Impaired Cytogenetic Damage Repair and Cell Cycle Regulation in Response to Ionizing Radiation in Human Fibroblast Cells with Individual Knock-down of 25 Genes

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ABSTRACT

Changes of gene expression profile are one of the most important biological responses in living cells after ionizing radiation (IR) exposure. Although some studies have demonstrated that genes with up-regulated expression induced by IR may play important roles in DNA damage sensing, cell cycle checkpoint and chromosomal repair, the relationship between the regulation of gene expression by IR and its impact on cytogenetic responses to ionizing radiation has not been systematically studied. In our present study, the expression of 25 genes selected based on their transcriptional changes in response to IR or from their known DNA repair roles were individually knocked down by siRNA transfection in human fibroblast cells. Chromosome aberrations (CA) and micronuclei (MN) formation were measured as the cytogenetic endpoints. Our results showed that the yield of MN and/or CA formation were significantly increased by suppressed expression of 5 genes that included Ku70 in the DSB repair pathway; XPA in the NER pathway; RPA1 in the MMR pathway; RAD17 and RBBP8 in cell cycle control. Knocked-down expression of 4 genes including MRE11A, RAD51 in the DSB pathway, and SESN1 and SUMO1 showed significant inhibition of cell cycle progression, possibly because of severe impairment of DNA damage repair. Furthermore, loss of XPA, p21 and MLH1 expression resulted in both enhanced cell cycle progression and significantly higher yield of cytogenetic damage, indicating the involvement of these gene products in both cell cycle control and DNA damage repair. Of these 11 genes that affected the cytogenetic response, 9 were up-regulated in the cells exposed to gamma radiation, suggesting that genes transcriptionally modulated by IR were critical to regulating the biological consequences after IR. Failure to express these IR-responsive genes, such as by gene mutation, could seriously change the outcome of the post IR scenario and lead to carcinogenesis.

INTRODUCTION

Living organisms are constantly threatened by environmental DNA-damaging agents including ultra-violet (UV) and ionizing radiation (IR). The major forms of DNA damage caused by exposure to IR include single-strand breaks (SSB), double-strand breaks (DSB), hydrolytic depurination, apyrimidinic sites, and oxidative damage to the bases and the phosphodiester backbone of DNA (1). These types of DNA damage are repaired following lesion-specific repair pathways, such as DSB repair, nucleotide excision repair (NER), mismatch repair (MMR) or base excision repair (BER) (2). Specific groups of proteins from these pathways recognize DNA damage sites, and repair and induce cell cycle checkpoints. After the primary event of causing DNA damage, ionizing radiation initiates a series of signal transduction cascades responsible for arresting the cell cycle of DNA damaged cells, maintaining cellular homeostasis and sending signals to interact with neighboring cells.

As an early response to DNA damage caused by IR, the activation of protein kinases ATM/ATR (ataxia telangiectasia mutated/ATM and Rad3-related) and the down-stream P53 signaling pathway have been well studied (3,4,5). Subsequently, transcriptional regulation of various genes and post-translational modification of proteins involved in DNA damage sensing, repair and cell cycle regulation occur so that subsequent cascades of signaling pathways are initiated (6,7). Due to the regular occurrence of exposure to IR in human beings through occupational, medical, environmental and/or other sources, the biological effects of low-dose radiation is a great concern for the general population especially on mutagenesis and carcinogenesis. Many studies focused on gene expression in cells after radiation exposure have demonstrated that global gene expression profile is markedly affected by IR (8, 9, 10), and the majority of changes belong to cell-cell signaling and signal

transduction in response to very low doses (7, 11, 12, 13). Although it has been suggested that cellular responses to IR may differ qualitatively between low and high doses, the majority of genes that respond to radiation of different doses are common to both. (11).

Impaired expression of genes involved in DNA repair or other systems, especially those inducible by IR, are likely to alter the cellular response to radiation insult. Previous studies have shown that certain IR inducible genes, such as p21, may play crucial roles in regulating cell cycle events as a CDK (cyclin-dependent kinase) inhibitor (6,10) in response to radiation insult. Mutations of specific genes, such as MSH2 in MMR, can also lead to impaired DNA repair and increased genomic instability in response to DNA damage mediated by radiation (14, 15). Many of these gene mutations have been found in cancer patients as well (16). Here we have studied the relationship between the transcriptional regulation of gene expression and its impact on the cytogenetic response to IR exposure, and systematically identified the genes that affected the cytogenetic endpoints.

In this study, we first performed gene expression analysis on normal human fibroblasts exposed to 2 cGy gamma rays at a low dose rate using PCR arrays containing a set of genes involved in the DNA damage signaling pathway. 25 genes, were selected for their transcriptional changes in response to IR or their known DNA repair roles, were individually knocked down by siRNA transfection in the human fibroblast cells. After siRNA treatment, the cells were then exposed to gamma rays and analyzed for cytogentic damage by the cytokinesis-block micronucleus (MN) assay and fluorescent in situ hybridization (FISH) chromosome aberration (CA) assay. While the CA and MN formation are associated with DNA damage and repair, the percentages of bi-nucleated cells were also recorded to provide information on cell cycle progression. Some genes, such as P53, which have been

intensively studied, were not included in our gene selection, but certain genes that are modulated transcriptionally by P53, such as p21, Gadd45A and DDit3, were chosen for this study.

MATERIALS & METHODS

Cell culture and gamma radiation

Normal human foreskin fibroblasts (AG1522, National Institute of Aging) of less than 20 passages were routinely cultured at 37 ºC, 95% humidity, and 5% CO_2, in α–MEM medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen) and 50mg/ml penicillin-streptomycin (Invitrogen). The exposure to gamma rays at a low dose rate of 1 cGy/hr or a high dose rate of 0.2 Gy/min was performed at the NASA Johnson Space Center (JSC, Houston, TX).

RT2 Profiler PCR Array Assay

Cells were grown in T-75 flasks until 80% of the cells were in G1 phase of the cell cycle, and then exposed to a total of 2 cGy of gamma rays at a dose rate of 1 cGy/hr. Immediately after exposure, the irradiated and control cells were washed with cold phosphate buffered saline (PBS), and the total RNA was isolated using RNeasy Kit (Qiagen, Valencia, CA) and treated with DNase A (Ambion, Austin, TX). The cDNA was synthesized from 1μg total RNA of each sample using a RT2 PCR array first strand kit (SuperArray, Frederick, MD). The RT2 Profiler PCR Array for the DNA damage signaling pathway was then analyzed using an ABI7900HT real time PCR instrument (Applied Biosystems, Foster City, CA) to examine the expression of 84 genes according to the manufacturer's protocol. The fold-change of gene expression after the low dose-rate IR was calculated using the Excel-based PCR Array data analysis program (Applied Biosystems).

siRNA Transfection

Since the discovery of RNA interference (RNAi) by Fire et al in 1998 (17), this technology, which allows highly selective post-transcriptional gene silencing, has been proven to be a powerful tool for the inhibition of gene expression in cell culture. However, due to the polyanionic nature of siRNA, which triggers the RNAi response, delivery systems are required for naked siRNA freely cross cell membranes (18). Lipofectamine 2000 (Invitrogen) transfection system has been used in our studies and proven to reach 70-90% transfection rate in our preliminary study (data not shown). A battery of siRNA for genes of ATM, BRCA1, BRCA2, CIB1, DDIT3, GADD45A, H2AX, IHPK3, KU70, MAP2K6, MLH1, MRE11A, p21, PRKDC, RAD1, RAD17, RAD51, RBBP8, RPA1, SESN1, SUMO1, XPA, XPC, XRCC1 and XRCC3, along with positive and negative controls, was purchased from Ambion. The siRNA were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Briefly, siRNA, negative control RNA or water was mixed with Lipofectamine 2000 reagent for 20 min and added to 30-50% confluent cells in serum- and antibiotic-free media at a final concentration of 33.3 nM siRNA. FBS was added to a final concentration of 10% after 5 hrs and the cells were exposed to 0.1-2 Gy gamma ray for 48 hrs after siRNA transfection. For P21, XPA and MLH1, the cytogenetic damages were also studied for 5-75 nM siRNA.

Micronuclei Assay

A cytokinesis blocking technique was performed according to the method of Fenech and Morley (19) and described previously (20). Briefly, after acute irradiation, siRNA treated cells were incubated at 37 ºC for 5 hrs, detached with 0.01% trypsin-EDTA (Invitrogen), and plated in chamber slides with fresh medium containing 2.5 µg/ml cytochalasin B (Sigma-Aldrich, St. Louis, MO). After further incubation in cytochalasin B for 48 h, cells were washed with PBS and fixed with

methanol/acetic acid (3:1, vol/vol, Fisher Scientific, Pittsburgh, PA) for 15 minutes. For MN analysis, the cell nuclei were stained with 1μg/ml DAPI (Abbott Molecular Inc., Des Plaines, IL), and MN formation was scored in bi-nucleated cells and classified according to standard criteria (21, 22). Approximately 1000 bi-nucleated cells collected from two independent experiments were scored for each data point. The MN frequencies presented in the figures are either the percentage of BN (bi-nucleated) cells containing MN or the average number of MN per bi-nucleated cells in total of 1000 counted BN cells.

Since the MN cytokinesis-block technique is based on the observation that cytochalasin B does not interfere with cell cycle progression until immediately after nuclear division when the cells appear binucleated under the microscope, cells that do not progress through DNA replication or mitosis due to reasons such as gross DNA damage will thus appear mono-nucleated. We have recently reported that the ratio of mono- to bi-nucleated cells can be used to indicate the cell cycle progression, and such a progression is closely correlated to the cell death assayed by other techniques such as the clonogenic assay (23). In the present study, we also assessed the percentage of bi-nucleated cells as a marker of cell progression through the entire cell cycle.

Aberration Assay

The aberration assay was performed using a premature chromosome condensation (PCC) technique with calyculin-A (Sigma) and in situ hybridization according to the protocol described previously (24, 25). After 2 Gy gamma exposure, cells were incubated at 37 ℃ for 5 hrs to allow DNA repair and then subcultured at low density at 37 ℃ for another 24 hrs. The microtubule inhibitor colcemid (Invitrogen) was then added at a final concentration of 0.03 μg/ml to arrest cells at mitosis. After

further incubation at 37 ºC for 8 hrs, the chromosomes were condensed by treatment with calyculin-A at a concentration of 50 nM for 30 min. The cells were then treated with 0.075 M KCl solution at 37 ºC for 20 min, and fixed with methanol:acetic acid (3:1 v/v).

Chromosome spreads were prepared by dropping fixed cells onto clean slides. After the slides were completely dried, the chromosomes were hybridized in situ by using kits for human chromosome 1 in Texas Red and chromosome 2 in FITC (Meta Systems, Watertown, MA) according to the manufacturer's protocol, and counterstained with 1μg/ml DAPI. Chromosome aberrations were scored by using a Zeiss Axioplan2 microscope. All types of detectable aberrations, especially deletions and translocations, were scored.

Western Blot Analysis

Cells harvested at different time points post-irradiation were resuspended in lysing buffer with cocktail proteinase inhibitor and phosphatase inhibitor (Sigma). Equivalent amounts of protein (50μg) in cell lysates were separated by gel electrophoresis at a 4-12% gradient SDS-PAGE gel, transferred to nitrocellulose membranes, and probed with anti-human P21 or anti-human MLH1 antibodies (1:200, Santa Cruz Biotechnology Inc, Santa Curz, CA). Donkey anti-rabbit Abs (1:1000, Amersham, Buckinghamshire, England) coupled to horseradish peroxidase were used to visualize bands with an ECL Plus kit (Amersham, Uppsala, Sweden) and Storm 840 PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). ß-actin was simultaneously immunodetected to verify loading of similar amounts of cell lysates.

RESULTS

Gene expression profile in low dose/low dose-rate gamma-irradiated cells

The pathway-specific PCR array for DNA damage signaling contains 84 relevant genes involved in DNA damage sensing, cell cycle regulating, damaged DNA binding and main lesion specific DNA repair mechanisms. Of the 84 genes, the expression level of 8 genes showed greater than 2 fold increase after continuous exposure for 2 hr to 1 cGy/hr low dose-rate gamma rays. The genes with altered expression levels included genes in cell cycle regulation (CHEK1, CHEK2, SESN1 and RBBP8), damaged DNA binding (XPA and XRCC2), double strand break repair (MRE11A) and mismatch repair (MLH1). The other 8 genes that had a fold-increase between 1.5 and 2 were RAD1, RAD17, RAD51, XPC, CIB1, RAD50, ATM and SUMO1 (Table 1a). Decreased expressions of XRCC3 and FANCG were also observed in irradiated cells. Although expression of p21 is not included in array analysis, western blot analysis showed that there was an increased expression of P21 protein by IR (table 1b) consistent with the result from other reported studies (8, 12). These IR-responsive genes identified by our studies belong to DNA damage sensing, DNA repair and cell cycle control gene groups, which have been recognized as IR-responsive gene categories, previously reported by Zhou et al. after analyzing global gene expression in 1.5 Gy gamma ray exposed fibroblasts (9). The IR-induced upregulation of SESN1 and genes involved in NER pathway genes have also been demonstrated in other studies (8,9,11,12).

Based on these results, we selected 25 genes, of which 13 were up-regulated in fibroblasts exposed to IR to systematically identify functions of these genes on the cytogenetic response of the cells to IR. In Table 1b, we present the list of the genes that can be categorized into different functional

groups including DSB repair (MRE11A, RAD51, Ku70, PRKDC, BRCA1, BRCA2, CIB1), NER (XPA, XPC), MMR (MLH1), other DNA repair related (RPA1, H2AX, XRCC1 and XRCC3, SUMO1 and SESN1), and cell cycle arrest/checkpoint (ATM, RAD1, RAD17, RBBP8, p21, DDIT3, MAP2K6, GADD45A and IHPK3). In the following experiments, these genes were individually knocked-down by siRNA in human fibroblasts and cytogenetic markers were measured after the cells were exposed to gamma rays.

Micronuclei formation in knocked-down cells

MN are formed during cell division when the nuclear envelope is reconstituted around chromosome fragments resulting from an inability to repair IR-induced DNA lesions, particularly DSBs. Micronuclei are excluded from the main nuclei, and MN frequently contain either parts of the chromosomes or even whole chromosomes that have been detected by fluorescence in situ hybridization (FISH) using DNA probes specific for centromeres, telomeres, and whole chromosomes. The formation of MN is therefore considered as a reliable marker for IR-induced chromosome breakage and loss in a wide variety of cell systems. Also, cells with MN typically can not reproduce due to their disability of passing through cell cycle checkpoint. To generate a significantly high frequency of MN formation in these studies, fibroblast cells with individual gene knock-downs were exposed to 2 Gy gamma radiation at 0.2 Gy/min. This treatment commonly generated 20% MN in normal AD1522 fibroblast cells compared with 1-2% MN generated by 2cGy low dose gamma rays that may be difficult to distinguish from background.

Without radiation, siRNA alone caused very small changes in the basal level (2-6%) of MN yield among various gene knock-down treatments. However, after 2 Gy gamma irradiation, MN yield

significantly increased in cells with impaired expression of 5 genes (Figure 1) compared with 20% in non-siRNA (NS) and control siRNA (CS) treated cells. These five genes and their respective MN yields were Ku70 (36%, P<0.02), RAD17 (33%, P<0.01), RBBP8 (29%, P<0.05), RPA1 (33%, P<0.02) and XPA (44%, P<0.005) (Figure 1a). XPA knocked-down cells showed the greatest induction of MN production and had the highest fraction of cells with multiple micronuclei (Figure 1b,c).

Percentage of bi-nucleated (BN) cells in gene knocked-down cells

While the MN assay is known as a reliable measurement of radiation damage, the percentage of bi-nucleated cells can also be used as an indicator of cell cycle progression as cells that are heavily damaged from radiation will be less likely to reach the bi-nucleated stage of the cell cycle (23). Here, we measured the percentage of BN cells to identify candidate genes that may also play a role in cell cycle regulation (Figure 2).

Several genes in the present study appeared to play different roles in cell cycle progression even without radiation damage. In un-irradiated cells, siRNA treatment targeting RPA1 and RAD51 alone lowered the percentage of BN cells by from 52% in CS treated cells to 30% whereas XPA knock-down increased the BN cell percentage to approximately 70%. After 2 Gy gamma irradiation, the percentage of BN cells in CS cells was reduced to 20%, as expected in cells with damaged DNA. We also identified several genes that affected the cell cycle progression after irradiation. In comparison to that of CS for irradiated cells, the percentage of BN cells was measured to be 39% (p<0.002) for p21, 36% (p<0.01) for MLH1 and 59% (p<0.008) for XPA, indicating faster growth in the cells with reduced gene expression levels. On the contrary, siRNA treatment on RAD51 (p<0.005), MRE11A

(p<0.01), SESN1 (p<0.02) and SUMO1 (p<0.01) yielded a percentage of bi-nucleated cells of 8.8%, 11.6%, 9.7% and 11.3% respectively, that were lower than that of the control.

SiRNA Dose dependence of MN formation and percentage of BN cells

The effects of partial knock-down expression of XPA, P21 and MLH1 by 5 to 10 nM siRNA treatment on MN formation and percentage of BN cells have been determined. Western blot analysis confirmed that transfection of 40nM siRNA was enough to successfully knock down the expression of P21 and MLH1 (data not shown). The increase of BN cell percentage was observed in partial XPA, P21 or MLH1 knock-down cells by transfection with only 5 or 10nM of siRNA (Figure 3A). Cells with XPA knock-down by transfection with different concentrations of siRNA treatment ranging from 5 to 75nM showed a similar increase in MN formation induced by IR (Figure 3B). These results suggested that a slightly suppressed expression of these three genes dramatically changed both the efficiency of cell cytogenetic repair and the regulation of cell cycle so that the level of IR-inducible gene expression might be an important factor to regulate post radiation cellular events.

Raidation Dose dependence of MN formation and percentage of BN cells

We also studied the radiation dose response of MN formation in cells with siRNA treatment for XPA, MLH1 and p21, as they showed the highest MN frequency after 2 Gy gamma irradiation. These cells were exposed to doses of 0.1, 0.5, 1 and 2 Gy at 0.2 Gy/min, and the MN yield and the percentage of BN cells are shown in Figure 3. The dose response for MN formation for XPA and MLH1 targeted cells were similar to that of the control, but the curvature of the dose response for p21 targeted cells appeared to be different (Figure 4A). The data of BN percentage presented interesting dose responses for different gene targeted cells (Figure 4B). The cells treated with CS

showed decreased BN percentage as the dose increased. The BN percentage for XPA targeted cells was significantly higher than that of the control even without radiation damage, and remained around 60-70% for the dose range between 0 and 2 Gy. The BN percentage for MLH1 treated cells decreased only slightly as a function of dose. The background level of BN cells after p21 targeted siRNA treatment was similar to that of the control, but the growth appeared to be affected by radiation even at the lowest dose of 0.1Gy (38%). However, increasing the dose further beyond 0.1Gy did not seem to have an effect on the BN percentage as shown in Figure 4A. Figure 4B showed well dividing XPA, MLH1 and p21 knock-down cells. These data suggests that XPA, MLH1 and p21 gene knock-down may allow either unchecked progression in cell cycle or a shortcut through cell cycle checkpoint.

Chromosome Aberration

Chromosome aberration and micronuclei formation are two common cytogenetic markers of radiation exposure. In the present study, we analyzed chromosome aberrations using whole chromosome specific painting technique with Chromosome #1 painted in Texas red and Chromosome #2 in FITC (Figure 5). Such an analysis allows for identification of complicated chromosomal damage, such as chromosomal translocations resulting from misrejoining of DSBs, that are not detectable by MN assay.

Although 2 Gy gamma rays did not significantly increase the MN frequency in MLH1 and p21 siRNA transfected cells, it did increase the percentage of cells containing chromosome aberrations from 8% of CS control cells to 17%. The frequency of chromosomal translocations was increased in all three gene knock-down cells (Figure 5). XPA suppressed cells had elevated number of cells with

more than two aberrations indicating possible more severe damage.

DISCUSSION

Within a cell, IR-induced DNA DSBs are probably the most serious form of the many different types of DNA damage that occur. The two distinct pathways of DSB repair, Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) have been investigated extensively in recent years (26). The HR repair mechanism corrects DSBs in an error-free manner by retrieving genetic information from a homologous, normally undamaged sister chromatin as a repair template. Cytogenetic endpoints, such as MN and CA, are believed to be induced mainly from the inability to correctly repair DBSs. As MN and PCC chromosome aberrations were analyzed in cells after cell division and in G2/M phase respectively, the yield of these cytogenectic endpoints is affected by the cells' DNA damage repair capability, the likelihood of unrepair/misrepaired cells to reach the respective phase of the cell cycle, or both. The percentage of BN cells is clearly an indicator of cell cycle progression, as the cells completed almost an entire cycle from G1 at time of irradiation to the stage of post-division. In the present study, we identified among 25 genes, the ones that may play a role in DNA DSB repair and/or cell cycle regulation in radiation insult.

RAD51 and MRE11A are among a group of proteins that function in the initial steps of meiotic recombination (27) and HR in response to DSBs (28,29,30). Knock-down of either of these two genes both affected cell cycle progression, which agreed with a recent report indicating G2-M phase blockage and lower clonogenic survival rate in gamma radiated stable RAD51kd and MRE11Akd cells (31). RAD51 has been also found to interact with RPA, RAD52 and ssDNA generated during DNA damage, indicating this protein may play roles in homologous pairing and strand transfer of DNA (32,33,34). Silencing of these two genes did not alter the MN yield, but reduced the percentage of BN cells by approximately 50% in comparison to that of NC cells. It has been reported that

BRCA1 and 2 may be key regulators of both the intracellular localization and DNA-binding ability of RAD51 (35,36). However, we did not observe any changes for either MN yield or the percentage of BN cells in BRCA1 or BRCA2 knocked-down cells in comparison to those for NC, suggesting a more complicated regulative role of RAD51.

In NHEJ, where the two DNA ends are connected without the need for longer stretches of homology, the repair of a DSB is error-prone and frequently leads to small deletions (37). The activity of the Ku70/Ku80, a nuclear complex consisting of two subunits and functioning as a single-stranded DNA-dependent ATP-dependent helicase, is essential for initiating NHEJ by binding to the free DNA ends and recruiting other necessary factors such as PRKDC as well as Rad50 and MRE11. As predicted, knocked-down expression of Ku70 (G22P1) resulted in a higher yield of MN, but did not appear to influence cell cycle regulation. The impaired expression of PRKDC did not show any impact on MN formation and cell cycle progression. However, we can not rule out that a possible small amount remaining PRKDC after siRNA knock-down due to its relatively high basal expression level, may be enough for its biological functions.

Damages to the DNA that do not produce strand breaks are repaired by BER, NER or MMR pathways. Interestingly, genes associated with the nucleotide excision repair (NER) pathway, such as XPC, have been found to be consistently up-regulated by IR (8). The prototype repair disorder, XP (xeroderma pigmentosum), which causes mutation in one of seven NER genes (XPA-XPG), is characterized by sun sensitivity and proclivity toward sun-induced skin cancer. However, most studies on genes involving in NER pathway like XPA and XPC focused on the cellular response to UV radiation but not gamma radiation. Our data support that NER proteins, especially XPA, formerly recognized as the main repair mechanism in UV caused DNA damage, are highly involved

in the repair of IR-induced damages. The expression of two NER genes, XPA and XPC, were induced in response to low dose-rate gamma radiation. To our surprise, cells with knocked-down expression of XPA showed a phenotype of elevated cell cycle progression and higher micronuclei background. The MN frequency after gamma exposure was the highest at all dose points from 0 to 2 Gy compared to that in cells knocked-down for other genes. More importantly, after 2 Gy gamma irradiation, XPA knocked-down cells progressed through the cell cycle at a rate similar to that of the unirradiated cell. Reported studies on XPA have demonstrated that XPA is involved in UV-induced ATR activation and the activation of Chk1 and RPA. Depletion of XPA resulted in decreased phosphorylation of Chk1 in Hela cells with treatment of UV or UV like agents but not with gamma radiation (38). However, due to the malignant nature of Hela cells, XPA in fibroblast cells may operate through a different pathway to regulate cell cycle progression. Although the mechanism on XPA mediated cell cycle regulation needs to be further investigated, our findings suggested that lesion bypass and not lesion repair may raise the level of UV damage that can be tolerated before checkpoint activation, and that XPA plays a critical role in this activation.

The MMR system is responsible for the post-replicative repair of mismatches and small single strand DNA loops. Defects in this system may increase mutation rates and initiate the process of oncogenesis, such as frequent mutation in MLH1 locus found in hereditary nonpolyposis colon cancer (39). Other studies have also shown that MLH1 is involved in G2 phase cell cycle progress suggesting that MLH1-mediated cell cycle delay may be important for MMR proofreading of DNA damage repaired lesions prior to chromosome segregation to eliminate carcinogenic lesions in daughter cells (40). Our data showed that the expression of MLH1 was induced by IR, and loss of

MLH1 expression not only elevated cell cycle progression but also increased the yield of IR-induced chromosomal translocations, although without changes on MN formation in comparison to that of the irradiated NC cells, suggesting that this gene is involved in mostly MMR and may play a role in DSB break repair and cell cycle regulation. The disagreement of MN formation data and CA data shows us that sole measurement of MN formation may not thoroughly reflect DNA damage and misrepair. This may be simply due to the ability of CA assay to indicate more profound damage than estimated by MN assay, even regardless of MN amount. Thus, CA and other markers must also be measured to confirm the intact cytogenetic damage repair in gene knock-down cells that have not shown induction of MN formation in future studies.

Three gene products, RPA1, SUMO1 and SESN1, that affected either the IR-induced MN yield or the percentage of BN cells, may have essential functions involved in all repair mechanisms in response to gamma-induced DNA damage. RPA1 is a 70kDa subunit of heterotrimer RPA and a single strand DNA binding protein that is involved in diverse DNA metabolic activities and almost every repair mechanism (41). In the present study, loss of RPA1 expression impaired DNA repair, showing an increased MN formation, indicating the important function of RPA1 during the process of DNA repair. SUMO1 is a member of the SUMO (small ubiquitin-like modifier) protein family, which modify and protect target proteins from degradation by the proteosome pathway. SUMO1 has been proven to bind with important proteins involved in DNA damage sensing, cell cycle progress and DNA repair system, such as RAD52 and XPC, thus to modulate cellular response to external stimuli (42,43). Our study showed that suppressed SUMO1 expression resulted in a slowdown of cell cycle progression as well as abnormal nucleus shape (data not shown) after IR in comparison to that for NC cells indicating possible severe impaired DNA repair in response to IR. Our observation

may explain that by the loss its protective modification of essential protein and its ability to maintain large multiprotein complexes to stay together, especially during DNA repair process, may severely impair DNA repair system. Inhibition of SESN1 expression that encodes an antioxidant modulator of peroxiredoxins resulted in more cell cycle blockage. This may be due to more DNA damage caused by increased ROS levels generated by impaired ROS metabolism without expression of SESN1 (44). It suggests that a successfully damaged DNA repair involves a much more complicated cellular mechanism, other than a solo traditional DNA repair system.

Many of the checkpoint proteins are activated in response to DNA damage by initiating gene transcription and modification, such as phosphorylation. These events in DNA repair mechanisms depend on activation of lesion-specific kinases in the ATM/ATR family, which act early in the checkpoint pathways in response to DNA-damage caused by ionized radiation with ATM being specific for agents that induce DNA-DSB, and ATR, probably responding to UV-induced damage. The tumor suppressor p53 is subsequently activated and is central to checkpoint controls, especially in DNA damage-induced G1 arrest through transcriptional induction of the cyclin-dependent kinases Chk1 and Chk2, and inhibitor p21, which binds to and inhibits G1 cyclin-dependent kinases (GADD45) (45). Cells under low dose and low dose-rate gamma radiation have dramatic transcriptional induction of Chk1, Chk2 and P21. Other genes upregulated in cells after IR exposure that are involved in cell cycle control are RAD1, RAD17 and RBBP8. Knocked-down expressions of P21, RAD17 and RBBP8, but not of ATM and RAD1 showed a significant increased production of MN indicating a possible premature repair process and unbalanced repair and cell cycle network. Only P21 knock-down showed an impact on cell cycle regulation. Moreover, suppression of P21 expression dramatically increased the rate of chromosomal aberration; especially translocation

forming aberration complexes involving more than two chromosomes. These data suggest that loss of cell cycle regulatory proteins may not only result in failure of cell cycle arrest on DNA damaged cells, but also subsequently generate more misrepaired DNA damaged cells.

Individual depletion of three genes MLH1, XPA and P21 affected both the cell cycle progression and the cytogenetic responses, and the effects of these genes were analyzed in detail for a set of dose points between 0 and 2 Gy. The yield of IR-induced MN formation showed a clear increase as a function of dose similar to that for most of the biological endpoints. However, the dose response of the percentage of BN cells shown in Figure 3 is interesting in that the percentage presented little changes as the dose increased from 0.1 to 2Gy, i.e., heavily IR-damaged cells possibly progressed through almost the entire cell cycle at the same rate as less damaged cells. More importantly, the loss of XPA in the cells apparently stimulated cell growth as the percent BN cells was 70% in XPA silenced cells compared to 53% in NC without radiation exposure. This growth stimulation due solely to the loss of expression of a single gene was not found for MLH1 and CDKN1A. The stimulated growth rate was little changed by the increased IR-induced damage in the cells, suggesting that XPA may be a critical cell cycle control switch linking DNA repair process and cell cycle progression so that without XPA, cells with a certain degree of DNA damage, without repair or partially repaired, may go through the cell cycle checkpoint. The possible ability of these defective cells to amplify and pass defective genetic information through generations may result in an increased possibility of carcinogenesis.

Inability to repair DSBs in cells with impaired DNA repair mechanisms can cause senescence or be lethal; whereas misrepaired DSBs lesions like chromosomal fragmentation, translocations and deletions may be potential inducers of carcinogenesis through malfunctions of mutated proto-oncogenes and tumor suppressor genes, abnormal cell cycle controls and failure of essential protein modulation causing impaired pathway signaling. Our data suggests that cells with suppressed expression of RAD51, SESN1, SUMO1 and MRE11A may severely impair DNA repair systems causing a lower cell survival rate. On the other hand, failure of expression of Ku70, RBBP8, RAD17, RPA1, P21, XPA and MLH1 may impair damaged DNA repair as well, but generate more misrepaired lesions after exposure to IR. Furthermore, most of the genes that were found to influence the cytogenetic response to 2 Gy gamma radiation at high dose rates, were transcriptionally induced during cellular response to low dose and low dose-rate gamma irradiation, suggesting that expression up-regulated genes have critical functions affecting the biological consequences after radiation exposure. Thus, failure to express these responsive genes could change the outcome of post IR scenario, whether cell cycle arrested or apoptosis; damaged DNA repaired or misrepaired; or even carcinogenesis

ACKNOWLEDGEMENTS

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Table 1

A.

Cell Cycle Checkpoint	Damaged DNA Binding	DNA Repair	Apoptosis	Other
CHEK1	RAD1	MRE11A	PDCD8	SUMO1
CHEK2	RAD51	MLH1	IHPK3	SESN
RAD17	XPA	RAD50		
RBBP8	XPC			
ATM	XRCC2			

B

| Gene Category	Gene Name	Fold Induction	Gene Bank Number	Sense siRNA Sequence
DSBs Repair	MRE11A	2.7	NM_005591	GGAAAAUGAAGUGGAUUUUtt
	RAD51	1.6	NM_002875	CCUAUUGGAGGAAAUAUCAtt
	Ku70	--	NM_001469	GCUCUAUCGGGAAACAAAUtt
	PRKDC		NM_006904	GGUUUACAAAGCUAAAUGAtt
	BRCA1		NM_007303	GGACUUCUGGCUAUGCAAGtt
	BRCA2	--	NM_000059	GGCAAAUGUUGAAUGAUAAtt
	CIB1	1.6	NM_006384	GGGAUGGAACCAUCAACCUtt
NER	XPA	3.1	NM_000380	GGAGGAGGCUUCAUUUUAGt
	XPC	1.8	NM_004628	GCGGCAUCUCCUGAAAUAUtt
MMR	MLH1	2.2	NM_000249	GGCAUUAGUUUCUCAGUUAtt
Repair Related	RPA1		NM_002945	GGAUUUUGAAGCCUUUUGUtt
	H2AX	--	NM_002105	GGCCUUUCACAUCAGCUCUtt
	XRCC1		NM_006297	CCGGGUCAAAAUUGUUUGCtt
	XRCC3		NM_005432	GGAGGUUUUACACUUUUCUtt
	SUMO1	1.8	NM_001005782	GGAUAGCAGUGAGAUUCACtt
	SESN1	2.1	NM_014454	GUCGGGUUAUCAAAUUUGAtt
Cell Cycle Control	ATM	1.7	NM_000051	GCAACAUUUGCCUAUAUCAtt
	RAD1	1.9	NM_133377	GGUCAUGUACCCUAAUAGUtt
	RAD17	1.8	NM_133344	CCUGGUUAAAAGCUCAAGUtt
	P21	--1.8	NM_000389	GGCGGUUAUGAAAUUCACCtt
	GADD45A		NM_001924	GCCGAAAGGGUUAAUCAUAtt
	RBBP8	2.2	NM_203292	GGGAACAGCAGAAAGUCCUtt
	DDIT3		NM_004083	GGUCUCAGCUUGUAUAUAGtt
	MAP2K6		NM_002758	GGAAGAACAGAGGGUUUUGtt
	IHPK3	2.6	NM_054111	GCGGCAUCGGUUCUUGUUGtt

Figure 1

A
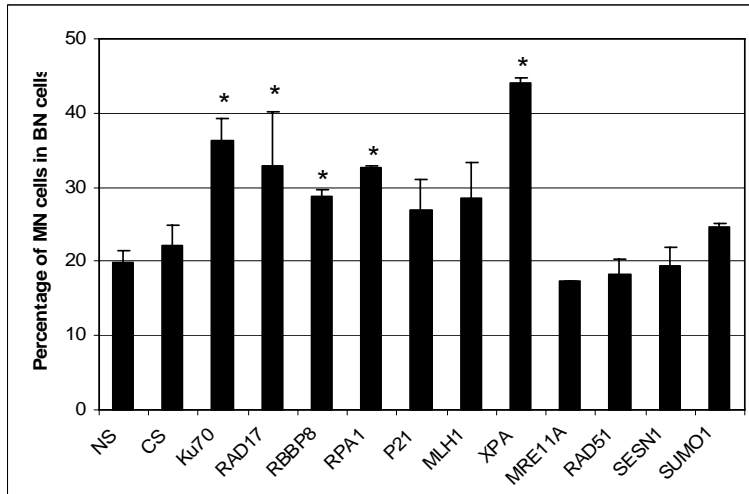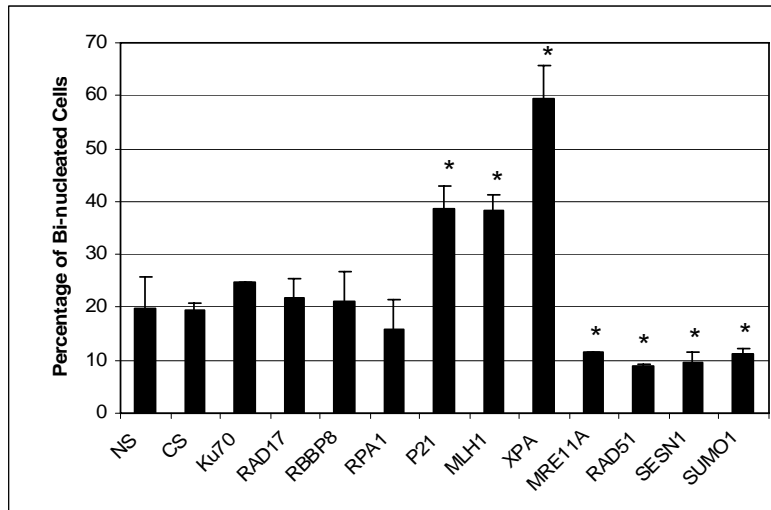

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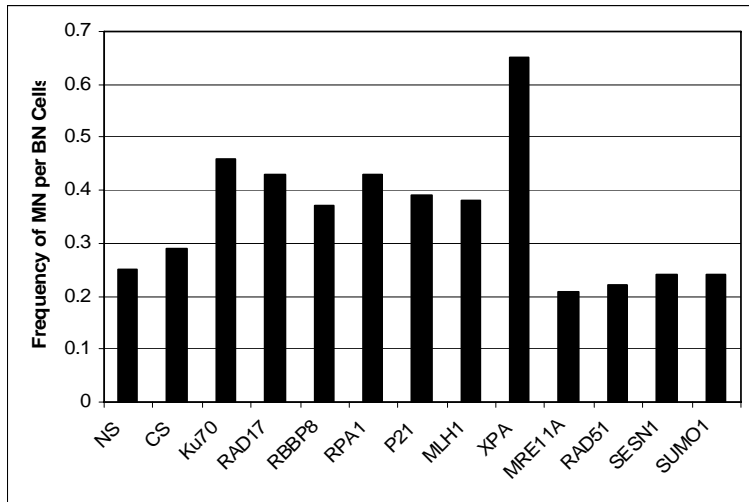
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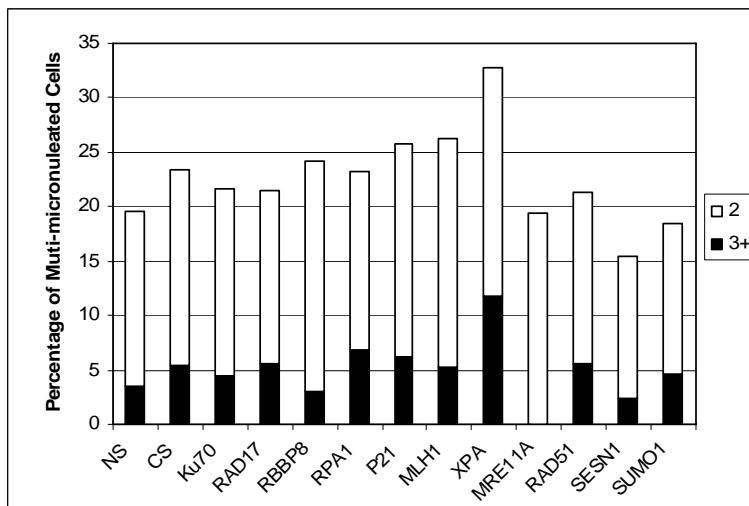
Figure 2

A

B

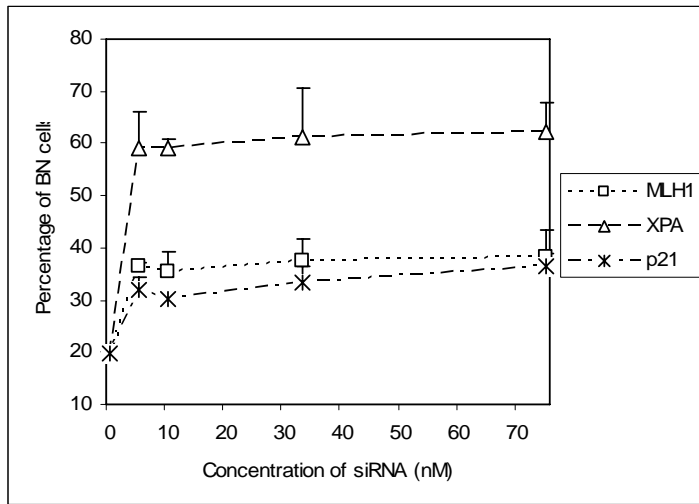
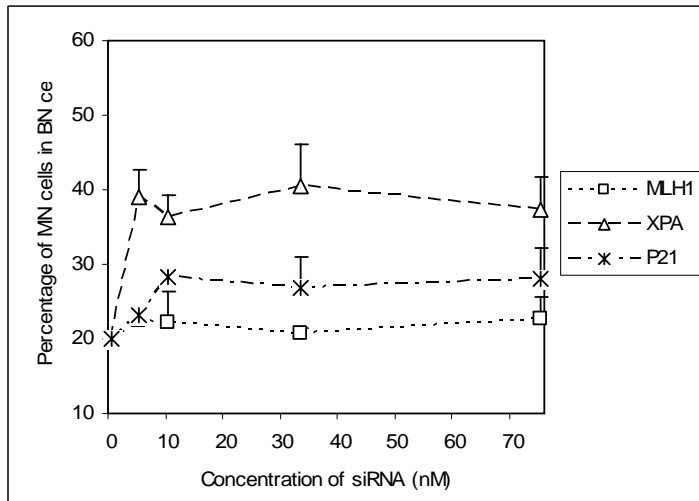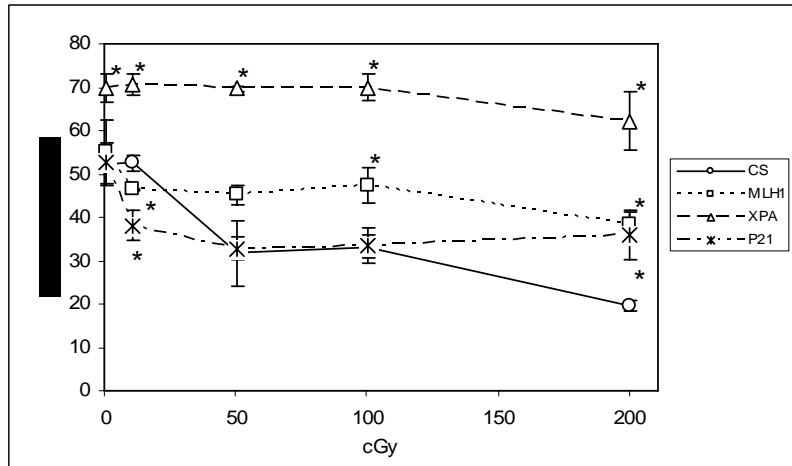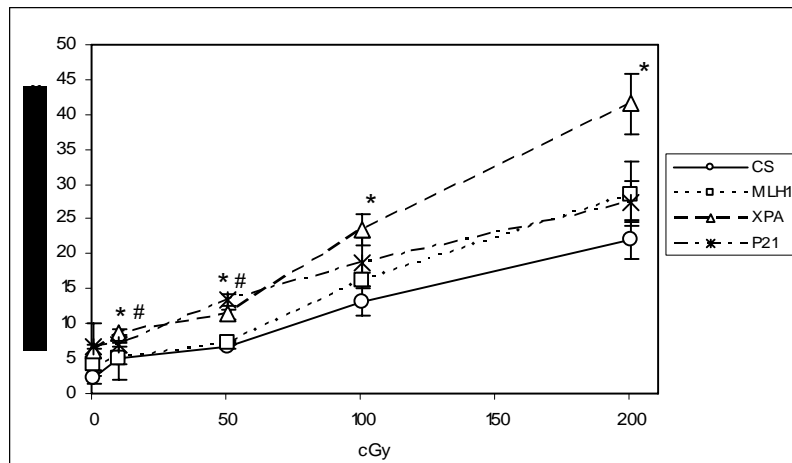1. P21   2. XPA   3. MLH1
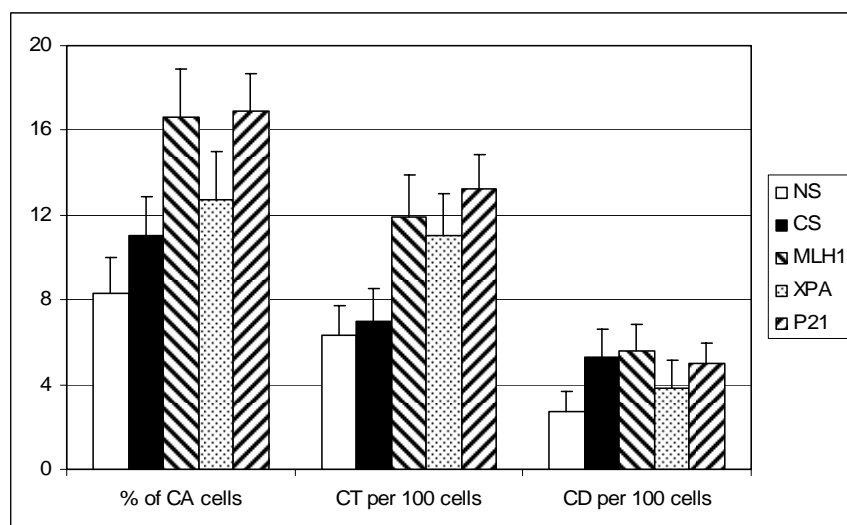
Figure 3

A.

B.

Figure 4

A

B

Figure 5

A

B

	Cell Number	Cells with NC	Cells with One CA	Cells with Two CA	Cells with CT	Cells with CD	CT per cell	CD per cell
NS	301	276	23	2	19	8	0.063	0.027
CS	300	267	29	4	21	16	0.07	0.053
P21	319	265	52	2	42	16	0.132	0.05
MLH1	302	252	46	4	36	17	0.119	0.056
XPA	395	345	41	9	44	15	0.11	0.038

FIGURE CAPTIONS

Table 1. A. The list of genes with induced expression in cells exposed to 2cGy low-rate gamma rays. B. The list of 25 genes selected for siRNA knock-down assays with fold increase induced by 2cGy low-rate gamma rays and siRNA sequence. (--not included in array assay; protein induction rate of p21 at 6hr post radiation shown in the table)

Figure 1. Frequency of MN formation in various gene knock-down cells induced by exposure to 2Gy gamma radiation presented in percentage of MN containing cells in BN cell population (A. *$P<0.05$ compared with CS treated cells), yield of micronuclei per BN cell (B) and percentage of multi-micronucleated cells (C).

Figure 2. The comparison of percentage of bi-nucleated cells in 2Gy gamma ray treated gene knock-down fibroblast cells (*$P<0.05$ compared with CS treated cells).

Figure 3. The impact of siRNA dose (0-75nM) on BN percentage (A) and MN formation (B) in XPA, P21 and MLH1 knock-down cells in response to 2 Gy gamma radiation.

Figure 4. The impact of radiation dose on BN percentage (A) and MN formation (B) in XPA, P21 and MLH1 knock-down cells in response to 0, 0.1, 0.5, 1 and 2 Gy gamma radiation (*# $P<0.05$ in XPA, P21 siRNA treated cells compared with CS treated cells respectively).

Figure 5. Frequency of chromosome aberration (CA) in 2 Gy gamma radiated XPA, P21 and MLH1 knock-down cells. NC: norma chromosome; CT: chromosomal translocations and CD: chromosomal deletions.