Ectopic Expression of Human MutS Homologue 2 on Renal Carcinoma Cells Is Induced by Oxidative Stress with Interleukin-18 Promotion via p38 Mitogen-activated Protein Kinase (MAPK) and c-Jun N-terminal Kinase (JNK) Signaling Pathways*

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Background: Ectopically expressed MutS homologue 2 (hMSH2) is a ligand of γδ T cells.

Results: Oxidative stress induces ectopic expression of hMSH2 on renal carcinoma cells with IL-18 promotion via p38 MAPK and JNK pathways, promoting γδ T cell-mediated lysis of renal tumor cells.

Conclusion: Ectopically expressed hMSH2 is induced under stressful conditions.

Significance: We reveal a mechanism of ectopic hMSH2 expression and its contribution to γδ T cell-mediated immunosurveillance in stress.

Human MutS homologue 2 (hMSH2), a crucial element of the highly conserved DNA mismatch repair system, maintains genetic stability in the nucleus of normal cells. Our previous studies indicate that hMSH2 is ectopically expressed on the surface of epithelial tumor cells and recognized by both T cell receptor γδ (TCRγδ) and natural killer group 2 member D (NKG2D) on Vγ2 T cells. Ectopically expressed hMSH2 could trigger a γδ T cell-mediated cytolyis. In this study, we showed that oxidative stress induced ectopic expression of hMSH2 on human renal carcinoma cells. Under oxidative stress, both p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways have been confirmed to mediate the ectopic expression of hMSH2 through the apoptosis-signaling kinase 1 (ASK1) upstream and activating transcription factor 3 (ATF3) downstream of both pathways. Moreover, renal carcinoma cell-derived interleukin (IL)-18 in oxidative stress was a prominent stimulator for ectopically induced expression of hMSH2, which was promoted by interferon (IFN)-γ as well. Finally, oxidative stress or pretreatment with IL-18 and IFN-γ enhanced γδ T cell-mediated cytolyis of renal carcinoma cells. Our results not only establish a mechanism of ectopic hMSH2 expression in tumor cells but also find a biological linkage between ectopic expression of hMSH2 and activation of γδ T cells in stressful conditions. Because γδ T cells play an important role in the early stage of innate anti-tumor response, γδ T cell activation triggered by ectopically expressed hMSH2 may be an important event in immunosurveillance for carcinogenesis.

The DNA MMR2 system plays a central role in maintaining genetic stability by inhibiting base-base mismatches and insertion/deletion mispairs generated during DNA replication and recombination (1, 2). Homologs of bacterial MMR proteins MutS and MutL in eukaryotes have received much attention because of many discoveries showing a linkage between human cancer and MMR defects (3). The critical role of the MMR system in carcinogenesis is exemplified by the fact that hereditary nonpolyposis colorectal cancer occurs primarily due to heterozygous germ line mutations in the MMR genes: hMLH1 (MutL homolog 1), hMSH2, hMSH6 (MutS homologue 6), and hPMS2 (MutL homologue) (4). The hMLH1 promoter hypermethylation has been observed in ovarian, endometrial, gastric, and colorectal carcinoma (5). The hMSH2 mutations have a higher incidence of extracolonic tumors (6). The defects of both MMR genes are involved in renal carcinogenesis and correlate with the occurrence of microsatellite instability (7).

Under physiological conditions, hMSH2 is mainly synthesized in cytoplasm and transported into the nucleus together with MutS homologue 3 (hMSH3) or hMSH6 to maintain the DNA replication fidelity (8, 9). Increased expression of hMSH2 RNA and/or protein has been reported in various malignancies (10–14). However, the underlying mechanism that causes increased levels of hMSH2 in malignancies is yet unknown. Previously, we found an unusual expression of hMSH2 on the surface of human ovarian carcinoma cells, and ectopically expressed hMSH2 promoted the lysis of ovarian carcinoma cells by γδ T cells (15). We also identified that ectopically expressed hMSH2 could be recognized by γδ T cells through the interaction with both TCRγδ (T cell receptor γδ) and

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NKG2D (natural killer group 2 member D) (77). However, the mechanism of ectopic expression of hMSH2 in tumor cells is unclear, and ectopic hMSH2 expression may be important for the recognition of potential damage-associated molecular patterns (DAMPs) by human γδ T cells (16–18).

Oxidative stress, defined as the imbalance between the generation of reactive oxygen species (ROS) and scavenging by antioxidant defenses of the cell, is involved in carcinogenesis because it induces DNA damage and causes oxidative modification (19). The tumorigenicity of oxidative stress has been observed among various carcinomas, especially in RCC cells, which possess low antioxidant capacities as a consequence of a deficiency of peroxisomes and the changed redox status (20, 21). The defective antioxidant capacity also produces a pro-oxidant environment in RCC cells and promotes the process of renal tumor genesis (20, 22).

Because hMSH2 or hMSH6 participates in removing complex DNA lesions produced in oxidative stress (23), we hypothesize that oxidative stress may be responsible for ectopic expression of hMSH2 on tumor cells. In the present study, we established a glucose oxidase (GO)-mediated oxidative stress model in RCC cells through oxidizing β-D-glucose and generating hydrogen peroxide (H2O2) continuously at a relatively low level (24). We found that hMSH2 was constitutively expressed at a low level on the surface of RCC cells. However, ectopic expression of hMSH2 was increased in GO-mediated oxidative stress through p38 MAPK and JNK pathways in RCC cells. The regulating role of p38 MAPK and JNK pathways was mediated by a node kinase, ASK1, upstream and the stress-sensitive activating transcription factor 3 (ATF3) downstream of both pathways. Moreover, proinflammatory cytokine IL-18 produced by RCC cells was a crucial stimulator inducing ectopic expression of hMSH2 in oxidative stress. Meanwhile, exogenous IFN-γ further promoted ectopic expression of hMSH2 on the surface of RCC cells, and γδ T cells produced a high level of IFN-γ during oxidative stress. We also showed that oxidative stress or IL-18 enhanced γδ T cell-mediated lysis of RCC cells. Taken together, our findings provide a better understanding of oxidative stress-induced ectopic expression of hMSH2 on the surface of RCC cells. As a result, ectopically expressed hMSH2 may act as a candidate molecule of DAMPs recognized by γδ T cells, which indicates that γδ T cells may perform immunosurveillance of hMSH2-bearing tumor cells in an oxidative stress environment.

EXPERIMENTAL PROCEDURES

Chemicals—The glucose oxidase (G7141) catalyzes the oxidation of β-D-glucose into α-D-glucono-δ-lactone and H2O2, with molecular oxygen as an electron acceptor. N-Acetyl-L-cysteine (NAC) (A7250), a ROS scavenger, was selected to block the H2O2 effect. Calyculin A from Discodermia calyx (C5552) was used as an inhibitor of protein phosphatases types 1 and 2A. The reagents above were purchased from Sigma–Aldrich. Pharmacological inhibitors of p38 MAPK (SB203580) and JNK (420119) were purchased from Merck, and the inhibitors of the MAPK/ERK kinase (MEK) and ERK pathway (U0126) were purchased from Selleck. The 1-methylpropyl 2-imidazolyl disulfide (PX-12) was purchased from TOCRIS.

Cell Culture and Treatment—Human renal carcinoma cell line G401, melanoma cell line A375, lymphoma cell line Jurkat, and leukemia cell line K562 were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Invitrogen). Human renal carcinoma cell line A498 was maintained in minimum Eagle’s medium supplemented with 10% FBS (HyClone). Human renal proximal tubule epithelial cell line HK-2 was maintained in DMEM/F-12 medium supplemented with 10% FCS (Invitrogen). All cell lines were obtained from the Cell Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). For constructing the oxidative stress model, the cells were activated by different concentrations of glucose oxidase (G7141; Sigma) in medium at 37 °C, 5% CO2 for prospective times (24).

γδ T Cell Expansion In Vitro—Peripheral blood mononuclear cells from healthy donors were isolated by Ficoll–Hypaque (TBD, Tianjin, China) centrifugation. γδ T cells were expanded as described previously (25). Briefly, 24-well plates were coated with 500 μl of purified anti-TCR-γδ antibody (Ab) (IMMU510; 1 μg/ml; Immunotech, Beckman Coulter) at 37 °C, 5% CO2 for 2 h. Peripheral blood mononuclear cells were transferred to the plate and cultured in RPMI 1640 medium supplemented with 10% FCS and recombinant human IL-2 (200 IU/ml; Beijing Read United Cross Pharmaceutical Co., Ltd., Beijing, China). After 2 weeks of culture, the purity of γδ T cells was more than 90% as assessed by flow cytometry analysis and ready for further use.

Cytotoxicity Assay— Cell cytotoxicity was determined by the Cytotox 96® non-radioactive cytotoxicity assay kit (Promega). G401 cells were preincubated with the indicated amounts of GO, IL-18, or IL-18 plus IFN-γ. γδ T cells of appropriate number were incubated with G401 cells (1 × 10^5/ml) in triplicates for 4 h at 37 °C. The release of lactate dehydrogenase and percentage of cytotoxicity were determined by the absorbance of supernatants at 490 nm.

RNA Interference—A pool of three specific 19–25-nt small interfering RNA (siRNA) designed to knock down IL-18Rα expression (sc-45285), mock control siRNA-a (sc-37007), and the transfection reagent (sc-29528) was purchased from Santa Cruz Biotechnology, Inc. Three pairs of Stealth™ RNAi-specific ATF3 siRNA were purchased from Invitrogen: sensel, 5′-GAGGCCGACGAGAAAAAUAAGAUU-3′; antisenel, 5′-AAUCUAUAUUCUCUUCGCCUC-3′; sensel, 5′-GCAGUCGAAAAAGUGCCGAAAAG-3′; antisenel, 5′-UCUCUUGUCCUGCGCAUUCCGU-3′; senselIII, 5′-GGAGGCACCGAGAAAGAAGA-3′; antisenelIII, 5′-UCUCUCCACGUCCAGGAGA-3′. Stealth™ RNAi universal negative control Med GC duplex (Invitrogen) were used as mock control. All of these siRNA duplexes were transfected using Lipofectamine™ RNAiMAX according to manufacturer’s instructions (Invitrogen). The delivery of siRNAs was measured by transfection with BLOCK-i™ Alexa Fluor® Red Fluorescent Oligo (Invitrogen) and analyzed at 12 h after transfection. Efficiency of target genes knockdown was evaluated by quantitative real-time RT-PCR and Western blot.

Real-time RT-PCR—Total RNA was isolated from G401 cells with the TRizol reagent (Invitrogen). The cDNA was syn-
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The expression of hMSH2 was analyzed using oligo(dT) as primers and the Moloney murine leukemia virus reverse transcriptase (Promega) in the reverse transcription reaction. The specific primers used were as follows: for IL-18Ra, 5'-ACCCCTTGGTGTTATATCTGT-TAA-3' (forward) and 5'-GGTCCCCCTCAACCACAGTA-ATG-3' (reverse); for hMSH2, 5'-TTCATGGCTGAAATGT- TGGA-3' (forward) and 5'-ATGCTAAAACATCTCATC-3' (reverse); for ATF3, 5'-GCCATGGAGACAGCTGTTCTT-3' (forward) and 5'-AGGGCCTGCTGAACCACC-3' (reverse); and for β-actin, 5'-AGAAAATCTGGCACCACACC-3' (forward) and 5'-TAGCACAGCCTGGATAGCAA-3' (reverse). The quantitative PCR was performed in an Applied Biosystems 7500 Fast Real-time PCR System to quantify the levels of IL-18Ra, hMSH2, and ATF3 mRNA. The amplification reaction was performed using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Cycling conditions were at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and at 58 °C for 45 s. Data were analyzed using Sequence Detection Software (version 1.2; Applied Biosystems).

Enzyme-linked Immunosorbent Assay (ELISA) Analyses—The IL-18 in the oxidative-stress-stimulated RCC, A375, Jurkat, or K562 cell culture supernatants and IFN-γ or tumor necrosis factor-α (TNF-α) in the oxidative-stress-stimulated γδ T cells culture supernatants were determined by ELISA kits (R&D Systems). The assay procedure was performed according to the manufacturer’s instructions.

Western Blotting—Cell lysates were prepared by CytoBuster™ protein extraction reagent (71009, Novagen) for total protein and by the NucBuster protein extraction kit (71183-3, Novagen) for nucleoprotein. Halt Protease and Phosphatase Inhibitor Single-Use Mixture, EDTA-Free (Thermo) were used to prevent enzymatic protein degradation during extraction procedures. The protein concentration was quantified by the Pierce BCA protein assay kit (23227, Thermo). An equal amount of proteins per sample was separated by 12% SDS-PAGE and blotted onto nitrocellulose membranes. The blots were treated with PBS containing 5% skim milk or bovine serum albumin overnight and probed with primary Ab at room temperature for 2 h, followed by the relevant secondary Ab for 1 h. Protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (NCI5079, Thermo). Monoclonal antibody (mAb) against phospho-JNK/phospho-p38/phospho-c-Jun (Cell Signaling Technology), anti-human IL-18Ra (R&D Systems), ATF3 (Abgent), hMSH2 (ProteinTech Group, Inc.), or β-actin (Signalchem) and polyclonal antibody to histone H3 (Abcam) were used as primary Abs.

Flow Cytometry—Cells were washed with PBS containing 1% BSA, stained with relevant Abs, and incubated at 4 °C for 30 min. Then the cells were washed and resuspended in 300 µl of PBS containing 1% formaldehyde. FITC-conjugated anti-human IL-18Ra Ab (catalog no. 313810), APC-conjugated anti-human MICA/B Ab (catalog no. 320908), and the respective isotype control mAbs were purchased from Biolegend (San Diego, CA). Ab FITC-conjugated anti-TCRγδ (IMMU510) was purchased from Immunotech. When purified anti-MSH2 Ab (H-300) (sc-22771) and the isotype control polyclonal Abs (Santa Cruz Biotechnology, Inc.) were used as primary Abs, FITC-conjugated goat anti-rabbit secondary Ab (Zhongshan, China) was then added and incubated for 30 min at 4 °C. For intracellular staining, the cells incubated with 20 ng/ml phorbol 12-myristate-13-acetate (Sigma), 0.5 µg/ml ionomycin (Sigma), and 3 µg/ml brefeldin A (eBioscience, San Diego, CA) acted as the positive control. After 4 h, the cells were collected and stained for surface markers. Then the cells were fixed, permeabilized, and incubated with intracellular Abs. All samples were analyzed on an Accuri C6 flow cytometer (BD Biosciences). Data analysis was carried out with FlowJo Software (version 7.6.4, Tree Star, Inc.).

Statistical Analysis—Data were expressed as the mean ± S.D. and were representative of at least three independent experiments. One-way analysis of variance (SPSS version 16.0 software) was used to determine significant differences between groups. Differences were considered statistically significant at a p value of less than 0.05.

RESULTS

Oxidative Stress Induces Ectopic Expression of hMSH2 on Surface of RCC Cells—To determine the ectopic expression of hMSH2 in stress, renal carcinoma cell lines G401 and A498 and a renal proximal tubular cell line, HK-2, were chosen because these cell lines from kidney exhibited a constitutive ectopic expression of hMSH2 on surfaces in normal cultures (Fig. 1A). In response to oxidative stress, ectopically expressed hMSH2 was induced on G401 cells treated by different concentrations of GO for 24 or 36 h, respectively (Fig. 1B). HK-2 and A498 cells showed similar results (data not shown). GO stimulation at 100 ng/ml for 36 h induced the strongest expression in all three cell lines (Fig. 1, B and C). A down-regulation of GO-induced hMSH2 ectopic expression was observed on G401, A498, and HK-2 cells by the addition of NAC, a ROS scavenger that strongly blocks the H₂O₂ effect (Fig. 1, B and C). Because G401 cells exhibited the most obvious changes of ectopically expressed hMSH2 mediated by GO or NAC, we subsequently chose G401 cells as the cell model to investigate the mechanisms underlying ectopic expression of hMSH2 in oxidative stress. An increase of hMSH2 mRNA was observed as early as 1 h and lasted to 48 h by exposing G401 cells to 100 ng/ml GO-mediated stress (Fig. 1D). Accordingly, the total protein level of hMSH2 was up-regulated in G401 cells stimulated by GO (Fig. 1E). These results demonstrate that ectopic expression of hMSH2 on the surface of RCC cells is induced in response to oxidative stress.

We also found that oxidative stress-induced ectopic expression of hMSH2 showed a similar change pattern to surface expression of MICA/B, classical stress-sensitive ligands of NKG2D in oxidative stress (Fig. 1, F–I), and both of them could be reduced markedly by NAC processing (Fig. 1, F–I). Because expression of MICA/B is restricted or absent in normal tissues and up-regulated in stress and diseases (26, 27), it further confirms the stress-inducible characteristic of ectopic expression of hMSH2 on RCC cells.

Both p38 MAPK and JNK Pathways Are Involved in Regulating Ectopic Expression of hMSH2 in RCC Cells in Oxidative Stress—The MAPK family plays an important role in oxidative stress-induced cellular events (28). Among the MAPK family, the activation of ERKs usually causes cell survival or differenti-
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Figure 1. Ectopic expression of hMSH2 is inducible on human renal proximal tubule epithelial cell line HK-2 and renal carcinoma cells in oxidative stress. A, surface expression of hMSH2 on normal cultured HK-2 and renal carcinoma cells G401 and A498 (labeled as Normal Ctrl), B and C, ectopic expression of hMSH2 on the surface of HK-2, A498, and G401 cells treated with GO for 24 or 36 h. The black lines represent surface expression of hMSH2 on GO-stimulated cells. Surface expression of hMSH2 on GO-stimulated HK-2, A498, and G401 cells treated by NAC (5 mM) is displayed as dotted lines, and the respective isotype controls are shown as solid gray. D, real-time RT-PCR analysis for transcriptional expression of hMSH2 in normal or GO (100 ng/ml)-stimulated G401 cells. Data are expressed as fold changes (mean ± S.D. [error bars]) compared with the transcriptional level of hMSH2 in normal cultured G401 cells. *, p < 0.05. E, total expression of hMSH2 in whole cell extracts of normal or GO (100 ng/ml)-treated G401 cells. The total expression of hMSH2 in A375 cells is used as a positive control (lane 1); the total hMSH2 expression in normal control G401 cells is displayed in lane 2; and G401 cells GO-stimulated for 24 or 36 h, respectively, are shown in lanes 3 and 4. β-Actin was used as a control of protein loading (10 μg/lane). F–I, comparison of ectopic expression of hMSH2 with MICA/B in oxidative stress (GO, 100 ng/ml) or adding with NAC (5 mM) into stressful cultures. Mean fluorescence intensity (MFI) values are shown as mean ± S.D. n = 4 independent experiments. *, p < 0.05.
Quantitative RT-PCR detection showed that GO-induced hMSH2 mRNA was decreased in G401 cells when pretreated with either the specific inhibitor of p38 MAPK (SB203580) or JNK pathway (420119) (Fig. 2D). However, there was no significant changes of GO-induced hMSH2 mRNA pretreated with the MEK/ERK pathway inhibitor (Fig. 2D). Importantly, pretreatment with either specific inhibitor for the p38 MAPK or JNK pathway suppressed ectopic expression of hMSH2 on the surface of G401 cells induced by GO (Fig. 2, E and F), whereas inhibition of the MEK/ERK pathway showed no obvious effects on ectopic expression of hMSH2 in oxidative stress (supplemental Fig. 1). The total expression of hMSH2 was also down-regulated when blocking the role of p38 MAPK or JNK pathway in GO-treated G401 cells (Fig. 2, G–J). The above data demonstrate the important roles of p38 MAPK and JNK pathways in regulating ectopic expression of hMSH2 during oxidative stress.

p38 MAPK and JNK Pathways Regulate Ectopic Expression of hMSH2 through ASK1, Node Kinase of Both Pathways

To further validate the functional involvement of the p38 MAPK and JNK pathways in regulating ectopic expression of hMSH2 during oxidative stress, we performed immunoblot analysis. As shown in Fig. 2A and B, we detected the phosphorylation (P-) of the p38 MAPK and JNK pathways in G401 cells at several time points after GO-mediated oxidative stress. β-Actin was used as the protein loading control (30 μg of total proteins/lane). C, expression of phospho-c-Jun in G401 cells treated with GO. Histone H3 was used as the protein loading control (30 μg of total proteins/lane). D, transcription of hMSH2 in G401 cells during oxidative stress in the presence or absence of the p38 MAPK pathway inhibitor (SB203580), the JNK pathway inhibitor (420119), or the MEK/ERK pathway inhibitor (U0126) was assessed by real-time RT-PCR. Data were normalized as -fold changes (mean ± S.D.) compared with the transcriptional level of hMSH2 in G401 cells without GO treatment. n = 5 independent experiments. *, p < 0.05. E and F, surface expression of hMSH2 on GO (100 ng/ml)-treated G401 cells in the presence or absence of p38 MAPK or JNK pathway inhibitor (n = 5 independent experiments). Error bars, S.D. *, p < 0.05. G–J, total expression of hMSH2 in GO (100 ng/ml)-stimulated G401 cells treated with or without specific inhibitors for the p38 MAPK (labeled as GO p38-) or JNK (displayed as GO JNK-) pathway (n = 4 independent experiments). Error bars, S.D., *, p < 0.05. K and L, total protein expression of hMSH2 in PX-12-stimulated G401 cells. Lane 1, positive control; lanes 2 and 3, expression of hMSH2 in G401 cells treated by PX-12 (50 μM) for 24 or 36 h, respectively (labeled as PX-12 24h and 36h). Lane 4, G401 cells treated with a combination of PX-12 (50 μM) and NAC (5 mM). Lane 5, normal control.
JNK pathways in regulating ectopic expression of hMSH2 in stress, we next examined the role of ASK1, a member of the mitogen-activated protein kinase kinase (MAP3K) family that could activate both JNK and p38 MAPK pathways in response to oxidative stress (35). We used the combination of PX-12 and a low level of glucose (1 g/liter) to activate ASK1 by disrupting the interaction between thioredoxin and ASK1 (36–38) and used NAC as an antioxidant to inhibit the PX-12-induced disassociation of thioredoxin from ASK1 and JNK activation (38). As shown in Fig. 2, K–M, both the total and ectopic expression of hMSH2 were enhanced in G401 cells when ASK1 was activated after treatment with PX-12. Meanwhile, this increase was suppressed substantially by inactivating ASK1 with NAC. To further confirm the role of activated ASK1 in enhancing ectopic expression of hMSH2, we used calycin A, an inhibitor of protein phosphatase type 1 and 2A to inhibit the primary activation of ASK1 through inhibiting H$_2$O$_2$-induced dephosphorylation of ASK1 Ser-967 (39). It showed that PX-12-induced surface expression of hMSH2 was suppressed when dephosphorylation of ASK1 was inhibited by calycin A (Fig. 2M). The effect of ASK1 on ectopic expression of hMSH2 further demonstrates the role of p38 MAPK and JNK pathways in regulating ectopic expression of hMSH2 in RCC cells.

**ATF3 Acts as Executor Inducing Ectopic Expression of hMSH2 Regulated by p38 MAPK and JNK Pathways in Oxidative Stress**—Activities of the activator protein 1 (AP-1) family can be stimulated by many environmental and cellular factors, including oxidative stress (40, 41). The synthesis of ATF3, a member of the AP-1 subfamily activating transcription factors, is quite robust relative to the levels of other oxidative stress-induced AP-1 proteins (42). We found that transcription factor binding sites for the AP-1 family presenting in the regulatory element of hMSH2 were matched to ATF3 transcription factor binding sites (43–45): AP-1, −611 to −605 (5’-TGAATCA-3’) and −297 to −291 (5’-TGAATTC-3’); ATF/cAMP-response element, −1156 to −1149 (5’-TGACGTCA-3’). Therefore, we tested if ATF3 is involved in the regulation of ectopic expression of hMSH2 during oxidative stress. Interestingly, GO treatment enhanced the transcription of ATF3 significantly at 1–3 h compared with the level in control G401 cells (Fig. 3A). When the GO-mediated oxidative stress was blocked by NAC, the ATF3 transcription was down-regulated (Fig. 3A). Consistent with it, the total expression of ATF3 was also up-regulated in G401 cells exposed to oxidative stress (Fig. 3B). To investigate the potential role of ATF3 in regulating ectopic expression of hMSH2 in response to oxidative stress, ATF3 was knocked down in G401 cells by siRNAs (Fig. 3, C and D). In response to GO-mediated oxidative stress, knocking down ATF3 for 48 h blocked the most induction of ectopically expressed hMSH2 on G401 cells (Fig. 3, E and F). Similar ATF3-RNAi effects under GO-mediated oxidative stress were also observed after transfecting with ATF3 siRNAs for 24 or 36 h (supplemental Fig. 2). However, the surface expression of MICA/B induced by oxidative stress was not affected significantly by ATF3 knockdown (Fig. 3, G and H). Although hMSH2 and MICA/B are both stress-inducible proteins recognized by γδ T cells, they show different responses of surface expression mediated by ATF3 in stress, and ATF3 appears to play a specific role in regulating ectopic expression of hMSH2 in oxidative stress.

**Proinflammatory Cytokine IL-18 Produced by RCC Cells in Oxidative Stress Is Prominent Stimulator Inducing Ectopic Expression of hMSH2**—To further seek the crucial factors inducing ectopic expression or distribution of hMSH2 during oxidative stress, we noted that ROS could trigger the activation of inflammasome NLRP3/NALP3 and recruited the caspase-1 (46). The inflammasome/caspase-1 axis is essential for the maturation and release of proinflammatory cytokines, such as IL-1β and IL-18 (46, 47). Importantly, IL-18 treatment enhances the expression of UL-16-binding protein 2 (ULBP2), a stress-inducible ligand for NKG2D in leukemia cells (48). Here, we examined whether IL-18 is a potential factor inducing ectopic expression of hMSH2 in oxidative stress. The present study showed that IL-18 was released increasingly into supernatants of GO-stimulated RCC cells (Fig. 4, A and B). IL-18α was constitutively expressed on normal cultured G401 cells and increased in oxidative stress (Fig. 4C). Similar results were shown in A498 cells (data not shown). Exogenous IL-18 alone could up-regulate ectopic expression of hMSH2 in a dose-dependent manner (Fig. 4D). All these data indicate the potential ability of IL-18 in inducing ectopic expression of hMSH2 on RCC cells during oxidative stress. To assess the role of endogenous IL-18 in oxidative stress, siRNA-IL-18Ra was used to knock down the expression of endogenous IL-18Ra in G401 cells. Both levels of IL-18Ra mRNA and protein were significantly reduced after transfection with siRNA-IL-18Ra (Fig. 4, E–G). Of note, no significant up-regulation of hMSH2 transcription or surface expression on G401 cells was found in siRNA-IL-18Ra-treated cells (Fig. 4, H and I). The total protein level of hMSH2 in G401 cells treated with siRNA-IL-18Ra also showed a down-regulated pattern after GO treatment (Fig. 4, J and K). These results suggest that endogenous IL-18 released by RCC cells during oxidative stress has a direct effect on inducing ectopic expression of hMSH2.

**Ectopic Expression of hMSH2 Is Promoted by IFN-γ and Proinflammatory Factors Enhance Lysis of RCC Cells by γδ T Cells in Oxidative Stress**—Our previous studies have demonstrated that hMSH2-bearing tumor cells were susceptible to Vδ2 γδ T cell-mediated cytolysis, in which IFN-γ and TNF-α were primary factors produced by γδ T cells (15, 25, 49). It led us to examine the level of these proinflammatory cytokines produced by GO-stimulated γδ T cells and their effects on ectopic expression of hMSH2. When anti-pan-TCR γδ mAb-expanded γδ T cells were treated with different concentrations of GO, an increased amount of IFN-γ was released into supernatants compared with γδ T cells in normal cultures (Fig. 5A). The largest amplitude of IFN-γ in supernatant was increased from 205.78 (in normal culture) to 1027.09 pg/ml (treated with GO 50 ng/ml for 36 h), about a 5-fold increase (Fig. 5A). However, there was no obvious change of TNF-α upon GO treatment (Fig. 5B). Consistent results of the intracellular staining assay showed that the increased level of IFN-γ was mainly produced by γδ T cells in GO-mediated oxidative stress (Fig. 5C). Then exogenous IFN-γ alone promoted GO-induced ectopic expression of hMSH2 on G401 cells, and this up-regulation was inhibited by the administration of NAC (Fig. 5, D and E). Moreover,
the lysis of G401 cells mediated by γδ T cells could be enhanced by treatment with GO, IL-18, or IL-18 plus IFN-γ (Fig. 5F). These data suggest that IFN-γ released by γδ T cells in oxidative stress might promote ectopic expression of hMSH2, and the proinflammatory cytokine IL-18 or IFN-γ from oxidative stress promotes the lysis of RCC cells by γδ T cells.

Ectopically Expressed hMSH2 Is Also Induced on Several Other Cancer Cell Lines in Oxidative Stress or Treatment by IL-18—To further investigate inducible ectopic expression of hMSH2 in other tumor cells, we chose the melanoma cell line A375, the lymphoma cell line Jurkat, and the leukemia cell line K562 to analyze the surface expression of hMSH2, especially under oxidative stress or treatment with exogenous IL-18. Here, the autocrine of IL-18 in normal or stressful conditions was detected in supernatants of A375 and RCC cell lines (Fig. 6A), and all of the above tumor cells exhibited a constitutive ectopic expression of hMSH2 on their surfaces (Fig. 6B). Importantly, oxidative stress further enhanced the ectopic expression of hMSH2 on these tumor cells (Fig. 6D).

FIGURE 3. Stress-sensitive ATF3 located downstream of both p38 MAPK and JNK pathways participates in inducing ectopic expression of hMSH2 in oxidative stress. A and B, mRNA (A) and protein (B) expression of ATF3 in G401 cells at several time points after GO treatment. n = 4 independent experiments. Error bars, S.D. *, p < 0.05. Histone H3 was used as the protein loading control (30 μg/lane). C and D, mRNA and protein expression of ATF3 in G401 cells after transfection with siRNAs for ATF3. siATF3I, siATF3II, and siATF3III represented three different siRNA duplex oligoribonucleotides for ATF3. E and F, surface expression of hMSH2 on GO-stimulated G401 cells after treatment with siRNAs for ATF3. siATF3III (si2) and siATF3III (si3) were chosen for their good efficiency to knock down ATF3 expression. GO-stimulated G401 cells treated with Stealth™ RNAi universal negative control duplex are depicted with black lines, GO-stimulated G401 cells treated with siRNAs for ATF3 (labeled as GOsi) are displayed in dotted lines, and the isotype controls are in solid gray. Mean fluorescence intensity (MFI) is shown as mean ± S.D. (n = 3 independent experiments). *, p < 0.05. G and H, MICA/B expressions on GO-stimulated G401 cells (black line) or ATF3-knocked down G401 cells (dotted line) and the isotype controls (solid gray). ns, no statistical significance.
These data suggest that ectopic expression of hMSH2 is broad in different tumor cells, and IL-18 induced by oxidative stress may be an important stimulator to promote ectopic hMSH2 expression on tumor cells.

**DISCUSSION**

Because hMSH2 participates in the removal of mispairs originating from damaged DNA in an oxidative environment (50), we have attempted to find a linkage between oxidative stress and ectopic expression of hMSH2 on tumor cells. To mimic the stressful status in patients with several types of tumors, we chose GO-mediated oxidative stress, which is characterized by forming a continuous H2O2 stream into cultures at constant levels (24, 51–53). Our present findings demonstrate that expression of hMSH2 mRNA could be enhanced in G401 cells exposed to oxidative stress. Accordingly, ectopic expression of hMSH2 is induced by GO treatment in a dose-dependent manner and blocked by a ROS scavenger, NAC. It suggests that...
hMSH2 possesses the characteristic of inducible ectopic expression on the surface of RCC cells during oxidative stress. In addition, inducible ectopic expression of hMSH2 was also observed on other tumor cells, including melanoma A375, lymphoma Jurkat, and leukemia K562 cells (Fig. 6B). As a tumor-associated protein promoting Vδ2 γδ T cell-mediated lysis (15), the inducibility of ectopically expressed hMSH2 in tumor cells indicates that it may activate many more γδ T cells in certain pathological conditions, such as the oxidative stress status in patients with renal tumors, and these activated γδ T cells could further recognize other ligands presenting on the surface of tumor cells and display anti-tumor cytotoxic activity. Here, the enhanced anti-tumor immunity of γδ T cells mediated by ectopic hMSH2 expression is consistent with our observation that the lysis of RCC cells by γδ T cells is enhanced in oxidative stress or by proinflammatory cytokines, which could induce ectopic expression of hMSH2. Therefore, ectopically expressed hMSH2 might act as one of potential DAMPs recognized by γδ T cells in stressful conditions.

Stressful states, including oxidative stress, can activate MAPK pathways (54–56). Exposure of retinal pigment epithelial cells to H2O2 induces apoptosis through a Racl/JNK/p38 signaling cascade (57), and H2O2 also causes apoptosis by up-regulating both JNK and p38 in testicular germ cells (58).
Oxidative Stress Induces Ectopic Expression of hMSH2

shown in this study that transcription factor binding sites of the AP-1 family presented in the regulatory element of hMSH2, and p38 MAPK and JNK pathways were activated during oxidative stress. Phosphorylated c-Jun indicated that the MEK/ERK pathway might also be active in GO-mediated oxidative stress. However, p38 MAPK and JNK but not MEK/ERK pathways indeed promoted surface expression of hMSH2 on RCC cells. The regulatory effect of p38 MAPK and JNK pathways was further confirmed by stimulating or inhibiting of ASK1, a kinase activating both MKK3/MKK6-p38 and MKK4/MKK7-JNK signaling cascades in environmental stresses (59, 60).

On the other hand, we showed that ATF3, a stress-sensitive transcription factor locating downstream of the p38 MAPK and JNK pathways (42, 61), played a crucial role in ectopic expression of hMSH2 in oxidative stress. When endogenous ATF3 is knocked down in G401 cells, there is no significant up-regulation of ectopically expressed hMSH2 even in oxidative stress, suggesting that ATF3 may act as an executor to promote ectopic expression of hMSH2 in an oxidative environment, although the specific mechanism needs to be clarified.

To further determine key factors regulating ectopic expression of hMSH2 on the surface of RCC cells in oxidative stress, we examined the role of IL-18, which could be produced largely by ROS-activated inflammasome NLRP3/NALP3 (46). Meanwhile, IL-18 shows the central role in promoting the inflammation and oxidative stress that lead to renal dysfunction and damage (62). Our data show that, accompanied by more IL-18 secretion, the expression of IL-18Rα is up-regulated on the surface of GO-stimulated RCC cells, which leads these cells to be more sensitive to endogenous IL-18 during oxidative stress. Meanwhile, exogenous IL-18 alone effectively up-regulates surface expression of hMSH2, and GO-induced ectopic expression of hMSH2 could be inhibited by knockdown of IL-18Rα in G401 cells. In order to identify the regulating effect of IL-18 on ectopic expression of hMSH2, we also tested the change of ectopically expressed hMSH2 on other tumor cell types, such as A375, Jurkat, and K562 cells, when treated with IL-18. Several reports have mentioned that these tumor cells could produce IL-18 under certain conditions (63–66). Interestingly, although the autocrine of IL-18 is only observed in supernatants of normal or GO-stimulated G401 and A375 cells in our study, treatment with IL-18 could induce significantly ectopic expression of hMSH2 on all of the above tumor cells. Thus, we envision that abundant IL-18 in the supernatants of stress-stimulated RCC or melanoma cells is an important stimulator inducing ectopic expression of hMSH2 on multiple types of tumor cells, including RCC, lymphoma, and leukemia. Because most hematological cancers are sensitive to the anti-tumor cytotoxicity of γδ T cells (67–69) and the production of IFN-γ, GM-CSF, and TNF-α by γδ T cells is strongly enhanced when treated with IL-18 (70), it suggests that IL-18 released by tumor cells in oxidative stress may promote both recognition and clearance of tumor cells by γδ T cells.

We also consider proinflammatory cytokines produced by γδ T cells during lysis of tumor cells (25, 49, 71, 72) and investigate their potential effects on regulating ectopic expression of hMSH2. Here, IFN-γ but not TNF-α is highly produced by GO-treated γδ T cells, and exogenous IFN-γ indeed amplifies GO-induced ectopic expression of hMSH2 on G401 cells. The fact that proinflammatory cytokines IL-18 and IFN-γ in combination or alone are involved in the production of ROS in various cell types (73–76) indicates that a potential positive feedback by ROS and proinflammatory cytokines may regulate integrally ectopic expression of hMSH2 in oxidative stress. As shown in Fig. 7, oxidative stress induces ectopic expression of hMSH2 on RCC cells. Meanwhile, ROS existing in oxidative stress promote the production of IL-18 and IFN-γ by tumor cells and γδ T cells, respectively. IL-18 alone could induce ectopic expression of hMSH2 on RCC cells, and both proinflammatory cytokines in turn may maintain the high level of ROS, which subsequently induces highly ectopic expression of hMSH2 presenting on the surface of RCC cells.

In conclusion, ectopically expressed hMSH2 on tumor cells possesses the inducibility in oxidative environments. The p38 MAPK and JNK pathways mainly control ectopic expression of hMSH2 on RCC cells through ASK1 upstream and stress-sen-
Oxidative Stress Induces Ectopic Expression of hMSH2

![Diagram of Oxidative Stress Induces Ectopic Expression of hMSH2](image)

**FIGURE 7.** A positive feedback model for regulating ectopic expression of hMSH2 in RCC cells during oxidative stress. A high level of ROS in oxidative stress causes excessive production of proinflammatory cytokines IL-18 and IFN-γ by RCC and γδT cells, respectively. IL-18 is shown as a stimulator inducing ectopic expression of hMSH2 in oxidative stress. IFN-γ promotes the above trend presenting on the surface of RCC cells. The combination of both proinflammatory cytokines in turn maintains a high level of ROS, which may further induce ectopic expression of hMSH2. B, in oxidative stress-stimulated RCC cells, ectopic expression of hMSH2 is controlled by p38 MAPK and JNK pathways. A node kinase ASK1 upstream and stress-sensitive ATF3 down-stream of both pathways participate in the intracellular regulation of ectopic expression of hMSH2. Because hMSH2-bearing RCC cells are susceptible to γδT cell-mediated lysis, this positive feedback indicates a potential contribution of ectopically expressed hMSH2 to γδT cell-mediated immunosurveillance of tumor cells in stressful conditions.

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