G_0/G_1 phase arrest which was confirmed by increased expression of β-galactosidase, a senescence induced marker. The G_2/M phase arrest and apoptosis were found to be associated with increased levels of p53 protein, but the senescence-like G_0/G_1 phase arrest was dissociated with p53 protein levels, since the p53 protein levels decreased in senescence-like arrested cells. We further, determined whether the decreased level of p53 was a transcriptional or a translational phenomenon. The results revealed that the decreased level of p53 protein in senescence-like arrested cells was a transcriptional phenomenon since *p53* mRNA levels simultaneously decreased after treatment with higher concentrations of MNNG. We also examined the effect of MNNG treatment on other cell cycle-related proteins such as p21, p27, cyclin B1, Cdc2, c-Myc and max. The expression levels of these proteins were increased in cells treated with lower concentrations of MNNG, which supported the G_2/M phase arrest. However, cells treated with higher concentrations of MNNG showed decreased levels of these proteins, and hence, may not play a role in cell cycle arrest. We then examined a possible association of the expression of APC protein and telomeric DNA signals with cellular senescence in MNNG-treated cells. We found that protein and mRNA levels of *APC* were drastically reduced in cells treated with higher concentrations of MNNG. The loss of *APC* expression might lead to chromosomal instability as well as microtubular disorganization through its dissociation with tubulin. In fact, the protein level of α-tubulin was also drastically decreased in senescence-like arrested cells treated with higher concentrations of MNNG. The levels of telomeric DNA also decreased in cells treated with higher concentrations of MNNG.

Conclusions: These results suggest that in response to DNA alkylation damage the senescence-like arrest of HCT-116 cells was associated with decreased levels of APC protein, microtubular organization, and telomeric DNA.

Background

Cellular senescence is a biological process leading to irreversible arrest of cell division. It was initially described in cultures of human fibroblast cells that lost the ability to divide indefinitely [1]. The proliferative life-span of normal human cells is limited by the replicative or cellular senescence [2,3]. The major feature of senescent phenotype includes an irreversible arrest of cell division, resistance to apoptotic cell death, specific changes in cellular functions, and senescent associated secretion of a variety of molecules such as proteases, cytokines and growth factors [4]. Phenotypically, similar processes can be achieved by accelerating senescence using various DNA-damaging agents such as γ-radiations [5,6], oncogenic stimulations [7,8], and genetic or pharmacological manipulations [9]. It is evident from the literature that the loss of tumor suppressor function is one of the major causes of transformation and immortalization of normal cells. Inactivation of tumor suppressor gene *p53* or Adenomatous polyposis coli (*APC*) are among the most common causes of colon cancer development [10-12]. Very often, the defective expressions of tumor suppressor genes with cancer development are linked with genetic instability. In colorectal cancer, genetic instability occurs in two forms – microsatelite instability (MSI) and chromosomal instability (CIN) [13]. In MSI instability, there is a defect in mismatch repair machinery that consequently results in the instability of repetitive DNA sequences [14]. In CIN, tumors exhibit a defect in chromosomal segregation, which results in variation of chromosome numbers among individual cells [15]. Recently, mutations in the *APC* gene have been linked with CIN [16]. Mutations in the *APC* gene produce truncated proteins. Many of the somatic mutations in the *APC* gene are located in the central region of the gene which is called as mutation cluster region (MCR) [12]. Cellular levels of APC are critical for maintaining cytoskeletal integrity, cellular adhesion, and Wnt signaling [17-19]. APC also binds and stabilizes microtubules in vivo and in vitro [17] and clusters at the ends of microtubules near the plasma membrane of interphase cells [16].

Another important aspect of *APC* is its transcriptional activation by p53 in response to DNA-damaging agents [20,21]. The activation of p53 by DNA-damaging agents induces cell cycle arrest, apoptotic cell death [22], or senescence [23]. The role of p53 in cell cycle arrest in G_1 phase is mediated by transcriptional activation of cyclin dependent kinase (CDK) inhibitor p21(Waf-1/Cip1), whereas in apoptosis it is mediated by transcriptional activation of mediators including p53 upregulated modulator of apoptosis (PUMA) and p53-induced gene 3 (PIG3) [24]. p53 also plays a role in cell cycle arrest in G_2 phase by transcriptionally activating the expression of *14-3-3σ* gene [25]. Biochemical and functional analysis of *p53* has also demonstrated its participation in the regulation of DNA damage-induced senescence and DNA repair, which can suppress tumorogenesis [22]. During cellular senescence, the length of telomeric DNA also decreases, possibly due to the absence of telomerase activity [26]. Senescent cells remain viable indefinitely and express specific phenotype markers, such as senescence-associated β-galactosidase [27]. In the present study, we investigated whether DNA-damaging agent MNNG can induce senescence-like cell cycle arrest in colon cancer cells. Data is presented to determine whether in these cells the expression levels of p53, APC, α-tubulin and telomeric DNA are associated with senescence-like cell cycle arrest.

Results

G_2/M phase arrest, apoptosis, and senescence in HCT-116 cells treated with different concentrations of MNNG

Cellular senescence is a process of irreversible arrest of cell division that can be induced by DNA-damaging agents [6,28]. DNA-damaging agents can also activate p53-dependent and -independent pathways leading to cell cycle arrest and apoptosis. Since HCT-116 cells contain a wild-type *p53* and *p21* genes, their role in G_2/M phase arrest and apoptosis is highly likely [29].

In order to asses the effect of DNA-damaging agent MNNG on cell cycle arrest and apoptosis of HCT-116 cells, we treated the synchronized cells with varying concentrations of MNNG (0 to 100 μM) for 50 h. Cells were harvested and analyzed for their distribution into different phases of cell cycle by FACS analysis. We found an increased G_2/M phase arrest in cells treated with lower concentrations of MNNG (0–25 μM), which dropped to the control level in cells treated with higher concentrations of MNNG (50 and 100 μM) (Figure 1). The number of cells in the G_0/G_1 phase decreased in a dose-dependent manner as concentrations of MNNG treatment increased to 25 μM, but beyond this concentration of MNNG treatment, an increased G_0/G_1 phase arrest in HCT-116 cells was evident. Interestingly, at 100 μM of MNNG treatment, the HCT-116 cells showed a major change in their cell cycle arrest profile in which the G_0/G_1 phase arrest was significantly increased, and the G_2/M phase arrest was decreased as compared to control cells. Furthermore, the sub-G_1 cells, which represent the apoptotic cells, increasingly accumulated until 50 μM of MNNG treatment and then drastically decreased to the control level at 100 μM of MNNG treatment (Figure 1). These results suggest that HCT-116 cells exhibit a dual response, i.e., a G_2/M phase arrest and apoptosis and G_0/G_1 phase arrest, respectively, after treatment with lower or higher concentrations of MNNG.

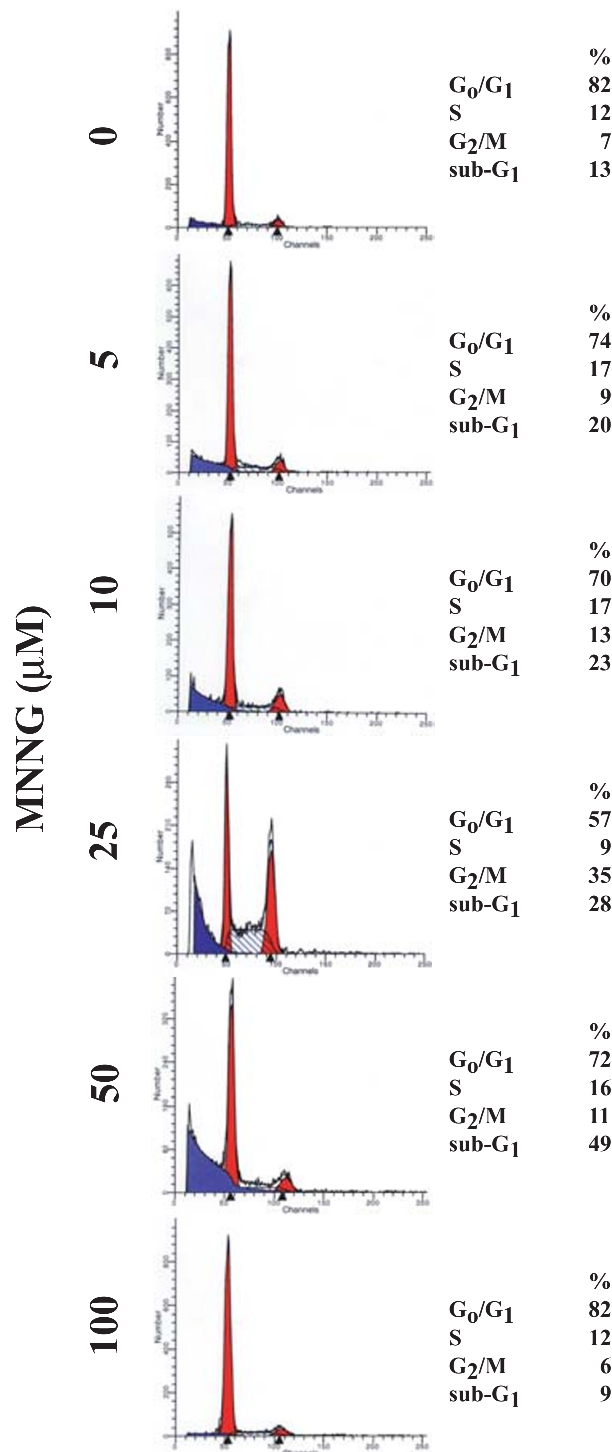

Figure 1
Cell cycle analysis in HCT-116 cells treated with MNNG. HCT-116 cells were treated with different concentrations of MNNG for 50 h. Cells were harvested and analyzed for cell cycle profile by FACScan analysis. The ranges of G_0/G_1, S, G_2/M, and sub-G_1 phase cells were established on the basis of the corresponding DNA content of the histograms. The data is representative of three different experiments carried out independently. The G_0/G_1, S, G_2/M and sub-G_1 cells are given as a percentage of the total counted cells.

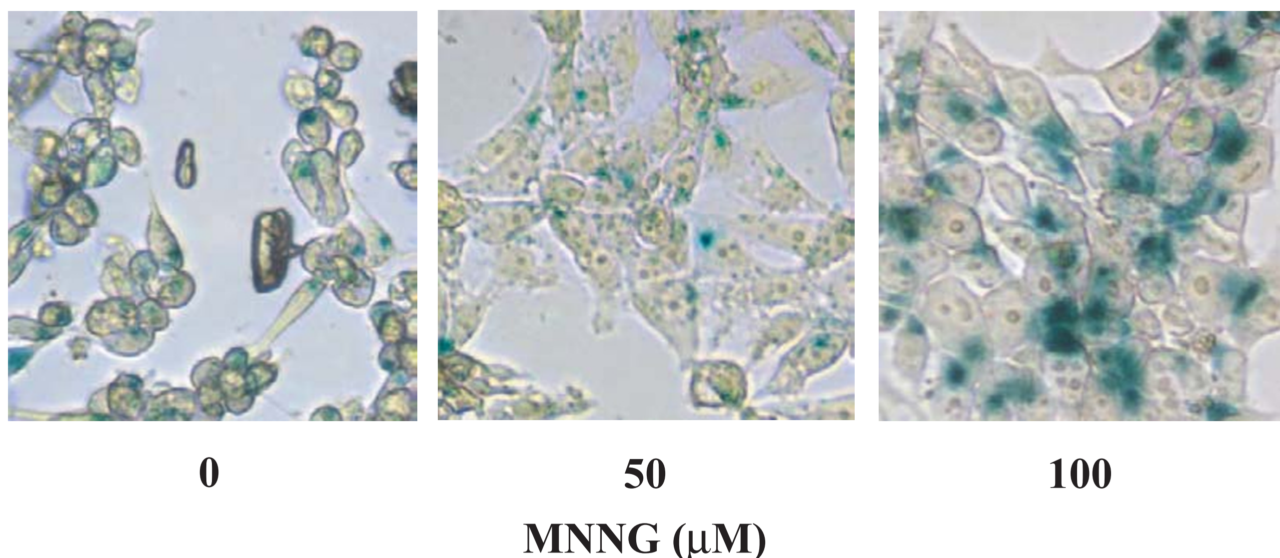

Figure 2
Senescence associated β-Galactosidase staining in HCT-116 cells treated with MNNG. HCT-116 cells were fixed after treatment either with 50 or 100 μM MNNG and processed for β-galactosidase staining as described in Materials and Methods. Stained cells were observed under the microscope and images were captured.

Senescence-like growth arrest in HCT-116 cells treated with higher concentrations of MNNG

To examine whether increased G_0/G_1 phase arrest of HCT-116 cells treated with higher concentrations of MNNG was due to increased senescence, we analyzed senescent-associated β-galactosidase expression in untreated- and MNNG-treated HCT-116 cells. Our result showed that senescence-associated β-galactosidase staining increased in cells treated with 100 μM MNNG as compared to untreated cells (Figure 2). We found no change in β-galactosidase staining in HCT-116 cells either untreated or treated with lower concentrations of MNNG (Figure 2). These results suggest that treatment of HCT-116 cells with higher concentration of MNNG caused senescence-like G_0/G_1 phase arrest.

G_2/M phase arrest and apoptosis, but not senescence-like G_0/G_1 phase arrest, is associated with increased levels of p53, p21(waf-1/cip-1), Cdc2/cyclin B1, and c-Myc proteins in HCT-116 cells treated with higher concentrations of MNNG

Since p53 is one of the important mediators of senescence response, we analyzed the level of p53 protein in HCT-116 cells treated for 50 h with varying concentrations of MNNG. The increased p53 protein level was seen and correlated with G_2/M phase arrest and apoptosis in HCT-116 cells treated with lower concentrations of MNNG. However, at higher concentrations of MNNG treatment, a drastically reduced level of p53 protein was observed. Since an increased level of p53 protein is often associated with senescence, the decreased level of p53 protein and increased level of senescence in HCT-116 cells treated with higher concentrations of MNNG suggests that the role of p53 is probably not involved in MNNG-induced senescence-like growth arrest in HCT-116 cells. We further tested whether the decrease in the p53 protein level at 100 μM MNNG treatment was a transcriptional or a post-translational phenomenon. We carried out the Northern blot analysis for *p53* mRNA levels in HCT-116 cells treated with different concentrations of MNNG. Results showed an increased level of *p53* mRNA up to 10 μM MNNG treatment. However, at higher concentrations of MNNG treatment, a reduced level of *p53* mRNA level was observed (Figure 3). The decreased level of *p53* mRNA at 25 μM or higher concentrations of MNNG treatment did not correlate with the increased level of p53 protein. These results suggest that the treatment of HCT-116 cells with 0–10 μM MNNG is a transcriptional phenomenon, while the treatment with 25–50 μM MNNG is a post-translational protein stabilization effect of p53. However, treatment with 100 μM MNNG caused a very low level of both *p53* mRNA and protein. We expected a post-translational stabilization of p53 at 100 μM MNNG treatment, as it was found after lower concentrations of MNNG treatment, but this was not the case. It appears that both transcription-mediated down-regulation of *p53* gene

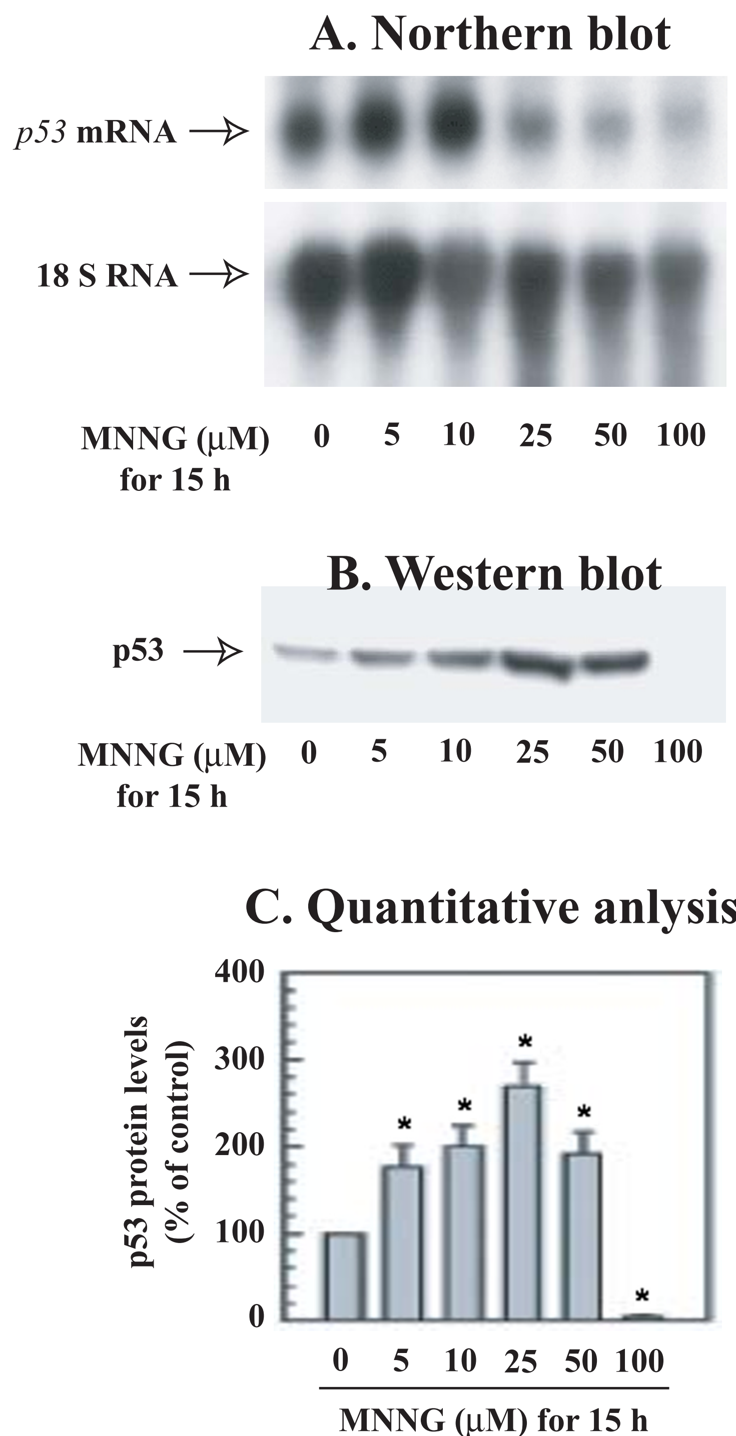

Figure 3
***p53* mRNA and protein levels in HCT-116 cells treated with MNNG.** HCT-116 cells were treated with different concentrations of MNNG (0–100 μM) for 15 h, and total RNA was isolated for Northern blot analysis as described in Materials and Methods. After MNNG, treatment cells were also harvested for the preparation of whole cell lysate. **Panel A** shows a representative autoradiogram of the *p53* mRNA and 18 S RNA. **Panel B** shows the p53 protein level in HCT-116 cells after treatment with 50 μM MNNG treatment for different periods. **Panel C** is a quantitative analysis of the p53 protein level. The data are mean ± SE of three different experiments. * = significantly different than untreated cells.

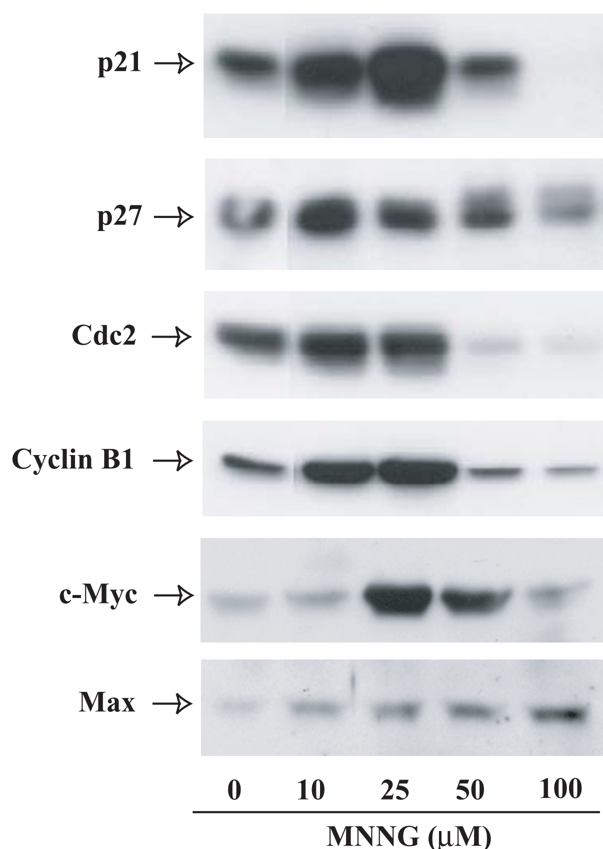

Figure 4
Western blot analysis of cell cycle-related proteins. HCT-116 cells were treated with different concentrations of MNNG and then processed for Western blot analysis of cell cycle related proteins such as p21(Waf-1/Cip1), p27, Cdc2, Cyclin B1, c-Myc and Max. Arrows indicate the position of the bands. The molecular sizes of p21(Waf-1/Cip1), p27, Cdc2, cyclin B1, c-Myc and Max proteins on the Western blots were identified as 21, 27, 34, 51, 67, and 41 kDa, respectively.

expression and de-stabilization of p53 protein are playing a role to exhibit reduced levels of p53 protein at 100 μM MNNG treatment (Figure 3).

We further examined the involvement of other cell cycle related proteins in the senescence-like G_0/G_1 phase arrest in HCT-116 cells treated with 100 μM MNNG. In these experiments, we analyzed the protein levels of cyclin-dependent kinase inhibitors p21(Waf1/Cip1) and p27 in MNNG-treated HCT-116 cells. Our results showed that the protein levels of p21(Waf1/Cip1) and p27 were increased up to 25 μM MNNG treatment and then decreased after higher concentrations of MNNG treatment (Figure 4). Since Cdc2 and cyclin B1 are associated with G_2/M phase arrest [30], we also determined their protein levels in HCT-116 cells treated with MNNG. The result showed an increased Cdc2 and cyclin B1 levels in cells treated with up to 25 μM MNNG and then decreased after higher concentrations of MNNG treatment as compared to control cells (Figure 4). The increased Cdc2/cyclin B1 levels correlated with G_2/M phase arrest in HCT-116 cells treated with lower concentrations of MNNG. However, in senescence-like arrested cells it appeared that Cdc2/cyclin B1 does not play any role, since the expression of these proteins was drastically decreased in these cells. We next determined whether the c-Myc and Max protein levels were altered after MNNG treatment. c-Myc transcription factor heterodimerizes with Max and regulates the expression of genes involved in cellular proliferation, differentiation and apoptosis [31]. It also regulates cell cycle progression and expression of other genes, which control cellular senescence [32]. In our previous studies, we found an increased level of c-Myc in HCT-116 cells treated with 25 and 50 μM MNNG as compared to control cells, which decreased after 50 μM MNNG treatment. On the other hand, the Max protein level increased at higher concentrations of MNNG treatment (Figure 3). These results suggest that Max, but not c-Myc, might be playing some role in MNNG-induced senescence-like growth arrest in HCT-116 cells, however, the mechanism is not yet clear.

Loss of APC protein level is associated with senescence-like G_0/G_1 phase arrest in HCT-116 cells treated with higher concentrations of MNNG

There are reports showing that the loss of *APC* function can result in chromosomal instability [12,16,33]. However, there are no reports available to suggest a clear involvement of APC in senescence. In our previous studies, we have indicated that treatment with lower concentrations of MNNG induces *APC* gene expression in HCT-116 cells [20]. The HCT-116 cells express a wild-type *APC* gene and translate a full-length APC protein [20]. In the present investigation, we tested whether the *APC* gene expression is affected at higher concentrations of MNNG treatment and whether the APC protein levels are associated with senescence-like growth arrest in HCT-116 cells. The results showed an increased APC protein level in HCT-116 cells treated with up to 25 μM MNNG and a decreased level of APC protein after treatment with higher concentration of MNNG (Figure 5). To further determine whether the decrease in the APC protein level after treatment with higher concentrations of MNNG was due to a transcriptional or a post-transcriptional effect, we measured the *APC* mRNA level in HCT-116 cells. The results showed an increased level of *APC* mRNA in a dose-dependent manner up to 50 μM MNNG treatment, while at the 100 μM MNNG treatment, the *APC* mRNA level was drastically decreased. In these experiments, the cells were

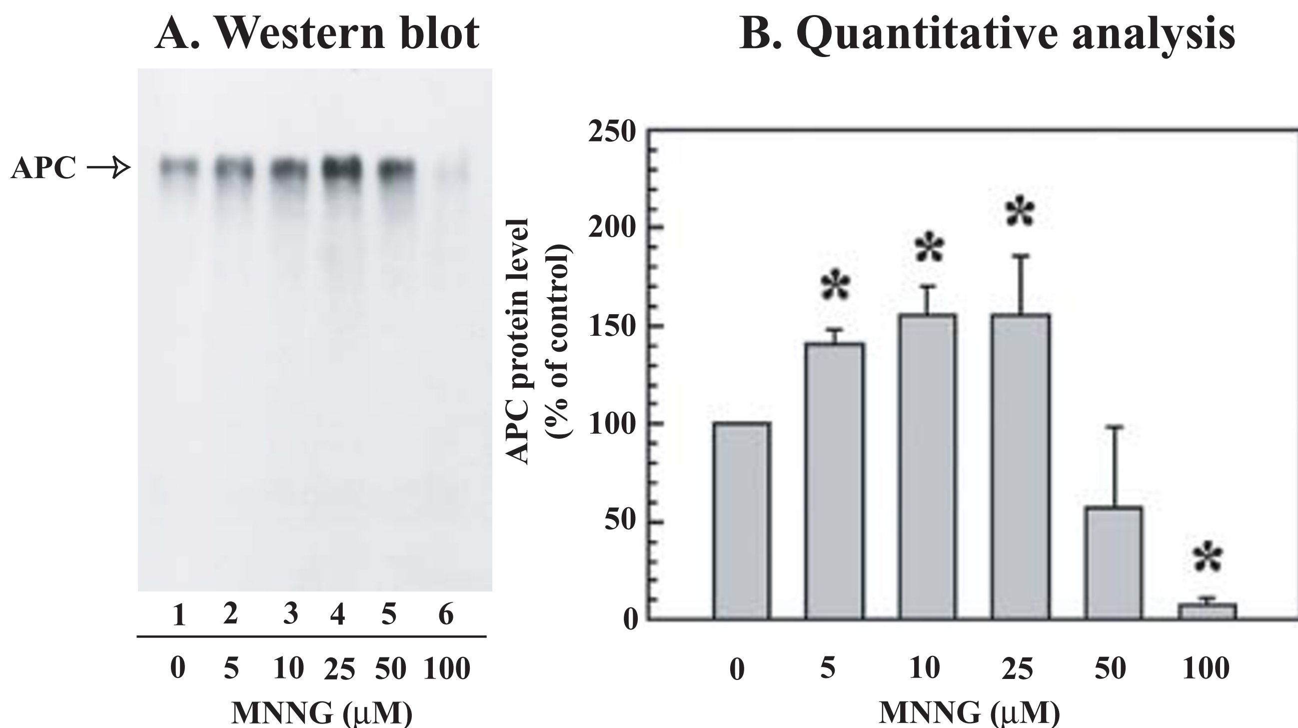

Figure 5
APC protein levels in HCT-116 cells treated with MNNG. HCT-116 cells were treated with different concentrations of MNNG (0–100 µM) for 15 h, and then APC protein was analyzed as described in Materials and Methods. **Panel A** shows a representative autoradiogram of the APC protein.**Panel B** is a quantitative analysis of the APC protein level in MNNG-treated HCT-116 cells. The data are mean ± SE of three different experiments. * = significantly different than untreated cells.

treated for 15 h with MNNG (Figure 6, Panel A). However, after 50 h of treatment with MNNG, the *APC* mRNA levels decreased (Figure 6, Panel B), which paralleled with the decreased levels of APC protein (Figure 5). These results suggest that the decreased level of APC protein is due to reduced *APC* gene expression in HCT-116 cells treated with higher concentrations of MNNG.

Loss of microtubule organization is associated with senescence-like G_0/G_1 arrest in HCT-116 cells treated with higher concentrations of MNNG

The sub-cellular distribution of APC, its interaction with microtubules [34] and its role in chromosomal segregation [16,33] has been reported. Microtubules are important components to keep kinetochores intact. It has been shown that a microtubule-depolymerizing agent can dissociate APC from kinetochores resulting in impaired segregation of chromosomes [16,33]. Based upon these findings, we suspected that the loss of APC in HCT-116 cells treated with higher concentrations of MNNG may lead to microtubule disorganization. For these experiments, the HCT-116 cells were grown on cover slips and treated with different concentrations of MNNG for 50 h. Cells were fixed and analyzed for APC and microtubule structural protein, α-tubulin, organization in HCT-116 cells by immunohistochemistry. After treatment with MNNG, cells were also examined for their morphological changes followed by immunostaining. Morphological observations suggested that cells were still viable, but their growth was drastically reduced at higher concentrations of MNNG treatment as compared to untreated cells. Immunostaining showed a reduced level of APC protein in HCT-116 cells treated with 100 µM MNNG. The analysis of α-tubulin after immunostaining also showed a loss of structural integrity of cells treated with 100 µM MNNG. A disrupted association of α-tubulin and APC can be seen in the cells treated at this concentration (Figure 7, see merged staining). These results indicate that the structural organization of APC and α-tubulin proteins is disrupted in MNNG-induced senescence-like G_0/G_1 phase arrested HCT-116 cells.

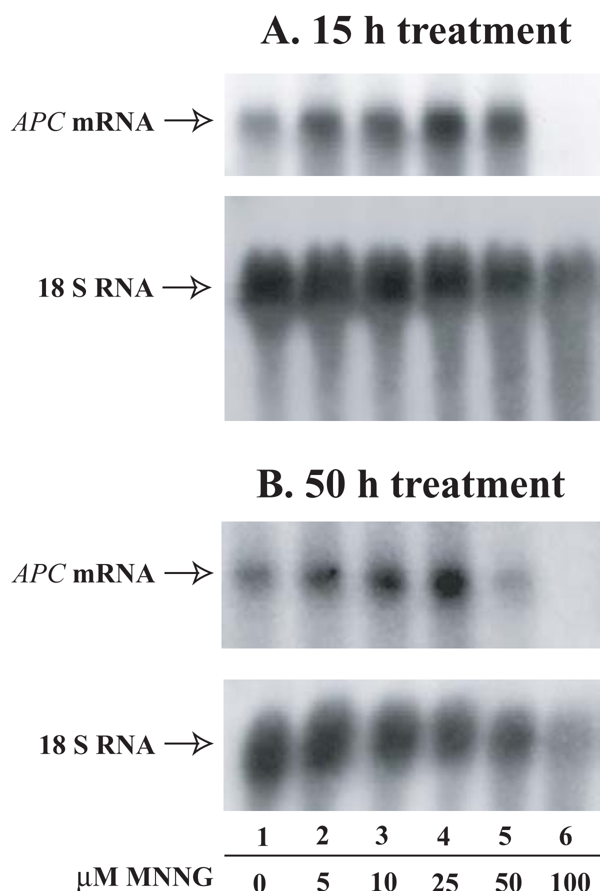

Figure 6
***APC* mRNA levels in HCT-116 cells treated with MNNG.** HCT-116 cells were treated with different concentrations of MNNG (0–100 μM) either for 15 h or for 50 h. After the treatment, the total RNA was isolated for Northern blot analysis as described in Materials and Methods. **Panel A** shows a representative autoradiogram of *APC* mRNA and corresponding 18 S RNA after 15 h of MNNG (0–100 μM) treatment. **Panel B** shows a representative autoradiogram of *APC* mRNA and corresponding 18 S RNA after 50 h of MNNG (0–100 μM) treatment. The level of 18 S mRNA was used for normalizing loading errors and transfer efficiency.

Loss of telomeric DNA is associated with senescence-like G_0/G_1 phase arrest in HCT-116 cells treated with higher concentrations of MNNG
It has been reported that telomere shortening triggers replicative senescence in human cells [3]. The exact relationship between DNA damage-induced telomere loss and cellular senescence has not been clearly defined. In the present study, we examined whether senescence-like G_0/G_1 phase arrest of HCT-116 cells after treatment with higher concentrations of MNNG was associated with the loss of telomeric DNA. HCT-116 cells were treated with 50 and 100 μM MNNG for 50 h and processed for Q-FISH analysis. Results showed a dose-dependent decrease in telomeric DNA signals after treatment with MNNG as compared to untreated cells (Figure 8). These results suggest that the decreased level of telomeric DNA is associated with MNNG-induced senescence in HCT-116 cells.

Discussion
Mutations in the Adenomatous polyposis coli (*APC*) gene are believed to be an early event in the tumorogenesis and results in the production of truncated APC protein [12,35]. Previously, it has been shown that the primary effect of the loss of expression of the *APC* gene in polyps is the accumulation and stabilization of β-catenin protein [19,36]. The stabilized β-catenin then translocates to the nucleus and binds to T-cell factor (Tcf)/lymphoid enhancer factor (Lef), a nuclear transcription factor, and induces target genes such as *cyclin D1* and the oncogene *c-myc* [37,38]. In the present study, we determined the involvement of the APC protein in DNA damage-induced senescence-like G_0/G_1 phase arrest of HCT-116 cells. Our results showed that the treatment of HCT-116 cells with lower concentrations of MNNG (0–50 μM) for 50 h resulted in a dose-dependent increase of the G_2/M phase arrest and apoptosis. The G_2/M phase arrest and apoptosis was associated with an increased level of cellular p53 protein, which was consistent with previous observations [22,29]. The p53 responds to DNA damage by activating transcription-dependent and -independent pathways leading to cell cycle arrest and/or apoptosis and preventing proliferation of cells with damaged genome [39]. The increased or stabilized level of p53 and p21(Waf-1/Cip1) has been suggested to play a critical role in the G_2/M phase arrest and apoptosis [24,40], which is consistent with our previous studies in HCT-116 cells treated with lower concentrations of MNNG [41]. However, HCT-116 cells treated with higher concentrations of MNNG showed a senescence-like G_0/G_1 phase arrest but did not show any increase in p53 protein level. These results suggest that the p53-mediated pathway was not involved in the senescence-like G_0/G_1 phase arrest of HCT-116 cells. It has been suggested that increased transcriptional activity of p53 protein also plays an important role in the senescence-like G_0/G_1 phase arrest via transactivation of *p21(Waf-1/Cip1)* gene [42]. Since p21(Waf-1/Cip1) protein level was decreased in HCT-116 cells treated with higher concentrations of MNNG, our findings further suggest that the p53/p21(Waf-1/cip1) pathway is not involved in senescence-like G_0/G_1 phase arrest of HCT-116 cells. Further, we found that other cell cycle related proteins such as p27, cdc2, Cyclin B1, c-Myc and Max were also not involved in senescence-like G_0/G_1 phase arrest in HCT-116 cells; although, their role in senescence-like G_0/G_1 arrest have

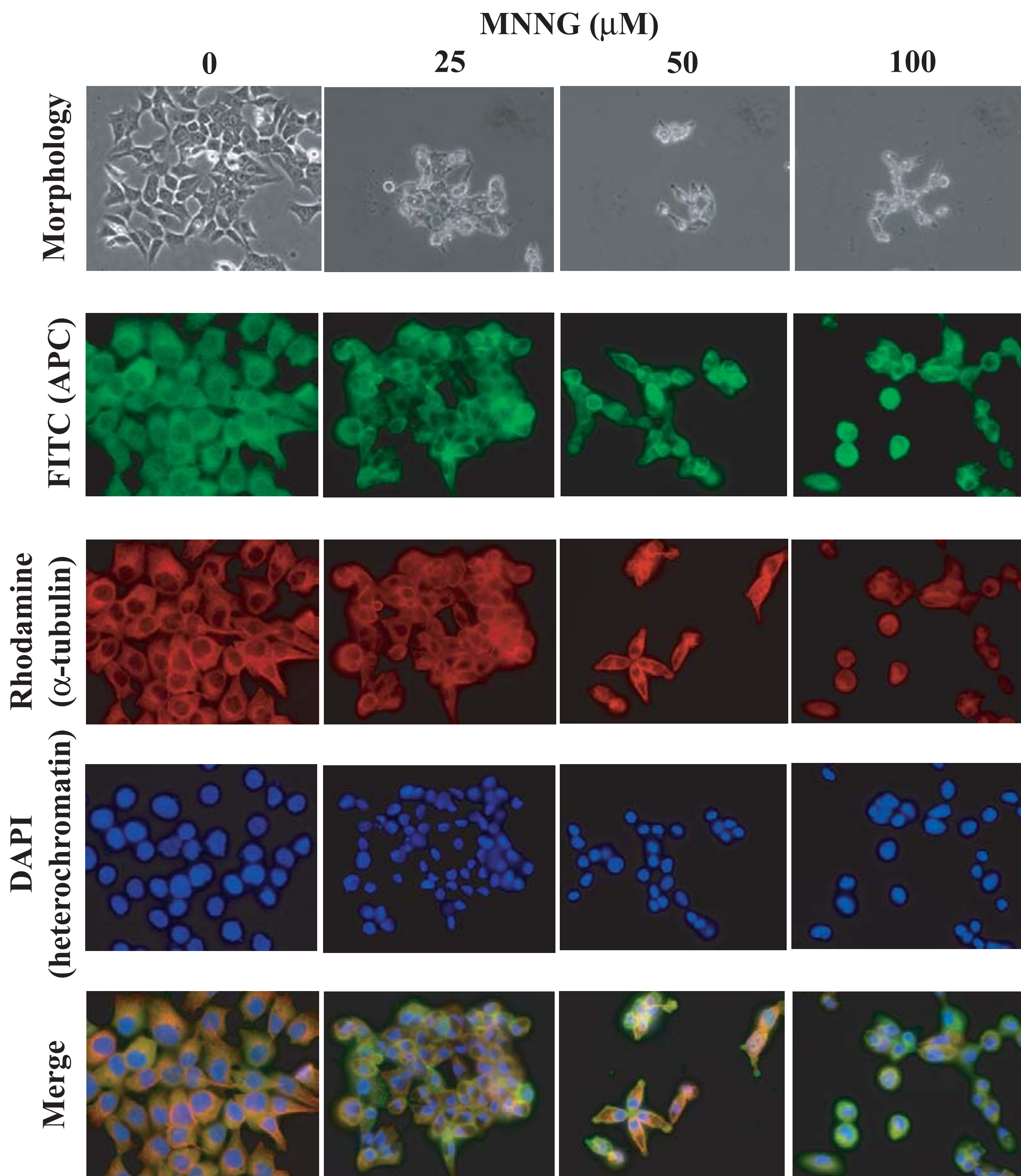

Figure 7
Immunohistochemical analysis of APC and α-tubulin proteins in HCT-116 cells after treatment with MNNG. Cells were grown in 0.5% FBS containing medium on cover slips and treated with different concentrations of MNNG for 50 h. Cells were fixed and processed for immunofluorescence staining as described in the Materials and Methods. Images were captured on the Zeiss upright microscope. Morphological changes were also recorded on an inverted Zeiss Microscope.

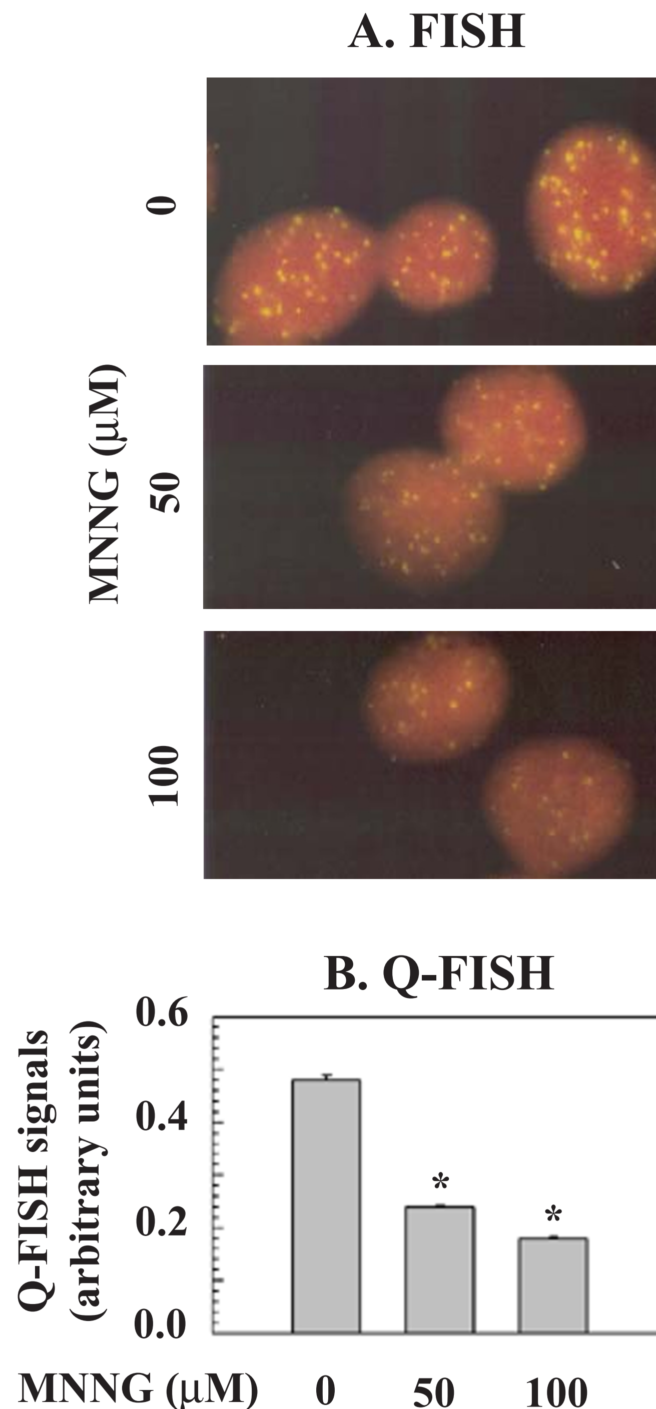

Figure 8
Determination of telomeric DNA levels by Q-FISH analysis in HCT-116 cells treated with MNNG. HCT-116 cells were treated with 50 and 100 μM of MNNG and then processed for Q-FISH analysis as described in Materials and Methods. **Panel A** shows the data of a FISH analysis. **Panel B** shows the quantitative analysis of the FISH data of the control and MNNG-treated cells. The percentage telomeric area of the interphase nuclei in FISH preparations was quantified using a software package (Metaview Imaging system-3.6a; Universal Imaging Co., Westchester, PA). From each sample at least 50 interphase nuclei were quantified, and mean values of percent telomeric area compared to the nuclear area were calculated. The values for the treated cells were compared to the control cells.

been shown in previous studies [32,43-45]. The difference in these and previous studies could have been due to differences in cell type and DNA damaging agents.

Once we determined that the p53/p21(Waf-1/Cip1) pathway was not involved in MNNG-induced senescence-like G_0/G_1 phase arrest of HCT-116 cells, then we looked at other parameters which are suggested to be associated with chromosomal abnormalities and likely with senescence. For this part of the study, we determined the expression level of APC, α-tubulin, and telomeric DNA. The loss of APC protein can cause chromosomal instability (CIN) and microtubule disorganization that can lead to aneuploidy [16]. The aneuploid cells can choose cell death, senescence, or cell survival pathways, depending upon the genetic pressure exerted upon these cells [46,47]. In the present study, we found that cells exhibiting senescence-like G_0/G_1 phase arrest after treatment with higher concentrations of MNNG showed a drastically reduced level of APC and α-tubulin proteins, which suggest their role in MNNG-induced chromosomal instability and perhaps senescence-like G_0/G_1 phase arrest of HCT-116 cells. This statement needs to be further verified by using the APC overexpression system to block DNA damage-induced senescence in these cells. In fact, the association of APC with microtubules plays an important role in chromosomal segregation in which the APC is specifically detected at the kinetochores, and the binding of APC at kinetochores requires intact microtubules [16,33].

Next, we examined whether the loss of MNNG-induced telomeric DNA was also associated with senescence-like G_0/G_1 phase arrest of HCT-116 cells. In previous studies, a DNA damage checkpoint response in telomere-initiated senescence has been described in human diploid fibroblast (HDF) cell lines [48]. Although we have not determined the activity of checkpoint responsive CHK1 and CHK2 kinase activities, the MNNG-induced level of telomeric DNA was significantly decreased in HCT-116 cells. In earlier studies, we have shown that HCT-116 cells treated with lower concentrations of MNNG showed a dose-dependent loss of telomeric DNA in a p53-independent manner [41]. In these studies, the loss in the amount of telomeric DNA at 50 μM MNNG treatment was approximately two-fold. However, in the present study, the loss in the amount of telomeric DNA at 100 μM MNNG treatment was more than two-fold. From these results, it appears that approximately two-fold loss of telomeric DNA favors G_2/M phase arrest and apoptosis, and more than two-fold loss of telomeric DNA after treatment with MNNG is linked with senescence-like growth.

Conclusions

Our results suggest that MNNG-induced senescence-like growth arrest of HCT-116 cells is associated with decreased levels of APC, α-tubulin, and telomeric DNA. Thus, DNA damage-induced senescence-like growth arrest can protect cells from abnormal growth and carcinogenesis.

Materials and Method

Maintenance and treatment of cells

Human colon cancer cell line HCT-116 was grown in McCoys 5a medium supplemented with 10% fetal bovine serum (FBS; Cell Grow, Mediatech, VA), 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. After cells reached 60% confluence, fresh medium containing 0.5% FBS and antibiotics were added to each plate and then further incubated for an additional 18 h. Treatment regimen with MNNG (Aldrich Chemical Co., Milwaukee, WI) is given in the figure legends.

FACS analysis

A detergent and proteolytic enzyme-based technique was used for nuclear isolation and DNA content analysis of cells in different phases of cell cycle. After treatment with different concentrations of MNNG for 50 h, cells were harvested and processed for staining of nuclei with propidium iodide [49]. The cellular DNA content was analyzed by the Becton-Dickinson FACScan flow cytometer (BD Biosciences, San Jose, CA). At least 10,000 cells per sample were considered in each gated region for calculations. The ranges for G_0/G_1, S, G_2/M and sub-G_1 phase cells were established based upon their corresponding DNA contents of histograms. Results were analyzed and expressed as a percentage of the total gated cells using the ModfitLT-V2.0 program.

Senescence-associated β-galactosidase staining

HCT-116 cells were treated with MNNG for 50 h and then washed three times with phosphate-buffered saline (PBS). Cells were fixed in 4% paraformaldehyde and again washed three times with PBS. Cells were incubated with freshly made β-galactosidase staining solution (1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM magnesium chloride in PBS at 37°C (without CO_2) as described earlier by Dimri et al. [27]. Staining of cells was observed under the microscope (Leica, Manheim, Germany), and images were captured using the magnifier program (Optelec US Inc., MA).

Western blot analysis

Changes in protein levels, subsequent to MNNG treatment, were determined using Western blot analysis of whole cell extracts as described previously [20]. In this study, the following antibodies were used to detect various cell cycle-related proteins: anti-APC (Ab-1) mouse monoclonal antibody from Oncogene Research Products

(Cambridge, MA), and anti-APC (N-15), anti-Cdc-2 p34 (17), anti-Cyclin B1 (GNS-1), anti-p21 (F-5), anti-p27, anti-c-Myc and anti-Max were from Santa Cruz Biotechnology (Santa Cruz, CA).

Northern blot analysis

For northern blot analysis, the total RNA from untreated- and MNNG-treated cells was isolated by TRIzol™ reagent as described by the manufacturer (Invitrogen Life Technologies, CA). Then 50 μg of total RNA were separated on 1% formaldehyde-agarose gel and transferred onto a Hybond-N+ membrane (Amersham Biosciences Corp., NJ). The membrane was prehybridized for 6 h at 65°C in 0.5 M sodium phosphate buffer (pH 7.2), 7% (w/v) SDS, 1 mM EDTA, and 1% (w/v) bovine serum albumin (BSA) and then hybridized with ^{32}P-labeled *APC* probe (*Eco*RI fragment of APC-HFBCI43; ATCC, Manassas, VA). Later the same membrane was reprobed with ^{32}P-labeled *Eco*RI fragment of 18 S RNA probe for normalization of RNA loading and transfer efficiency. The membranes were exposed to x-ray films for detection of specific mRNA signals.

Immunohistochemical analysis

Immunohistochemical analysis was performed to examine the localization of APC and α-tubulin proteins in untreated- and MNNG-treated cells. Briefly, 5×10^5 cells were grown on cover slips. Once cells reached 60% confluence, fresh medium containing 0.5% FBS and antibiotics were added to each dish and then further incubated for 18 h. Treatment regimen with MNNG is given in figure legends. After treatment with MNNG, cells were washed with PBS and fixed with 4% paraformaldehyde solution for 30 min at 22°C. After fixing cells, cover slips were washed again with PBS and incubated for 30 min with 50 mM NH_4Cl in PBS containing 0.2% triton X-100. After washing with PBS, cells were further incubated for 2 h at 22°C with either anti-APC (N-15) rabbit polyclonal or anti-α-tubulin antibody (dilution 1:100) in 5% goat serum containing 0.2% triton X-100. Unbound antibodies were washed with PBS buffer and antibodies were stained for 1 h at 22°C with anti-rabbit secondary antibody conjugated to FITC or rhodamine (dilution 1:200) in 5% goat serum, 0.2% triton X-100 in PBS. After washing, a drop of DAPI (in mounting solution) was added, and cover slips were sealed from sides using nail polish. Slides were viewed under Zeiss Axioplan-2 imaging upright microscope (Zeiss, Thornwood, NY) systems using different filters, and images were captured using the open lab program.

Quantitative fluorescence in situ hybridization (Q-FISH) analysis

The control and MNNG-treated HCT-116 cells were harvested and processed for cytological preparations for Q-FISH analysis as described previously [50].

Authors' contributions

ASJ and SN drafted the paper. AM and SP did the Q-FISH analysis. All authors read and approved the final draft of the manuscript.

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References

1. Hayflick L: **The limited in vitro lifetime of human diploid cell strains.** *Exp Cell Res* 1965, **37:**614-636.
2. Hayflick L, Moorhead PS: **The serial cultivation of human diploid cell strains.** *Exp Cell Res* 1961, **25:**585-621.
3. Campisi J, Kim SH, Lim CS, Rubio M: **Cellular senescence, cancer and aging: the telomere connection.** *Exp Gerontol* 2001, **36:**1619-1637.
4. Campisi J: **Aging, chromatin and food restriction – connecting the dots.** *Science* 2000, **289:**2126-2128.
5. Chen Q, Ames BN: **Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells.** *Proc Natl Acad Sci USA* 1994, **91:**4130-4134.
6. Robles SJ, Adami GR: **Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts.** *Oncogene* 1998, **16:**1113-1123.
7. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW: **Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a.** *Cell* 1997, **88:**593-602.
8. Zhu J, Woods D, McMahon M, Bishop JM: **Senescence of human fibroblasts induced by oncogenic Raf.** *Genes Dev* 1998, **12:**2997-3007.
9. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M: **The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus.** *Nature* 1999, **397:**164-168.
10. Kinzler KW, Vogelstein B: **Lessons from hereditary colorectal cancer.** *Cell* 1996, **87:**159-170.
11. Levine AJ: **p53, the cellular gatekeeper for growth and division.** *Cell* 1997, **88:**323-331.
12. Bienz M: **The subcellular destinations of APC proteins.** *Nat Rev Mol Cell Biol* 2002, **3:**328-338.
13. Narayan S, Roy D: **Role of APC and DNA mismatch repair genes in the development of colorectal cancers.** *Molecular Cancer* 2003, **2:**41.
14. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M: **Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis.** *Nature* 1993, **363:**558-561.
15. Lengauer C, Kinzler KW, Vogelstein B: **Genetic instability in colorectal cancers.** *Nature* 1997, **386:**623-627.
16. Fodde R, Kuipers J, Rosenberg C, Smits R, Kielman M, Gaspar C, van Es JH, Breukel C, Wiegant J, Giles RH, Clevers H: **Mutations in the APC tumour suppressor gene cause chromosomal instability.** *Nat Cell Biol* 2001, **3:**433-438.
17. Munemitsu S, Souza B, Muller O, Albert I, Rubinfeld B, Polakis P: **The APC gene product associates with microtubules in vivo and promotes their assembly in vitro.** *Cancer Res* 1994, **54:**3676-3681.
18. Smith KJ, Levy DB, Maupin P, Pollard TD, Vogelstein B, Kinzler KW: **Wild-type but not mutant APC associates with the microtubule cytoskeleton.** *Cancer Res* 1994, **54:**3672-3675.
19. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H: **Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma.** *Science* 1997, **275:**1784-1787.

20. Narayan S, Jaiswal AS: **Activation of adenomatous polyposis coli (APC) gene expression by the DNA-alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine requires p53.** *J Biol Chem* 1997, **272:**30619-30622.
21. Jaiswal AS, Narayan S: **p53-dependent transcriptional regulation of the APC promoter in colon cancer cells treated with DNA alkylating agents.** *J Biol Chem* 2001, **276:**18193-18199.
22. Vogelstein B, Lane D, Levine AJ: **Surfing the p53 network.** *Nature* 2000, **408:**307-310.
23. Itahana K, Dimri G, Campisi J: **Regulation of cellular senescence by p53.** *Eur J Biochem* 2001, **268:**2784-2791.
24. Vousden KH, Lu X: **Live or let die: the cell's response to p53.** *Nat Rev Cancer* 2002, **2:**594-604.
25. Chan TA, Hwang PM, Hermeking H, Kinzler KW, Vogelstein B: **Cooperative effects of genes controlling the G(2)/M checkpoint.** *Genes Dev* 2000, **14:**1584-1588.
26. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE: **Extension of life-span by introduction of telomerase into normal human cells.** *Science* 1998, **279:**349-352.
27. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi J: **A biomarker that identifies senescent human cells in culture and in aging skin in vivo.** *Proc Natl Acad Sci USA* 1995, **92:**9363-9367.
28. Chen Q, Fisher A, Reagan JD, Yan LJ, Ames BN: **Oxidative DNA damage and senescence of human diploid fibroblast cells.** *Proc Natl Acad Sci USA* 1995, **92:**4337-4341.
29. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW, Vogelstein B: **Requirement for p53 and p21 to sustain G2 arrest after DNA damage.** *Science* 1998, **282:**1497-1501.
30. Taylor WR, DePrimo SE, Agarwal A, Agarwal ML, Schonthal AH, Katula KS, Stark GR: **Mechanisms of G2 arrest in response to overexpression of p53.** *Mol Biol Cell* 1999, **10:**3607-3622.
31. Dang CV, Resar LM, Emison E, Kim S, Li Q, Prescott JE, Wonsey D, Zeller K: **Function of the c-Myc oncogenic transcription factor.** *Exp Cell Res* 1999, **253:**63-77.
32. Grandori C, Wu KJ, Fernandez P, Ngouenet C, Grim J, Clurman BE, Moser MJ, Oshima J, Russell DW, Swisshelm K, Frank S, Amati B, Dalla-Favera R, Monnat RJ Jr: **Werner syndrome protein limits MYC-induced cellular senescence.** *Genes Dev* 2003, **17:**1569-1574.
33. Kaplan KB, Burds AA, Swedlow JR, Bekir SS, Sorger PK, Nathke IS: **A role for the Adenomatous Polyposis Coli protein in chromosome segregation.** *Nat Cell Biol* 2001, **3:**429-432.
34. Nathke IS, Hinck L, Swedlow JR, Papkoff J, Nelson WJ: **Defining interactions and distributions of cadherin and catenin complexes in polarized epithelial cells.** *J Cell Biol* 1994, **125:**1341-1352.
35. van Es JH, Giles RH, Clevers HC: **The many faces of the tumor suppressor gene APC.** *Exp Cell Res* 2001, **264:**126-134.
36. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW: **Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC.** *Science* 1997, **275:**1787-1790.
37. Shutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Avri Ben-Ze'ev: **The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway.** *Proc Natl Acad Sci USA* 1999, **96:**5522-5527.
38. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: **Identification of c-MYC as a target of the APC pathway.** *Science* 1998, **281:**1509-1512.
39. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B: **WAF1, a potential mediator of p53 tumor suppression.** *Cell* 1993, **75:**817-825.
40. Gartel AL, Tyner AL: **The role of the cyclin-dependent kinase inhibitor p21 in apoptosis.** *Mol Cancer Ther* 2002, **1:**639-649.
41. Narayan S, Jaiswal AS, Multani AS, Pathak S: **DNA damage-induced cell cycle checkpoints involve both p53-dependent and -independent pathways: role of telomere repeat binding factor 2.** *Br J Cancer* 2001, **85:**898-901.
42. Bond J, Haughton M, Blaydes J, Gire V, Wynford-Thomas D, Wyllie F: **Evidence that transcriptional activation by p53 plays a direct role in the induction of cellular senescence.** *Oncogene* 1996, **13:**2097-2104.
43. Schwarze SR, Shi Y, Fu VX, Watson PA, Jarrard DF: **Role of cyclin-dependent kinase inhibitors in the growth arrest at senescence in human prostate epithelial and uroepithelial cells.** *Oncogene* 2001, **20:**8184-8192.
44. Richter KH, Afshari CA, Annab LA, Burkhart BA, Owen RD, Boyd J, Barrett JC: **Down-regulation of cdc2 in senescent human and hamster cells.** *Cancer Res* 1991, **51:**6010-6013.
45. Marcotte R, Qian JF, Chen J, Wang E: **hMad4, c-Myc endogenous inhibitor, induces a replicative senescence-like state when overexpressed in human fibroblasts.** *J Cell Biochem* 2003, **89:**576-588.
46. Breivik J, Gaudernack G: **Genomic instability, DNA methylation, and natural selection in colorectal carcinogenesis.** *Semin Cancer Biol* 1999, **9:**245-254.
47. Bardelli A, Cahill DP, Lederer G, Speicher MR, Kinzler KW, Vogelstein B, Lengauer C: **Carcinogen-specific induction of genetic instability.** *Proc Natl Acad Sci USA* 2001, **98:**5770-5775.
48. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP: **A DNA damage checkpoint response in telomere-initiated senescence.** *Nature* 2003, **426:**194-198.
49. Jaiswal AS, Bloom LB, Narayan S: **Long-patch base excision repair of apurinic/apyrimidinic site DNA is decreased in mouse embryonic fibroblast cell lines treated with plumbagin: involvement of cyclin-dependent kinase inhibitor p21Waf-1/Cip-1.** *Oncogene* 2002, **21:**5912-5922.
50. Multani AS, Ozen M, Narayan S, Kumar V, Chandra J, McConkey DJ, Newman RA, Pathak S: **Caspase-dependent apoptosis induced by telomere cleavage and TRF2 loss.** *Neoplasia* 2000, **2:**339-345.

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