Initiation of the ATM-Chk2 DNA damage response through the base excision repair pathway

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Abstract

The DNA damage response (DDR) is activated by various genotoxic stresses. Base lesions, which are structurally simple and predominantly fixed by base excision repair (BER), can trigger the ataxia telangiectasia mutated (ATM)–checkpoint kinase 2 (Chk2) pathway, a DDR component. How these lesions trigger DDR remains unclear. Here we show that, for alkylation damage, methylpurine-DNA glycosylase (MPG) and apurinic/apyrimidinic endonuclease 1, both of which function early in BER, are required for ATM-Chk2–dependent DDR. In addition, other DNA glycosylases, including uracil-DNA glycosylase and 8-oxoguanine glycosylase, which are involved in repairing deaminated bases and oxidative damage, also induced DDR. The early steps of BER therefore play a vital role in modulating the ATM-Chk2 DDR in response to base lesions, facilitating downstream BER processing for repair, in which the formation of a single-strand break was shown to play a critical role. Moreover, MPG knockdown rescued cell lethality, its overexpression led to cell death triggered by DNA damage and, more interestingly, higher MPG expression in breast and ovarian cancers corresponded with a greater probability of relapse-free survival after chemotherapy, underscoring the importance of glycosylase-dependent DDR. This study highlights the crosstalk between BER and DDR that contributes to maintaining genomic integrity and may have clinical applications in cancer therapy.

Introduction

DNA mutation and genomic instability are features of most types of cancer but are almost completely absent in healthy organisms (1). One reason for this distinct difference is that, in a normal cell, distortion of the DNA structure by events such as strand breaks, incomplete replication or intra- or inter-strand crosslinks triggers the DNA damage response (DDR), a specialized genome surveillance mechanism, to avoid further chromosomal aberration (2). In humans, two major DDRs of parallel kinase-to-substrate reactions that control signal transduction have been characterized. One of these pathways comprises ataxia telangiectasia mutated (ATM) and its downstream target, checkpoint kinase 2 (Chk2). ATM-Chk2 activation is associated with DNA double-strand breaks (DSBs) or chromatin relaxation by histone acetylation and can be divided into three events: ATM is autophosphorylated at S1981; ATM phosphorylates and activates Chk2 at T68; and both ATM and Chk2 phosphorylate downstream effector proteins, such as p53, E2F1, Artemis and BRCA1 (3). The second pathway comprises ATM- and Rad3-related (ATR) and checkpoint kinase 1 (Chk1), which have functions similar to ATM and Chk2, respectively, and is activated when DNA replication stalls (4). In addition to ATM and ATR as DDR initiators, there are many signal mediators that are also required for complete DDR activation (3,4).

In contrast with complicated types of DNA damage, which are toxic and harmful to cells, most lesions are much simpler and smaller and frequently persist in the nucleus (5,6). When base modifications occur near the hydrogen-bonding sites of purines and pyrimidines, as in the case of thymine glycol and N3-methyladenine, distortion of the DNA structure can occur, resulting in replication block and cell death (7,8). However, some base modifications do not distort DNA structure, and it is not known whether and how such DNA damage activates ATM or...
**Materials and methods**

**Cell culture and reagents**

**Cell lines**

Human 293T, HeLa and MCF7 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich). U2OS and MDA-MB-453 cells were cultured in McCoy’s 5A medium and RPMI 1640 medium (both from Sigma-Aldrich), respectively. All cells were maintained in medium supplemented with 10% fetal bovine serum (FBS). The MDA-MB-453 cell line was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan), and other cell lines were obtained as described (16). Cells used in the study were authenticated using the Promega GenePrint 10 System by Genelabs (Taipei, Taiwan) to ensure that no contamination had occurred. Transfection of 293T cells was performed as described (16). For the other transfections, transfection with plasmids was performed using TurboFect (Fermentas), and small interfering RNA (siRNA) or siRNA plus plasmid transfection was performed using Lipofectamine 2000 (Invitrogen).

**Treatment**

To induce DNA damage, 0.3 mg/mL MMS, 50 μM H₂O₂, or 100 nM 4-hydroxytamoxifen (4-OHT) was added for the indicated times before cell harvesting or further experiments. For ionizing radiation (IR), the exposure was 4 Gy as described (16). To induce relaxation of chromatin structure, histone deacetylase inhibitors (0.5 μg/mL Trichostatin A or 2 mM sodium butyrate) were added to the culture medium for 4 h as described (17). AP sites were blocked by adding methoxyamine (neutralized by 10 N NaOH addition before treatment) for 30 min. APE1 activity was inhibited by adding 20 μM AB03 (Axon Medchem) or APE1 inhibitor III (Merck-Millipore), and PARP1 activity was inhibited by adding 10 μM 4-amino-1,8-naphthalimide or ABT-888 (Selleck Chemicals). All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

**Antibodies**

The antibodies used in this study were anti-Chk1Ser173 (2348) and anti-phospho(S/T)Q (2851) from Cell Signaling Technology; anti-GFP (clone JL-8) from Clontech; anti-ATMSer1981 (2152-1) from Epitomics; anti-OGG1 (GTX101115), anti-UNG2 (GTX13860) and anti-ATM (GTX70103) from GeneTex; anti-Chk2 (K0088-3) from MBL International; anti-ATMIN (AB3271), anti-Mre11 (PC888), anti-acetyl histone H3 (K14) (07-353), anti-histone H3 pan (07-690) and anti-Tip60 (07-038) from Merck-Millipore and anti-Myc (M4439) from Sigma-Aldrich. The other antibodies were described previously (16). The primary antibodies that were bound to the nitrocellulose membrane were recognized by horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch); resulting binding was detected with chemiluminescent reagents (Merck-Millipore).

**Clinical datasets analysis**

For survival analysis, relapse-free survival, stratified by expression of the gene of interest, was presented as Kaplan–Meier plots and tested for significance using log-rank tests (18). In the study, the patients were split into two groups based on the median expression level of the gene of interest and were analyzed according to website instructions (www.kmplot.com). To compare MPG expression between normal tissues and cancers, RNA sequence (RNA-seq) data were retrieved from The Cancer Genome Atlas (TCGA) Data Matrix on 15 July 2014. Initially, RNAseqV2 data consisting of normalized expression values for fifteen cancer types with both normal and tumor samples were downloaded; however, the data for pancreatic ductal adenocarcinoma was restricted for use prior to initial TCGA global analysis and was removed before further analysis. The MPG expression value of each sample was normalized to that of TATA-box binding protein. Comparisons between the RNA-seq-derived normal and tumor expression values and statistical significance were determined using the Mann–Whitney U-test.

The other materials and methods, including plasmids, siRNAs, protein extraction, ATM kinase assay, indirect immunofluorescence, comet assay, colony formation analysis and the nucleotide sequence of the A3A-ER segment, can be found in Supplementary Materials and methods and Supplementary Figure 1, available at Carcinogenesis Online.

**Results**

MPG is involved in the MMS-induced DDR pathway

An important finding of our previous study (16) is that inactivation of MPG, but not XRCC1, inhibits the autophosphorylation of ATM S1981 that is caused by 30 min of treatment with 0.3 mg/mL MMS, suggesting that ATM-dependent DDR is activated during
the initial processing of damaged bases in BER. In the present study, siRNA knockdown of MPG markedly reduced MMS-induced phosphorylation of ATM S1981 and Chk2 T68 in human cells of different tissue origins (Figure 1A and Supplementary Figure 2) is available at Carcinogenesis Online. In contrast, phosphorylation of Chk1 S345, which is caused predominantly by ATR, was not affected as well as Chk2 T68 (Figure 1A), suggesting that the role of MPG in ATR-dependent DDR is ambiguous. In addition to the autophosphorylation of ATM, siRNA knockdown of MPG reduced the damage-induced accumulation of ATM on chromatin (high NP-40 fraction) caused by MMS treatment, but not that caused by IR (Figure 1B). We also found that after MPG knockdown, the levels of ATM-pS1981 bound to chromatin declined more than the levels in the cytoplasmic (low NP-40) fraction (Figure 1B).

Because MPG is chromatin bound (16,19) and because full activation of ATM following exposure to IR requires proteins that facilitate ATM binding to chromatin (17), it is conceivable that MMS-induced ATM activation is dependent on MPG. However, using coimmunoprecipitation with antibodies against MPG or ATM, we were unable to detect an MMS-induced association between MPG and ATM (data not shown). It is notable that none of the members of Mre11-Rad50-NBS1 complex, ATMIN or Tip60, any of which is known to promote IR-induced ATM activation (20), was involved in the effects of MMS (Supplementary Figure 3–6 is available at Carcinogenesis Online), suggesting that MPG triggers ATM activation by a mechanism unrelated to the conventional DDR pathway. Moreover, when control cells or cells transfected with siRNA targeting MPG were incubated for 4h with ethanol (vehicle control) or histone deacetylase inhibitors to increase histone H3 acetylation (17) before MMS treatment, MMS-induced ATM activation occurred independently of acetylation of histone H3 at lysine 14 (H3-acK14) (Figure 1C). This acetylation has been considered to be an indicator of chromatin relaxation during IR-induced DNA double-strand breakage in an HMGN1-dependent manner (17). Thus, ATM activation by MPG does not depend on proteins or pathways already known to be involved in DNA DSB repair and presumably results from the direct action of MPG.

**Active MPG is required for ATM-Chk2 activation**

To excise a damaged DNA base via the BER pathway, two DNA glycosylase activities are required: one is the ability of the molecule to bind to a damaged DNA site, and the other is the catalysis of the formation of AP sites. Structural analyses (21–23) have shown that three amino acid residues in the catalytic domain are needed for MPG activity: glutamic acid 125 (E125), aspartic acid 132 (D132) and asparagine 169 (N169). Mutation of N169 to aspartic acid 132 (D132) and asparagine 169 (N169) causes loss of binding to the damaged site, and mutation of E125 to glutamine (E125Q) or of D132 to aspartagine (D132N) causes loss of the ability to excise damaged DNA. To explore whether these residues of MPG are required for MMS-induced ATM activation, these MPG mutants were expressed in U2OS cells, which were reported to express a very low level of endogenous MPG (24) (Supplementary Figure 7 is available at Carcinogenesis Online) and thus are appropriate for this experiment. U2OS cells that expressed the MPG mutants showed markedly less MMS-induced phosphorylation of ATM and Chk2 than did those that expressed wild-type MPG (Figure 2A). Although overexpression of the N169D mutant increased activation of the DDR pathway slightly as compared with control vector transfection, overexpression of D132N or E125Q/N169D had no effect, suggesting that DNA excision by MPG, rather than its binding to alkyl-DNA sites, is more critical for ATM activation. These results were confirmed by an immunofluorescence study in which U2OS cells that were transfected with wild-type MPG, but not those transfected with the D132N mutant or an untransfected control, stained positively for ATM phosphorylated at S1981 (Figure 2B).

In addition, we needed to demonstrate the presence of MMS-induced DNA damage in these cells, and the formation of DNA SSBs and DSBs during BER would be one possible explanation for this MPG-induced ATM DDR in response to MMS. To examine this, these U2OS cells were subjected to alkaline comet analysis, which detects and measures a mixture of AP sites and DNA SSBs and DSBs within cells. We found that, during MMS treatment, cells transfected with wild-type MPG had extensive DNA damage, but this was not present in cells transfected with the D132N mutant or an empty vector-transfected control (Figure 2C). Importantly, this result correlates positively with the level of MMS-induced phosphorylation of ATM S1981 (Supplementary Figure 8 is available at Carcinogenesis Online).

APE1 and PARP1 activities modulate ATM-Chk2 activation

Both MMS-induced base damage and the AP site resulting from excision of this damage by glycosylase can interfere with DNA repair. To explore whether these residues of MPG are required for MMS-induced ATM activation, these MPG mutants were expressed in U2OS cells, which were reported to express a very low level of endogenous MPG (24) (Supplementary Figure 7 is available at Carcinogenesis Online) and thus are appropriate for this experiment. U2OS cells that expressed the MPG mutants showed markedly less MMS-induced phosphorylation of ATM and Chk2 than did those that expressed wild-type MPG (Figure 2A). Although overexpression of the N169D mutant increased activation of the DDR pathway slightly as compared with control vector transfection, overexpression of D132N or E125Q/N169D had no effect, suggesting that DNA excision by MPG, rather than its binding to alkyl-DNA sites, is more critical for ATM activation. These results were confirmed by an immunofluorescence study in which U2OS cells that were transfected with wild-type MPG, but not those transfected with the D132N mutant or an untransfected control, stained positively for ATM phosphorylated at S1981 (Figure 2B).

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replication, leading to a replication block, replication fork collapse and eventually the formation of DSBs during S phase of the cell cycle and activation of ATM-Chk2 (23). However, previous studies, including ours, have shown that MMS-induced ATM and Chk2 phosphorylation occurs during the G1 phase of the cell cycle (16). This suggests that these DDR mechanisms do not always occur because of DNA DSBs and might be caused by SSBs or other damage. In this study, we focused on the role of DNA glycosylases in activating ATM-dependent signaling and examined whether AP sites or extended DNA SSBs are critical for ATM activation. To this end, we used the chemical inhibitor methoxyamine, which reacts irreversibly with the aldehyde sugar group of an AP site and prevents its excision by APE1 (12).

We first tested the ability of methoxyamine to inhibit APE1 excision in an in vitro assay (supercollider relaxation assay) using recombinant human APE1. In this test system, the untreated circular plasmid forms a supercoil that migrates rapidly on an agarose gel, whereas the nicked circle formed by digestion with APE1 migrates more slowly. Pretreatment with 25 mM methoxyamine partly inhibited the effect of APE1, as shown by the residual lower supercoiled band on the gel (Supplementary Figure 9 is available at Carcinogenesis Online) and resulted in a decrease in MMS-induced phosphorylation of ATM S1981 and Chk2 T68 (Figure 3A), suggesting that APE1 may participate in the activation of ATM-dependent signaling by converting AP sites to DNA SSBs. Because inhibition of APE1 prevents DNA SSB formation, whereas inhibition of PARP1 leads to SSB accumulation, this was consistent with our observations in 293T cells that pretreatment with AR03 or APE1 inhibitor III before MMS treatment resulted in less phosphorylation of ATM S1981 and Chk2 T68, but pretreatment with 4-amino-1,8-naphthalimide or ABT-888 before MMS treatment led to more phosphorylation (Figure 3B). Furthermore, to confirm that ATM is indeed activated upon APE1 action, which leads to the formation of DNA breaks, an in vitro kinase assay was performed. ATM purified from 293T cell lysates was tested for kinase activity, in which GST-p53 was used as the substrate (Supplementary Figure 10 is available at Carcinogenesis Online). In this assay, consistent with what was reported previously (26), ATM kinase activity was obviously induced by incubating with Ncol-cut plasmid, as compared with the uncut control. It was also activated by the effect of APE1 excision on the AP-carrying plasmid (Figure 3C). We therefore propose that, in response to alkylating damage, APE1 acts together with MPG to activate MMS-induced ATM-Chk2 DDR. Along with the results from the comet analysis reported in the previous section, we further propose that production of DNA SSBs, which result from MPG-APE1 action as MPG in MMS-induced DNA damage, is required for ATM-Chk2 DDR and because another DNA glycosylase, UNG2, is important for the repair of DNA deamination, we thought it meaningful to determine whether UNG2, which has the same action as MPG in MMS-induced DNA damage, is required for DNA deamination-induced ATM-Chk2 DDR. According to previous studies (27), expression of A3A—a protein in the cytidine deaminase family that catalyzes the deamination of DNA by converting, for example, cytosine to uracil, to induce phosphorylation of H2AX S139, a marker of ATM activation, and ATM S1981 (27)—was used in the following experiments. To generate base deamination in damaged DNA, we initially constructed a mammalian expression vector to overexpress A3A fused to GFP.
in 293T cells. Cells expressing A3A showed markedly increased phosphorylation of ATM S1981 (Figure 4A), which is consistent with our findings concerning MPG expression in U2OS cells and previously reported data (27). To confirm that ATM phosphorylation is inducible by A3A activity, we generated a 4-OHT-inducible A3A vector, in which an ER-LBD was fused to the C-terminal end of A3A. Thus, A3A could be activated by 4-OHT binding to the ER-LBD (28,29). Cells expressing inducible GFP-A3A (GFP-A3A-ER) showed markedly increased 4-OHT-induced phosphorylation of ATM S1981 (Figure 4A).

Because DNA deamination requires specific binding and is repaired by UNG2 (30), it is conceivable that 4-OHT-induced ATM phosphorylation is dependent on UNG2. We found that UNG2 knockdown reduced A3A-induced phosphorylation of ATM S1981 caused by 4-OHT activation (Figure 4B), suggesting that, like MPG, UNG2 plays a role in DDR modulation. This finding is consistent with the observation that cells expressing the D154N mutant of UNG2 (31) showed markedly less A3A-induced phosphorylation of ATM S1981 than did those expressing wild-type UNG2 in the presence of 4-OHT (Figure 4C).

IR exposure has been reported to cause DNA deamination at cytosine or 5-methylcytosine (32). We found that UNG2 knockdown by siRNA reduced the phosphorylation of ATM S1981 caused by IR, but not by MMS (Figure 4D and Supplementary Figure 11) available at Carcinogenesis Online. Knockdown of OGG1, which encodes another DNA glycosylase that specifically removes products of oxidative DNA lesions such as 8-oxoguanine (11), showed markedly reduced H2O2-induced phosphorylation of Chk2 T68 in 293T cells (Supplementary Figure 12) available at Carcinogenesis Online. However, autophosphorylation of ATM S1981 was not affected after H2O2 treatment (Supplementary Figure 12) available at Carcinogenesis Online, because ATM is activated by oxidation at cysteine 2991 (33). Furthermore, H2O2-induced phosphorylation of Chk2 T68 was increased by pretreatment with ABT-888 but was repressed by pretreatment with APE1 inhibitor III (Supplementary Figure 13) available at Carcinogenesis Online, both of which are consistent with the finding in Figure 3B. Taken together, these data regarding a common role for individual glycosylases—including MPG, UNG2 and OGG1—in the regulation of ATM and Chk2.

**Figure 3.** APE1 and PARP1 contribute to the modulation of MMS-induced activation of the ATM-Chk2 pathway. (A) Immunoblot showing inhibition of MMS-induced ATM-pS1981 and Chk2-pT68 phosphorylation by methoxyamine pretreatment in 293T cells. Cells were incubated with the indicated doses of methoxyamine for 30 min before incubation with or without MMS for 30 min, and the cell lysates were immunoblotted to detect the indicated proteins. (B) Immunoblot analysis of 293T cell lysates that had been incubated, respectively, with 4-amino-1,8-naphthalimide, ABT-888, AR03, or APE1 inhibitor III for 1 h before incubation with or without MMS for 30 min. (C) Immunoblot analysis showing ATM kinase activity was stimulated when incubated with DNA that had DSBs or SSBs. Lane 1, supercoiled pcDNA3. Lane 2, supercoiled pcDNA3 cut by Ncol. Lane 3, depurinated pcDNA3. Lane 4, depurinated pcDNA3 cut by APE1. Flag-ATM and GST-p53 were incubated with the indicated 30 min pretreatment with APE1 inhibitor III. (D) Immunoblot showing ATM kinase activity was stimulated when incubated with DNA that had DSBs or SSBs. Lane 1, supercoiled pcDNA3. Lane 2, supercoiled pcDNA3 cut by Ncol. Ncol-cut fragments, DNA gel electrophoresis.

**Figure 4.** UNG2 promotes ATM activation in response to deamination-related base lesions. (A) Immunoblot analysis showing GFP-A3A-induced phosphorylation of ATM S1981 in 293T cells. Cells expressing GFP-A3A or GFP-A3A-ER were incubated with or without 100 nM 4-OHT for 20 h to promote A3A activity, and the cell lysates were immunoblotted to detect the indicated proteins. (B) Immunoblot analysis showing decreased 4-OHT-induced phosphorylation of ATM S1981 by GFP-A3A-ER activation in 293T cells transfected with siRNA targeting UNG2. Cells expressing GFP-A3A-ER were transfected with control siRNA or two different sets of siRNA targeting UNG2. After 24 h, the cells were left untreated or were incubated with 100 nM 4-OHT for 20 h to promote A3A activity and DNA damage, and the cell lysates were immunoblotted to detect the indicated proteins. (C) 293T cells expressing GFP-A3A-ER were transfected with empty vector, Myc-UNG2WT or Myc-UNG2D154N. After 24 h, the cells were left untreated or incubated with 100 nM 4-OHT for 20 h to promote A3A activity and DNA damage, and the cell lysates were immunoblotted. (D) Immunoblot analysis showing induced phosphorylation of ATM S1981 following IR exposure (4 Gy) in 293T cells transfected with control siRNA or siRNA targeting UNG2.
phosphorylation suggest that other glycosylases may also be involved in the modulation of the DDR in response to DNA damage.

**Elevated MPG expression causes cell lethality and confers sensitivity to chemotherapy**

Because MPG is required to initiate the BER and because we found that the catalytic activity of MPG is critical for induction of the ATM-dependent DDR, we hypothesized that ATM is important for the cell response to MMS-induced DNA damage and DNA SSBs resulting from MPG action. To test this hypothesis, we first tested the ability of MPG expression to modulate cell viability as measured by the colony formation assay. When MPG was knocked down using siRNA, HeLa cells were more likely to survive higher levels of MMS exposure (survival fractions in 0.12 mg/ml MMS treatment were 3.4 ± 0.9% for the control group and 10 ± 3.4% for the siRNA targeting MPG group; **Supplementary Figure 14** is available at Carcinogenesis Online). In contrast, U2OS cells that expressed wild-type MPG were more sensitive to MMS treatment and had greater cytotoxicity than did cells that expressed the D132N mutant or those transfected with the vector control (survival fractions in 0.04 mg/ml MMS treatment were 4 ± 1.2%, 9.4 ± 1.6% and 8.9 ± 2.7%, respectively; **Figure 5A**). Alternatively, when we overexpressed ATM in cells that expressed wild-type MPG, ATM rescued MMS-mediated cell death promoted by MPG expression (survival fraction in 0.04 mg/ml MMS treatment was 9 ± 1.7%; **Figure 5A**). This suggests that ATM activation participates in preventing MPG-mediated cell death after MMS-induced DNA damage.

Because many drugs commonly used in chemotherapy kill tumor cells by formation of DNA SSBs or DSBs leading to activation of DDR, it is intriguing to know whether our cell-based and molecular findings shown above are of important relevance in cancer therapy. Therefore, to further explore how the expression of MPG affects cell survival and to better understand its implicated antitumor role, we evaluated the prognostic value of MPG in conjunction with chemotherapy status in a large public clinical microarray database (18), which includes data from 3455 patients with breast cancer. In the breast cancer dataset, expression of MPG was not associated with prognostic outcome across all patients or among only the chemotherapy-excluded group (**Figure 5B** and **C**). In contrast, when data from those patients who received the cyclophosphamide, methotrexate and fluorouracil regimen, which is a commonly used chemotherapy for breast cancer, were analyzed, a trend toward a favorable prognosis for MPG-high patients was found. As cyclophosphamide,

**Figure 5.** Elevated MPG expression sensitized cells to DNA damage and served as a therapeutic target. (A) Colony survival of U2OS cells expressing the empty vector (Control), Myc-MPG<sup>WT</sup>, Myc-MPG<sup>D132N</sup> or Myc-MPG<sup>WT</sup> combined with Flag-ATM plasmid in response to a 1-h treatment with different concentrations of MMS. Bar, standard error. Transfection was confirmed by immunoblot analysis (lower) Asterisks indicate that the survival fractions in the indicated dosage of treatment for the empty vector, Myc-MPG<sup>D132N</sup>, or Myc-MPG<sup>WT</sup> combined with Flag-ATM plasmid groups are respectively different from those seen in the Myc-MPG<sup>WT</sup> group (P < 0.05). (B–D) Kaplan–Meier plots of relapse-free survival showing patient survival across all patients (B) in a breast cancer dataset and among those patients in the same dataset who did not (C) or did (D) receive chemotherapy. Data in B, C, and D were obtained from the Kaplan–Meier Plotter database (18), and P values were calculated by log-rank tests. HR, hazard ratio; n, number of patients. (E) MPG messenger RNA expression levels compared in various human cancer types. Each data point was queried from the TCGA RNA-seq database and represents one normal or tumor sample. Blue and red horizontal bars indicate the median of MPG levels relative to TATA-box binding protein levels for each normal tissue and cancer type. Asterisks indicate significant MPG upregulation in the indicated tumor type relative to the corresponding normal tissues at P < 0.0001 (by Mann–Whitney U-test).
methotrexate and fluorouracil regimen generates DNA DSBs by blocking DNA replication, elevated DNA SSBs casued by higher MPG expression lead to additional DNA lesions and more harmful to cancer cells (Figure 5D). In addition, meta-analysis data from 1171 patients with ovarian cancer showed that MPG-high patients displayed favorable prognoses among the individuals who were treated with those drugs that markedly block DNA replication and produce DNA SSBs or DSBs, such as Gemcitabine, platin and Topotecan (Supplementary Figure 15A–C is available at Carcinogenesis Online). In contrast, MPG expression was not associated with prognostic outcome among those individuals who were treated with an angiogenesis inhibitor, Avastin, or with mitotic inhibitors including Docetaxel, Paclitaxel and Taxol (Supplementary Figure 15D–G is available at Carcinogenesis Online). Taken together, these results suggest that higher MPG expression in tumor cells may confer greater sensitivity to chemotherapy and could lead to more favorable prognoses.

To determine whether MPG messenger RNA is more highly expressed in human cancers, we performed an analysis of MPG RNA-seq data available from the TCGA database. In fourteen distinct tumor types, we found eight cancer types that showed significant MPG upregulation across tumors from six tissues including breast, colon, kidney, lung, thyroid and uterus ($P < 0.0001$ by Mann–Whitney U-test; Figure 5E). Among other cancers, such as those of the bladder and rectum, there was a trend toward higher MPG levels in tumors relative to normal tissues (Figure 5E). Thus, these data support the idea that MPG status could be a predictive marker of prognostic outcome especially among patients who are receiving chemotherapeutic treatments that rely on the presence of DNA SSBs or DSBs.

Discussion

Maintenance of genomic integrity by efficient DNA repair prevents genetic message disruption. To this end, coordination between the DDR and DNA repair pathways is essential (34,35). Although ATM and ATR act as upstream kinases in genome surveillance to initiate their respective DDR pathways, recent studies suggest that they are regulated by DNA repair mechanisms (36,37). In this study, we found that base-lesion-specific DNA damage was not sufficient to activate the ATM-Chk2–dependent checkpoint and that base lesions are needed to be excised sequentially by DNA glycosylases and APE1 into DNA SSBs to result in the activation of ATM and Chk2. Notably, we found that overexpression of ATM protected cells that expressed active MPG, whereas ATM deficiency sensitized cells to MMS treatment (38). This suggests that activation of the ATM pathway upon MPG action in BER is an important event in the DDR to S$_2$-type alkylating DNA damage, specially caused by MMS treatment, by preventing the accumulation of DNA SSBs (15).

In contrast to MPG and APE1 promoting formation of DNA SSBs, downstream proteins of the BER, including PARP1, DNA polymerase $\beta$ and XRCC1, are important for SSB removal, and, as a result, blocking the function of these proteins leads to MMS-induced ATM activation as observed in the present study and in our previous study (16). In our previous study, however, activating the ATM-Chk2 DDR was shown to promote efficient BER by phosphorylating XRCC1 (16). Therefore, we suggest the following model regarding the interplay between BER and ATM-dependent DDR: once a base lesion occurs and is initially recognized by DNA glycosylases, ATM-Chk2 is then activated and phosphorylates XRCC1 at T284, which is important for recruiting XRCC1 and its partners (PARP1, DNA polymerase $\beta$ and DNA ligase III) to the site of DNA damage near the glycosylase, providing an efficient regulatory mechanism during BER processing (Figure 6). More recently, it was reported that ATM enhances MPG activity by phosphorylating MPG at S172, promoting a more direct regulation in initiating the BER pathway in response to DNA damage (39).

MPG is required for processing MMS-induced DNA damage to activate ATM-Chk2. In addition, UNG2, which is responsible for deaminating damaged DNA, promoted A3A-induced phosphorylation of ATM S1981 (Figure 4), and OGG1, which is required for oxidative DNA damage, promoted H$_2$O$_2$-induced phosphorylation of Chk2 T68 (Supplementary Figure 12 is available at Carcinogenesis Online). These findings suggest that, in addition to responding to DSBs, ATM is activated in the DDR of various kinds of base lesions. However, given that the first step to detect base lesions completely depends on BER, we suggest that the ATM-dependent DDR is not directly activated and that this activation depends on individual glycosylases acting in response to specific types of lesions. For example, MPG was specifically required for the MMS-induced ATM-Chk2 DDR but had no role in A3A- or H$_2$O$_2$-induced signaling. On the basis of these findings, we hypothesize that ATM might be activated under the conditions during which any type of DNA damages is processed to a common intermediate, i.e. a DNA SSB, by different DNA repair pathways. This hypothesis is consistent with findings from those
identified in other DNA repair pathways, such as mismatch repair proteins (MutSα and MutL) and nucleotide excision repair proteins (XPC and DDB2), which are reported to regulate N-methyl-N′-nitro-N-nitrosoguanidine-induced and ultraviolet-induced ATM-dependent DDR, respectively (36,40). Furthermore, our data also confirmed that the MRN complex, together with CtIP, which are known to facilitate ATM activation by promoting DNA end resection at the site of IR-induced DNA DSBs (26,41), do not participate in MMS-induced ATM phosphorylation, although MMS-induced Chk2 T68 phosphorylation was affected by reduced expression of either one of MRN complex. Furthermore, we did not detect the role of histone modification, i.e. histone acetylation, in MMS-induced ATM activation, though our finding shows that ATM was recruited to chromatin architecture independent from direct interaction with MPG. Therefore we suggest that there exist other players, especially those proteins, e.g. HP1 and KAP1, interacting with ATM on chromatin (42), may mediate MMS-induced ATM activation, and are involved in the mechanisms parallel to MPG-dependent pathway.

Inactivation of MPG increases the frequency of spontaneous mutation (43); overexpression of MPG causes breast cancer cells and gliomas to be more sensitive to MMS and temozolomide (the other DNA alkylator commonly used in clinic) treatment (44–46). This is because MPG removes O′-methylguanine adducts from DNA and leads to DNA SSB formation, which is harmful to the cell. On the other hand, temozolomide produces relatively abundant amount of O′-methylguanine, that is well-known to be repaired by MGMT (44,45), and is thus not appropriate to provide more straightforward results in this study to assess MPG-induced ATM activation. As a result, we used MMS as the source of DNA alkylating damage. Our findings also support the idea that overexpression of MPG promotes ATM activation by producing unexpected AP sites or SSBs, which are much more harmful than single-base lesions. Therefore, initiation of BER acts as a double-edged sword. Because the ATM-Chk2 pathway participates in promoting BER and cell cycle arrest (15), cells were more resistant to MMS treatment when ATM was induced (Figure 5A). In contrast, once the ATM pathway was already activated by chemotherapeutic drugs, the induction of glycosylases and base lesions in cancer cells may serve as a combinatorial regimen to cure cancers. For example, we reported that higher MPG expression in ovarian cancer tumors was correlated with more favorable prognoses in patients who were treated with Gemcitabine, platins or Topotecan, but no benefit was seen in patients treated with other drugs such as Avastin or taxoid-related drugs. Alternatively, KU55933, an ATM-specific small molecule inhibitor, in combination with inhibition of downstream BER proteins, such as PARP1 and XRCC1, has been suggested as a novel combination for cancer treatment (47,48). Based on our findings in the present study, MPG upregulation, as identified in at least eight distinct cancers, in consistent with PARP1 inhibition, may increase the accumulation of DNA SSBs and thus could increase the therapeutic capacity in combination with treatment with KU55933 as a clinical regimen.

In this study, we explored how base lesions initiate DDR. MPG,UNG2 and OGG1 promoted the ATM-Chk2 DDR, and APE1 and PARP1 participated in the regulation of DNA SSB production, which is essential for ATM-Chk2 activation. Considering that the combined inhibition of PARP1 activity and DSB repair, which blocks both repair pathways for DNA SSBs and DSBs, has emerged as a potential strategy for cell growth inhibition and cancer treatment (49,50), our findings provide important evidence in support of the induction of DNA SSBs through a specific DNA glycosylase as an alternative chemotherapeutic treatment for cancer.

Supplementary material

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References