Chronic Oxidative Stress Increases Resistance to Doxorubicin-Induced Cytotoxicity in Renal Carcinoma Cells Potentially Through Epigenetic Mechanism

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ABSTRACT
Renal cell carcinoma is the most common form of kidney cancer and is highly resistant to chemotherapy. Although the role of oxidative stress in kidney cancer is known, the chemotherapeutic response of cancer cells adapted to chronic oxidative stress is not clear. Hence, the effect of oxidative stress on sensitivity to doxorubicin-induced cytotoxicity was evaluated using an in vitro model of human kidney cancer cells adapted to chronic oxidative stress. Results of MTT- and anchorage-independent growth assays and cell cycle analysis revealed significant decrease in sensitivity to doxorubicin in Caki-1 cells adapted to oxidative stress. Changes in the expression of genes involved in drug transport, cell survival, and DNA repair-dependent apoptosis further confirmed increased resistance to doxorubicin-induced cytotoxicity in these cells. Decreased expression of mismatch repair (MMR) gene MSH2 in cells exposed to oxidative stress suggests that loss of MMR-dependent apoptosis could be a potential mechanism for increased resistance to doxorubicin-induced cytotoxicity. Additionally, downregulation of HDAC1, an increase in the level of histone H3 acetylation, and hypermethylation of MSH2 promoter were also observed in Caki-1 cells adapted to chronic oxidative stress. DNA-demethylating agent 5-Aza-2dC significantly restored the expression of MSH2 and doxorubicin-induced cytotoxicity in Caki-1 cells adapted to chronic oxidative stress, suggesting the role of DNA hypermethylation in inactivation of MSH2 expression and consequently MMR-dependent apoptosis in these cells. In summary, this study for the first time provides direct evidence for the role of oxidative stress in chemotherapeutic resistance in renal carcinoma cells potentially through epigenetic mechanism.

Introduction
Renal cell carcinoma (RCC) represents the most common form of kidney cancer and its incidence has been increasing (Ho et al., 2013; Wang et al., 2013; Araújo et al., 2015). Additionally, kidney cancer is highly refractory to chemotherapy. Though the reasons are not completely understood, the presence of a metastatic form during initial diagnosis and high resistance to conventional therapeutic strategies have been considered as main factors for the poor prognosis of RCC (Kojima et al., 2009; Li et al., 2014a). Even though improved clinical response has been achieved with a new class of drugs, the low response rate, resistance development, and adverse effects associated with new therapeutics restrict their clinical use (Mahalingam et al., 2009; Sankhala et al., 2009). Hence, understanding acquired resistance to therapy is of immense importance to improving clinical outcomes.

Multiple mechanisms, such as increased expression of efflux transporters (Gottesman et al., 2002; Huang et al., 2004), an increase in detoxification mechanisms (Stordal and Davey, 2007; Raguz and Yagüe, 2008), alteration in key cellular targets (Stavrovskaya, 2000; Singh et al., 2006; Housman et al., 2014), and evasion of apoptotic death (Scheltema et al., 2001; Igney and Krammer, 2002; Indrun et al., 2011), have been suggested for acquired resistance development. In addition, changes in cellular microenvironment, such as increased reactive oxygen species (ROS) leading to oxidative stress, may play an important role not only in kidney cancer development and increased metastatic potential but also in development of resistance against chemotherapeutic drugs (Spitz et al., 1993; Pelicano et al., 2004; Hwang et al., 2007). ROS-induced biochemical and molecular lesions might facilitate the development of a heterogeneous cancer cell population with drug-resistant clones. Thus, the induction of drug resistance depends on both the inherent genetic make-up of cells and the chemotherapeutic targeted action (Pelicano et al., 2004).

Extensive studies over the years have implicated alterations in various pathways for oxidative stress–induced drug resistance. For example, activation of redox-sensitive transcription factor Nrf2 can induce antioxidant defense system,
including MnSOD, thioredoxin, peroxiredoxins, catalase, and glutathione peroxidase (Osburn and Kessler, 2008), and consequently this can confer resistance to both oxidative stress and anticancer drugs by inhibiting apoptosis and offering cell survival mechanisms (Landriscina et al., 2009). Likewise, activation of nuclear factor κB has also been shown to be involved in chemotherapeutic resistance through various mechanisms, such as through upregulation of multidrug resistance (MDR) genes (Thévenod et al., 2000), increased expression of Bcl-2 (Viator et al., 2003), and increased angiogenic vascular endothelial growth factor (Morais et al., 2009). Adaptation to chronic oxidative stress has been shown not only to increase the growth of cancer cells but also to affect directly the response to chemotherapy, as many anticancer drugs exert their cytotoxicity by generating ROS (Spitz et al., 1993; Scheltema et al., 2001; Mahalingaiah and Singh, 2014).

In addition to genetic changes, ROS-induced epigenetic changes may also play a role in development of resistance to chemotherapeutic drugs. For example, increased effects of anticancer drugs (Hagiwara et al., 2008; Iwata et al., 2011; Ricketts et al., 2013) and suppression of cell growth in xenograft model of RCC (Hagiwara et al., 2008) following reversal of DNA hypermethylation by inhibition of DNA methyl transferases (DNMT) have been reported. An abnormally high level of DNMT1 activity in tumor tissues and its correlation with poor prognosis in RCC patients further support the role of an epigenetic mechanism in resistance to chemotherapy (Li et al., 2014b).

Therefore, the objective of this study was to identify the mechanism through which the exposure to chronic oxidative stress can lead to acquired drug resistance in renal cancer cells. To address this question, an RCC cell model of chronic exposure to oxidative stress was developed in which Caki-1 renal carcinoma cells were exposed to hydrogen peroxide–induced oxidative stress for a chronic period. The role of oxidative stress in sensitivity to the anticancer drug doxorubicin was evaluated by various parameters. To further evaluate the possible role of oxidative stress–induced epigenetic changes in cellular response to doxorubicin, these cells were first pretreated with demethylating agent (5-aza-2'-deoxycytidine) and then sensitivity to doxorubicin was measured.

**Materials and Methods**

**Reagents.** Doxorubicin HCl, cis-diamineplatinum (II) dichloride (cisplatin), 5-aza-2'-deoxycytidine (5-Aza-2’dC), hydrogen peroxide (H2O2) 30% solution (w/v), 2’, 7’-dichlorodihydrofluorescein diacetate (DCFH-DA), and 3’4’, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were procured from Sigma-Aldrich (St. Louis, MO). Cell cycle reagent, radiomunoprecipitation assay (RIPA) lysis buffer (1×), and polymerase chain reaction (PCR) reagents were obtained from EMD Millipore (Billerica, MA), Santa Cruz Biotechnology, Inc. (Dallas, TX), and Bio-Rad (Heracles, CA), respectively. McCoy’s 5A modified medium was purchased from Lonza (Walkersville, MD). Fetal bovine serum and antibiotic/antimycotic solution were obtained from Life Technologies (Grand Island, NY), and trypsin EDTA and Trizol reagent were purchased from Invitrogen (Life Technologies).

**Cell Line and Culture Conditions.** Human renal cell carcinoma cell line (Caki-1) was obtained from American Type Culture Collection (ATCC). After an initial procurement, subsequent in vitro expansion was followed and cell lines were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum and 1% antibiotic and antimycotic solution at 37°C in a humidified atmosphere containing 5% CO2.

**Exposure to Chronic Oxidative Stress.** Exponentially growing Caki-1 cells were seeded in 25-cm² flasks and at 60–70% confluence, treated with 25 μM (low-dose) and 200 μM (high-dose) concentrations of H2O2 in fresh culture media. H2O2 concentration of 25 μM as low, noncytotoxic concentration and 200 μM as high, significantly cytotoxic concentration were selected from an initial study with a range of concentration of 10–500 μM.

Caki-1 cells were chronically exposed to low- and high-dose H2O2 for 6 months. Briefly, Caki-1 cells were treated with H2O2 and allowed to grow up to 70–80% confluence and then subcultured and reseeded for H2O2 treatment. This process of treatment and subculture was repeated for 6 months continuously. All the treatment groups were maintained and treated in triplicate. Untreated parental cultures were grown and maintained as passage matched parallel controls. Before any further experiment and sample collection, chronically exposed Caki-1 cells were maintained in H2O2-free medium for at least one passage.

**Treatment of Cells with Doxorubicin, Cisplatin, and 5-Aza-2’-Deoxycytidine (Demethylation).** Stock solutions of doxorubicin and 5-Aza-2’dC were prepared in dimethyl sulfoxide (DMSO) and stored at −80°C for further use. On the basis of the initial pilot study using different concentrations (10 nM to 1 μM) of doxorubicin, 600 nM was found to be the EC50 concentration for Caki-1 cells, hence this concentration was selected for further study. Concentration of 5-Aza-2’dC (2 μM) was selected on the basis of the commonly used concentration for demethylation effects in cancer cells (Singh et al., 2012). After exposure to chronic oxidative stress, sensitivity of Caki-1 cells to doxorubicin was evaluated. To evaluate the effect of demethylation on doxorubicin sensitivity, Caki-1 cells were pretreated with 5-Aza-2’dC for 24 hours and then cotreated with doxorubicin and 5-Aza-2’dC for an additional 48 hours. Corresponding untreated parental control Caki-1 cells were treated with 0.001% DMSO vehicle.

To test the sensitivity of these cells to another class of chemotherapeutic agent, cisplatin was also used in this study. On the basis of the previous study on cisplatin cytotoxicity in Caki-1 cells (Hueber et al., 2006), a range of cisplatin concentrations (10, 40, and 80 μM) were used in this study for treatment of cells along with the vehicle-treated (0.9% NaCl solution) control cells.

**Measurement of Intracellular ROS Production.** To confirm H2O2-induced increase in intracellular ROS level in Caki-1 cells, 2’, 7’-dichlorofluorescein diacetate (DCFH-DA) assay was performed. Caki-1 cells were seeded in 96-well plates at a concentration of 1×10⁴ cells per well, and after overnight attachment, cells were washed with 1× phosphate-buffered saline (PBS) and incubated with DCFH-DA in a 10 μM final concentration for 30 minutes. Cells were then rinsed with 1× PBS and treated with different concentrations of H2O2 solution in PBS for another 30 minutes. Intensity of DCF fluorescence was detected using a microplate reader at maximum excitation and emission wavelength of 495 nm and 535 nm, respectively.

**MTT Assay for Evaluation of Cellular Sensitivity to Chemotherapeutic Drug (Doxorubicin and Cisplatin)–Induced Cytotoxicity.** The effects of chronic oxidative stress on cell viability as well as the sensitivity of Caki-1 cells to doxorubicin and cisplatin following exposure to chronic oxidative stress were determined by MTT assay. Briefly, Caki-1 cells exposed to chronic oxidative stress were seeded into 96-well plates at cell density of 4000 cells per well and allowed to attach and grow for 24 hours. To evaluate the sensitivity to doxorubicin with and without 5-Aza-2’dC pre- and co-treatment, a treatment protocol was followed as mentioned above. Likewise, to test the sensitivity to another class of chemotherapeutic agent, these cells were also treated with cisplatin alone. After 48 hours of treatment, media was removed for all treated and untreated control cells and replaced with fresh media containing MTT (1 mg/ml) and incubated for 3 hours at 37°C. This was followed by the removal of MTT solution and then addition of 150 μl of DMSO to dissolve
insoluble formazan crystals formed within mitochondria of viable cells. The plate was incubated with DMEM for 5 minutes with gentle shaking, and intensity of color formed was quantified using a plate reader at optical density wavelength of 570 nm and 630 nm. The experiment was performed in triplicate and was repeated twice. The absorbance value (after subtracting the reference background) was converted into percentage growth to estimate the change in cell viability for different treatment groups.

**Cell Cycle Analysis by Flow Cytometry.** To determine the effect of chronic oxidative exposure on Caki-1 cell cycle in response to doxorubicin treatment, cell cycle analysis was performed using flow cytometry. Ethanol (70%)–fixed cells from different treatment groups as stated earlier and untreated control group were collected by centrifugation, washed with 1× ice-cold PBS, stained with Guava Cell Cycle Reagent (Millipore), and then analyzed in Guava Easy-Cyte HT flow cytometer (Millipore). Cell cycle analysis was accomplished with total count of 5000 events, and data were acquired using Guava software (Millipore). All samples were evaluated in triplicate and experiment was repeated twice.

**Soft Agar Assay for Anchorage-Independent Growth.** To evaluate the effect of chronic oxidative stress on in vitro tumorigenic potential of Caki-1 cells, colony formation assay on soft agar was carried out. For this anchorage-independent growth assay, Caki-1 cells exposed to chronic oxidative stress were treated with doxorubicin and 5-Aza-2dC, as in the treatment protocol described for the cytotoxicity assay, then used for seeding on soft agar. The base layer of soft agar (0.5%) and the top layer of agar (0.35%) mixed with cells (5000 cells) in McCoy's medium were prepared as reported previously by us (Mahalingiah et al., 2015). Plates were incubated at 37°C in an incubator with 5% CO2 and observed daily for colony formation by microscopic observation until the soft agar colonies reach clearly visible size. The soft agar–grown colonies were counted, and representative photomicrographs were taken on day 14. The number of colonies in each treatment groups were expressed as a percentage value compared with untreated control by considering control as 100%. The size of colonies was also determined using Image J software and values were expressed as percentage of control.

**Wound Healing Cell Migration Assay.** Scratch wound healing assay was performed to determine the impact of chronic oxidative stress on migration potential of Caki-1 cells. Actively growing cells were seeded into six-well cell culture plates. After cells were fully attached, two parallel scratch wounds per well were created. After creating wounds, cell culture media was replaced with fresh media (to remove the floating cells) containing various drugs or vehicle only as mentioned earlier. The plates were then incubated at 37°C with 5% CO2 in humidified incubator and observed daily for cell migration into wounded area, and representative images were taken at different time points. Area of unoccupied wound gaps were quantified by using Image J software with MBI wound-migration quantification plug-in tool. Percentage of wound closure was calculated by subtracting unoccupied wound area at different time points from total wound gap on day 0 for each treatment group as reported earlier (Yue et al., 2010).

**Isolation of RNA and Real Time Quantitative PCR.** Gene expression was analyzed using total RNA from treatment and control groups of cells. Genes of interest were amplified by one-step RT-PCR kit with SYBR green (Bio-Rad) using 75 ng of total RNA. PCR amplifications were performed in Myq2 Real-time PCR detection system (Bio-Rad) programmed for reverse transcription at 50°C for 15 minutes, denaturation and reverse transcription enzyme inactivation at 95°C for 5 minutes, followed by 40 cycles, each containing 10 seconds for denaturation at 95°C and 30 seconds for annealing and extension at 60°C. Specificity of PCR products was confirmed by melt curve analysis. Cycle threshold (Ct) values of each gene were normalized to the Ct of GAPDH from each sample and the fold-changes in gene expression were computed by following delta-delta Ct method (Livak and Schmittgen, 2001). Primer sequences used for gene expression analysis are listed in Table 1.

**Western Blot Analysis.** To evaluate the changes in the levels of proteins and epigenetic modifications in histone H3, total cellular protein lysates from treated and untreated parental control Caki-1 cells were harvested by disrupting the cells using RIPA lysis buffer. Protein samples (30 μg) were subjected to electrophoresis separation on a 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. After incubation in blocking buffer (5% nonfat dried milk in 1× PBS), membranes were probed with primary antibody overnight at 4°C. Considering the reaction efficiency of antibodies, a dilution of 1: 5000 of α-tubulin (cat. no. 5063; Cell Signaling Technology, Danvers, MA), 1:500 of MDR1 (cat. no. 126838; Cell Signaling Technology), 1:1000 of Survivin (cat. no. 2808; Cell Signaling Technology), 1:100 of MSH2 (cat. no. SC-494; Santa Cruz Biotechnology), 1:200 of MBD4 (cat. no. SC-10753; Santa Cruz Biotechnology), 1:1000 of HDAC1 (cat. no. SC-6298; Santa Cruz Biotechnology), and 1:1000 of acetyl Histone H3Lys9 (cat. no. 9649P; Cell Signaling Technology), and 1:1000 of acetyl Histone H3Lys9/14 (cat. no. 06-599; Upstate Biotechnology, Lake Placid, NY) were used. Following incubation, membranes were washed thrice (each wash last for 10 minutes) with washing buffer (1× PBS with 0.05% Tween 20) and then incubated with suitable secondary antibody conjugated with horseradish peroxidase at a dilution of 1:1000 for an hour at room temperature. Membranes were thrice washed again using washing buffer and the signal was sensed by an enhanced chemiluminescence detection system (Amersham/GE Healthcare Life Sciences, Piscataway, NJ). Intensity of protein bands were measured using Image J software and normalized to α-tubulin.

**Analysis of MSH2 Promoter DNA Methylation by Pyrosequencing.** Methylation status of CpG sites in the promoter region of MSH2 was determined by the pyrosequencing method previously reported by us (Treas et al., 2013). Briefly, bisulfite conversion of DNA (500 ng) and its purification was performed using Epit取证 bisulfite conversion kit (Qiagen, Valencia, CA). PCR amplification was performed with bisulfite-treated and purified DNA as template and the PyroMark CpG assay primer set (cat. no. PM00007784; Qiagen) to amplify the 261-bp region containing six CpG sites from promoter region of MSH2 gene. To generate biotinylated single-strand template for pyrosequencing, the PCR products were first immobilized onto Sepharose beads coated with streptavidin and then passed through denaturation and washing buffer using PyroMark vacuum preparation workstation (Qiagen). Biotinylated single-stranded templates were then mixed with sequencing primer in a pyrosequencing plate and incubated at 80°C for 2 minutes followed by cooling at room temperature for 5 minutes to allow the annealing of template with the sequencing primer. PyroMark Q24 Pyrosequencer (Qiagen) was used for pyrosequencing and the percentage of methylation (C/T ratio) for each CpG site was obtained by analyzing the pyrogram using the PyroMark CpG software (Version 1).

**Statistical Analysis.** To define the statistical significance of the differences observed in different parameters, a two-tailed paired t test for means with hypothesized difference of 0 was carried out. An analysis of variance (ANOVA) was accomplished to estimate if the source of variation in the data were between or within treatment groups. A value of 0.05 was set as alpha level for all statistical tests, and data with value of P < 0.05 were considered as statistically significant.

**Results**

**Development of Caki-1 Cells Adapted to Chronic Oxidative Stress.** To develop the cell model adapted to chronic oxidative stress, Caki-1 cells were treated with H2O2 and allowed to grow up to 70–80% confluence and then subcultured and reseded for H2O2 treatment. The result of DCFH-DA assay confirmed the H2O2-induced intracellular reactive oxygen species (Supplemental Fig. 1A). This process of treatment and subculture was repeated for 6 months as...
mentioned in Materials and Methods. The result of cell viability by MTT assay revealed an increase by 36% in the growth of cells after chronic exposure to low-dose (25 μM) H2O2, and adaptive response to high-dose (200 μM) H2O2-induced cytotoxicity compared with the passage matched untreated control (Supplemental Fig. 1B). However, in acute exposure (48 hours) study, the same low dose of H2O2 had no significant effect on cell viability, whereas the high dose—induced a significant cytotoxicity. The comparison of cellular response to acute exposure to H2O2 with that of chronic exposure confirmed the adaptation of these cells to oxidative stress. From now onward the low-dose H2O2—adapted cells are referred as Caki-1LA and high-dose H2O2—adapted cells are Caki-1HA.

Doxorubicin Sensitivity in Caki-1 Cells Adapted to Chronic Oxidative Stress. Doxorubicin sensitivity of Caki-1 cells adapted to chronic oxidative stress was evaluated by various parameters, such as analysis of cytotoxicity, cell cycle, and tumorigenic and migration potential. The results from these parameters were further confirmed at the molecular level by gene and protein expression. Results of each parameter follow.

Chronic Oxidative Stress Causes Increased Resistance to Doxorubicin-Induced Cytotoxicity. To evaluate the sensitivity of doxorubicin in cells adapted to chronic oxidative stress, both Caki-1LA and Caki-1HA cells were treated with 600 nM doxorubicin for 48 hours and the cytotoxicity was measured by MTT assay. The result revealed that doxorubicin-induced cytotoxicity was significantly higher (35%) in Caki-1 cells, whereas only 16 and 25% in Caki-1LA and Caki-1HA cells, respectively, compared with their untreated controls (Fig. 1A). The comparison of cell viability after doxorubicin treatment revealed a decreased sensitivity to doxorubicin-induced cytotoxicity in Caki-1LA and Caki-1HA cells by 20.48 and 10%, respectively, compared with doxorubicin-induced cytotoxicity in Caki-1 cells (Fig. 1A). Therefore, the results of MTT assay suggest that kidney cancer cells adapted to chronic oxidative stress were less sensitive to doxorubicin-induced cytotoxicity. Moreover, the cells adapted to lower levels of oxidative stress (Caki-1LA) were more resistant to doxorubicin cytotoxicity than the cells adapted to relatively higher levels of oxidative stress (Caki-1HA).

Chronic Oxidative Stress Causes Increased Resistance to Cisplatin-Induced Cytotoxicity. To test whether the decreased sensitivity to chemotherapeutic drug-induced cytotoxicity as a result of adaptation to chronic oxidative stress is doxorubicin-specific or applicable to other class of chemotherapeutic drugs as well, cells were also treated with cisplatin. Interestingly, as with the results of doxorubicin treatment, a significant decrease in the sensitivity to cisplatin was also observed in Caki-1LA cells compared with Caki-1 cells. For example, 10 μM cisplatin treatment resulted in 51% cytotoxicity in Caki-1LA cells, whereas the same concentration of cisplatin caused only 17% cytotoxicity in Caki-1LA cells (Fig. 1B). Likewise, a significant decrease in sensitivity to higher concentrations (40 and 80 μM) of cisplatin in Caki-1LA cells compared with Caki-1 cells was also observed (Fig. 1B).

Chronic Oxidative Stress Abrogates Doxorubicin-Induced Apoptosis. Cell apoptosis (Pre-G1 cell population) analysis by flow cytometry revealed that doxorubicin treatment resulted in significantly less apoptosis (Pre-G1 cells) in Caki-1LA (11%) and Caki-1HA (22%) cells compared with Caki-1 (33%) cells (Fig. 2). This further suggests that Caki-1LA cells are relatively more resistant to doxorubicin-induced apoptosis than are Caki-1 cells.

Decreased Sensitivity of Doxorubicin As Evaluated by In Vitro Colony Formation Assay in Cells Adapted to Oxidative Stress. Colony formation assay on soft agar was carried out to evaluate the effect of doxorubicin on anchorage-independent growth (in vitro tumorigenic potential) of Caki-1, Caki-1LA, and Caki-1HA cells. The images of soft agar—grown colonies are given in Fig. 3A and the histogram of colony counts and size are shown in Fig. 3, B and C, respectively. In Caki-1LA and Caki-1HA cells, significant increases in soft agar—grown colonies by 49 and 28%, respectively, were observed compared with Caki-1 cells, indicating increased tumorigenic potential following exposure to chronic oxidative stress. In addition to the increase in number of colonies, the soft agar—grown colonies from Caki-1LA cells were also significantly bigger in size compared with Caki-1 cells and Caki-1HA cells (Fig. 3, A and C).

The inhibitory effects of doxorubicin on cell growth and colony formation was also significantly decreased in Caki-1LA and Caki-1HA cells, compared with Caki-1 cells. In Caki-1 cells, doxorubicin treatment resulted in 66.88% decrease in the number of soft agar— colony formation, compared with untreated cells. In contrast, doxorubicin treatment in Caki-1LA and Caki-1HA cells showed only 36 and 46.55% decreases in colony count, respectively, compared with untreated control Caki-1 cells (Fig. 3B).

Chronic Oxidative Stress Decreases the Efficacy of Doxorubicin to Inhibit Cell Migration. To evaluate the effect of doxorubicin on cell migration potential, scratch-wound healing assays were performed for Caki-1, Caki-1LA, and Caki-1HA cells. The results of microscopic evaluation of
Caki-1 cell migration into the wound area in different treatment groups at different time points are presented in Figs. 4 and 5. The data for the quantification of area in the wound gap covered by cell migration in various treatment groups at different time points are given in Supplemental Fig. 2. Chronic oxidative stress significantly increased the

Fig. 1. Bar graph depiction of cytotoxicity data as determined by MTT assay. Caki-1 cells adapted to chronic oxidative stress were treated with doxorubicin with or without demethylating agent 5-Aza-2dC (A) or different concentrations of cisplatin (B), and their cell viability by MTT assay was performed as mentioned in Materials and Methods. Values of MTT assay are given in percentage with control as 100%. The error bars represent the standard deviation of the mean (± S.D.). Statistically significant (P < 0.05) changes in treatment groups compared with their respective vehicle control are indicated by asterisk symbol (*). Hash symbol (#) in Fig. 1A indicates the significant (P < 0.05) changes in doxorubicin alone treated Caki-1LA and Caki-1HA cells compared with doxorubicin alone treated Caki-1 cells. Infinity symbol (∞) in Fig. 1B indicates the significant (P < 0.05) changes in cisplatin-treated Caki-1LA cells compared with Caki-1 cells treated with corresponding dose of cisplatin.

Fig. 2. Histogram representation of flow cytometry data for population of cells in Pre-G1, G0/G1, S, and G2/M phases of cell cycle from Caki-1, Caki-1LA, and Caki-1HA cells treated with doxorubicin and 5-Aza-2dC individually and in combination. Cells were collected after treatment, fixed, and stained for cell cycle analysis by flow cytometer as described in Materials and Methods. Percentage of cells in Pre-G1, G0/G1, S, and G2/M phases of cell cycle represent the mean value of triplicates. The error bars represent the standard deviation of the mean (± S.D.). An asterisk (*) indicates the statistically significant (P < 0.05) change in Caki-1LA and Caki-1HA cells compared with Caki-1 control cells for each treatment group. Hash symbol (#) indicates statistically significant (P < 0.05) change in 5-Aza-2dC pretreated and doxorubicin treated Caki-1LA cells compared with doxorubicin treatment alone.
migration potential of Caki-1 cells. Caki-1LA cells migrated much faster and the wound gap was closed completely on day 3, compared with Caki-1HA cells and Caki-1 cells (Fig. 4 and Supplemental Fig. 2).

Following doxorubicin treatment, visible differences in migration potential of Caki-1LA and Caki-1HA cells were observed compared with Caki-1 cells. In Caki-1 cells, doxorubicin treatment resulted in more cell death on day 3 and dead cells were still visible floating in between the wound edges, whereas in Caki-1LA and Caki-1HA cells, sensitivity to doxorubicin-induced cytotoxicity was decreased and cells migrated to the wounded area much earlier than did Caki-1 cells (Fig. 4 and Supplemental Fig. 2).

Evaluation of Molecular Markers for Doxorubicin Sensitivity/Resistance in Cells Adapted to Oxidative Stress. Real-time quantitative PCR analysis was performed to measure the gene expression changes at the transcript level to further confirm the response of cells to doxorubicin. Representative genes involved in mediating the doxorubicin effect, drug transporters, and mismatch repair (MMR)-dependent apoptosis were analyzed at transcript level.

**Changes in the Expression of Genes Involved in Drug Transport and Doxorubicin-Sensitive TOP2A.** The representative genes of drug transporters, such as MDR1 (ABCB1), MRP2 (ABCC2), BCRP (ABCG2), as well as TOP2A, a well known target gene for doxorubicin, were analyzed at transcript level and results are summarized in Table 2. The comparison of gene expression data from Caki-1LA, Caki-1HA, and Caki-1 cells revealed that MDR1 expression was upregulated by 4- and 2.4-fold in Caki-1LA and Caki-1HA cells, respectively, compared with Caki-1 cells (Fig. 6, A and B). Likewise, the expression of BCRP was also upregulated by 3.5- and 3.0-fold in Caki-1LA and Caki-1HA cells, respectively, compared with Caki-1 cells.
significant change in the expression of MRP2 in Caki-1LA and Caki-1HA cells compared with Caki-1 cells. Although the TOP2A expression was decreased in Caki-1LA and Caki-1HA cells compared with Caki-1 cells, these changes were statistically insignificant.

The comparison of gene expression data from Caki-1LA, Caki-1HA, and Caki-1 cells after doxorubicin treatment revealed increased expression of MDR1, MRP2, and TOP2A gene transcripts by 3.0-, 2.0-, and 3.4-fold, respectively, in Caki-1LA cells (Fig. 6A), whereas gene expression increased 9.0-, 6.0-, 4.3-fold, respectively, in Caki-1HA cells compared with Caki-1 cells (Fig. 6B). Therefore, these upregulations of drug transporters and TOP2A, a mediator of doxorubicin-induced cytotoxicity in both Caki-1LA and Caki-1HA cells, further confirmed the mechanistic basis for the decreased sensitivity to doxorubicin in cells adapted to chronic oxidative stress.

Changes in Expression of Genes Involved in Cell Survival and DNA Mismatch Repair–Dependent Apoptotic Pathway. Survivin was used as a representative gene to evaluate the cell survival and apoptotic response to chronic oxidative stress in Caki-1 cells. A statistically significant increase in expression of Survivin by 58.64-fold was observed in Caki-1LA cells, compared with Caki-1 cells (Fig. 6A). Following doxorubicin treatment, Caki-1LA cells expressed a further increase in survivin expression level by 709.77-fold compared with Caki-1 cells, indicating increased survival and antiapoptotic response against doxorubicin-induced cytotoxicity. However, there were no significant changes in expression level of Survivin in Caki-1HA cells.

The representative genes involved in DNA damage response like MSH2, MLH1, and MBD4 were analyzed at transcript level. A significant downregulation of MSH2 expression by 2.78- and 3.4-fold was observed in Caki-1LA and Caki-1HA cells, respectively, compared with Caki-1 cells (Table 2; Fig. 6, A and B). Doxorubicin treatment also resulted in reduced MSH2 expression by 1.6- and 2.7-fold in Caki-1LA and Caki-1HA cells, respectively, compared with Caki-1 cells treated with doxorubicin. In contrast, increase in the MLH1 expression by 1.76-fold was observed only in Caki-1LA cells, compared with Caki-1 cells. Following doxorubicin treatment, further increase in MLH1 expression by 3.18-fold was observed in Caki-1LA cells (Fig. 6A). There was no significant change in the expression of MBD4 in both Caki-1LA and Caki-1HA cells, compared with Caki-1 cells. However, doxorubicin treatment significantly increased MBD4 expression by 4.1-fold in Caki-1LA cells, compared with doxorubicin exposed Caki-1 cells.

Western Blot Analysis for Gene Expression Changes at Protein Level and Histone Modifications. Western blot analysis was performed to further confirm the gene expression changes at protein level as well as to determine transcriptional changes. Western blot analysis was performed to further confirm the gene expression changes at protein level as well as to determine transcriptional changes.
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Values represent mean fold-change (±S.D.) compared with respective control (control = 1 fold). An asterisk (*) indicates the statistical significance (P < 0.05) in Caki-1LA and Caki-1HA compared with Caki-1 with 5-Aza-2dC treatment. A hash symbol (#) indicates the statistical significance (P < 0.05) in Caki-1LA and Caki-1HA with doxorubicin treatment compared with Caki-1 with doxorubicin treatment. Likewise, a dagger symbol (†) indicates the statistical significance (P < 0.05) in Caki-1LA and Caki-1HA treated with 5-Aza-2dC and doxorubicin combination compared with Caki-1 treated with 5-Aza-2dC and doxorubicin combination.

The expression of various genes at transcript level as measured by real-time quantitative PCR using total RNA from Caki-1, Caki-1LA, and Caki-1HA cells.

### Changes in Expression of Proteins Involved in Cell Survival, Drug Transport, and DNA-Damage Mismatch Repair-Dependent Apoptotic Pathway

The expressions of cell survival and antipapoptotic protein Survivin and multidrug-resistant efflux transporter protein MDR1 were significantly upregulated in both Caki-1LA and Caki-1HA cells compared with Caki-1 control cells. Expression of Survivin protein was further upregulated and expression of MDR1 protein persisted following doxorubicin treatment in Caki-1LA and Caki-1HA cells (Fig. 7, A and B).

DNA damage response protein MSH2 was significantly downregulated in Caki-1LA cells compared with Caki-1 cells (Fig. 7, A and B). However, a significant increase in MSH2 protein expression was observed in all the groups (Caki-1, Caki-1LA, and Caki-1HA) of cells following doxorubicin exposure. Thus, results of Western blot analysis further confirmed the transcript level changes observed in Survivin, MDR1, and MSH2.

### Changes in Expression of Epigenetic Regulatory Proteins and Histone Acetylation

Histone deacetylase 1 (HDAC1) expression was significantly reduced in Caki-1LA and Caki-1HA cells, compared with Caki-1 cells (Figs. 7, A and B). The decrease in HDAC1 expression was also associated with significant increase in the level of histone acetylation (acetyl histone H3Lys9 and acetyl histone H3Lys9/14) in Caki-1LA cells. In addition, doxorubicin treatment increased the HDAC1 expression in Caki-1LA and Caki-1HA cells compared with their untreated control cells (Fig. 7, A and B). However, this doxorubicin-induced increase in HDAC1 expression observed in Caki-1LA and Caki-1HA cells was significantly lower compared with Caki-1 cells exposed to doxorubicin. Doxorubicin-induced HDAC1 upregulation was also associated with significant downregulation of acetyl histone H3Lys9 in both Caki-1LA and Caki-1HA cells. There was no significant change in methyl binding-domain protein (MBD4) expression in Caki-1LA and Caki-1HA cells compared with Caki-1 cells. However, doxorubicin treatment resulted in significant downregulation of MBD4 protein expression in Caki-1LA and Caki-1HA cells compared with Caki-1 cells (Fig. 7, A and B).

### Effect of DNA Demethylation on Doxorubicin Sensitivity in Chronic Oxidative Stress–Adapted Cells

To evaluate the potential role of DNA hypermethylation changes for decreased sensitivity to doxorubicin in Caki-1LA and Caki-1HA cells, these cells were pretreated with DNA demethylating agent 5-Aza-2dC (2 μM), and then the doxorubicin sensitivity was evaluated.

### Effect of DNA Demethylation on Doxorubicin-Induced Cytotoxicity and Cell Cycle Changes

Pre-treatment with DNA-demethylating agent significantly enhanced the doxorubicin-induced cytotoxicity in all the three groups (Caki-1LA, Caki-1HA, and Caki-1 cells). Compared with untreated cells, the pretreatment with 5-Aza-2dC resulted in improved doxorubicin cytotoxicity from 35 to 48% in Caki-1 cells, from 15 to 26% in Caki-1LA cells, and from 25 to 41% in Caki-1HA cells (Fig 1A). Resensitization to doxorubicin-induced cytotoxicity was much greater in Caki-1LA and Caki-1HA cells than in Caki-1 cells, suggesting the involvement of DNA hypermethylation in acquired doxorubicin resistance observed in Caki-1LA and Caki-1HA cells. Treatment with 5-Aza-2dC...
alone did not induce significant cytotoxicity in any of the three (Caki-1LA, Caki-1HA, and Caki-1) groups of cells (Fig. 1A).

To further confirm the DNA demethylation–mediated resensitization to doxorubicin-induced cytotoxicity, flow cytometry analysis on the apoptotic Pre-G1 cell population was performed. Demethylation prior to doxorubicin treatment resulted in an increase in percentage of cell population in Pre-G1 in all three groups. Treatment with doxorubicin alone resulted in 11 and 22% of cells in Pre-G1 from Caki-1LA and Caki-1HA cells, respectively, compared with Caki-1 control cells (33%), whereas demethylation using 5-Aza-2dC pretreatment before doxorubicin increased the Pre-G1 population to 34 and 24% in Caki-1LA and Caki-1HA cells, respectively (Fig. 2). This suggests that DNA demethylation by 5-Aza-2dC pretreatment improved the efficacy of doxorubicin in inducing cytotoxicity and growth inhibition in Caki-1 cells adapted to chronic oxidative stress.

Effect of DNA Demethylation on Anchorage-Independent Growth and Migration Potential. To evaluate the effect of demethylation on doxorubicin-induced inhibition of soft agar colony formation, Caki-1, Caki-1LA, and Caki-1HA cells were pretreated with 5-Aza-2dC and exposed to doxorubicin. Cells were then seeded on soft agar as described earlier under Materials and Methods. Significant increase in inhibitory effect of doxorubicin on growth and colony formation of both Caki-1LA and Caki-1HA cells was observed following 5-Aza-2dC pretreatment (Fig. 3A). Pretreatment with 5-Aza-2dC followed by doxorubicin exposure resulted in 83.80, 81, and 84.22% inhibition in Caki-1, Caki-1LA, and Caki-1HA cells, respectively, indicating the chemoresistance induced by chronic oxidative stress resulted in hypermethylated phenotype that was eventually resensitized by demethylation (Fig. 3B and C). Likewise, 5-Aza-2dC treatment also significantly restored the effect of doxorubicin on migration potential of Caki-1 cells exposed to chronic oxidative stress. As shown in Fig. 5 and Supplemental Fig. 2, pretreatment with 5-Aza-2dC followed by doxorubicin exposure significantly reduced the migration of and also induced more cell death in Caki-1LA and Caki-1HA treated with 5-Aza-2dC and doxorubicin combination compared with Caki-1 with 5-Aza-2dC and doxorubicin combination.

Effect of DNA Demethylation on Expression of Marker Genes Associated with Doxorubicin Resistance. To identify the potential role of DNA hypermethylation in the observed downregulation of MSH2, a gene involved in the DNA repair–dependent apoptotic pathway,
the expression of this gene was determined in oxidative stress–adapted Caki-1LA and Caki-1HA cells after 5-Aza-2dC treatment.

Compared with control Caki-1 cells, the expression of MSH2 was downregulated by 2.8- and 3.4-fold in Caki-1LA and Caki-1HA cells, respectively (Table 2; Fig. 6, A and B). The treatment with 5-Aza-2dC restored the expression of MSH2 gene with 2.2-fold upregulation in Caki-1LA cells. However, this recovery of expression was minimal by 0.4-fold (from 3.4-fold to 3.0-fold downregulation) in Caki-1HA cells (Fig. 6B). Restoration of MSH2 expression by 5-Aza-2dC persisted even after doxorubicin treatment as evident from its upregulation by 2.4-fold in Caki-1LA cells. Likewise, demethylation using 5-Aza-2dC followed by doxorubicin treatment resulted in downregulation of chronic oxidative stress–induced Survivin as well as BCRP expression in Caki-1LA cells.

**Effect of DNA Demethylation on Protein Expression and Histone Modifications.** To confirm the effect of demethylation on the expression of marker genes at protein level as well as to determine the changes in the levels of histone acetylation, Western blot analysis was performed.

**Effect of Demethylation on the Expression of Marker Proteins Involved in Drug Transport, Cell Survival, and DNA Mismatch Repair–Dependent Apoptotic Pathway.** The expression of MDR1 protein was significantly increased in Caki-1LA cells and Caki-1HA cells compared with Caki-1 cells. Treatment with either 5-Aza-2dC or doxorubicin alone had no effect on the level of MDR1 expression in Caki-1LA cells and Caki-1HA cells, compared with respective untreated groups. However, combination treatment of both 5-Aza-2dC and doxorubicin resulted in complete abolition of MDR1 expression in Caki-1LA cells and Caki-1HA cells, compared with respective untreated groups. However, combination treatment of both 5-Aza-2dC and doxorubicin resulted in complete abolition of MDR1 expression in Caki-1LA cells and Caki-1HA cells (Fig. 7, A and B). Likewise, a significant downregulation of Survivin expression was observed in Caki-1LA cells and Caki-1HA cells pretreated with 5-Aza-2dC and then cotreated in combination with doxorubicin.

Treatment with doxorubicin alone significantly downregulated the expression of MBD4 protein in both Caki-1LA and
Caki-1\(^{HA}\) cells. Interestingly, this decrease in MBD4 expression by doxorubicin-alone treatment was restored when these cells were first pretreated with 5-Aza-2dC and subsequently in combination with doxorubicin (Fig. 7, A and B).

The expression of MSH2, a MMR-related protein that also plays a role in MMR-dependent apoptosis, was significantly downregulated in Caki-1\(^{LA}\) cells, compared with Caki-1 cells. Treatment with demethylating agent 5-Aza-2dC alone significantly restored the MSH2 expression in Caki-1\(^{LA}\) cells (Fig. 7A). This suggests that development of doxorubicin resistance and loss of apoptotic potential in Caki-1\(^{LA}\) cells could potentially result from DNA hypermethylation–mediated loss of MSH2 expression.

Effect of DNA Demethylation on Epigenetic Regulatory-Protein Expression and Histone Acetylation. A significant decrease in HDAC1 expression was observed in Caki-1\(^{LA}\) and Caki-1\(^{HA}\) cells compared with their control Caki-1 cells. This decrease in HDAC1 expression was partially restored by treatment with 5-Aza-2dC alone as well as with a combination of both 5-Aza-2dC and doxorubicin in Caki-1\(^{LA}\) cells (Fig. 7, A and B).

Likewise, a significant increase in histone H3 lysine acetylation (H3K9 and H3K9/14) was observed in Caki-1\(^{LA}\) cells. However, these increases in H3 acetylation were reversed when Caki-1\(^{LA}\) cells were first pretreated with 5-Aza-2dC and subsequently with doxorubicin treatment (Fig. 7A). Treatment with 5-Aza-2dC alone did not significantly alter acetylation levels of H3 in Caki-1\(^{LA}\) cells.

Chronic Oxidative Stress-Induced Hypermethylation in MSH2 Promoter. Decreased expression of MSH2 expression in Caki-1\(^{LA}\) cells and its restoration by treatment with demethylating agent 5-Aza-2dC suggested the possible role of oxidative stress–induced DNA hypermethylation in silencing MSH2 with the consequent loss of MSH2-dependent apoptosis, followed by decreased sensitivity to chemotherapeutic drug-induced cytotoxicity. To further confirm the DNA hypermethylation of MSH2 promoter, site-specific CpG methylation analysis of MSH2 promoter was performed by DNA pyrosequencing. The result of pyrosequencing revealed a modest increase in methylation from 5% in Caki-1 to 9% in Caki-1\(^{LA}\) cells in one among the six CpG sites analyzed from MSH2 promoter region (Fig. 8).

Discussion

Kidney cancer is highly refractory to currently available therapeutics and the de novo or acquired resistance to the available therapeutic options is a major limitation in kidney cancer treatment. The mechanism of chemotherapeutic resistance in kidney cancer is not fully understood. It is in this context that the novel findings of this study suggest that chronic oxidative stress can lead to increased resistance to doxorubicin, a chemotherapeutic drug widely used for treatment of various cancers and currently under clinical trial for combination therapy in kidney cancer (Roubaud et al., 2011; Haas et al., 2012; Buti et al., 2013). Additionally, the data of this study also suggest that oxidative stress–induced resistance to doxorubicin in kidney cancer cells is, at least in part, mediated by epigenetic inactivation of MMR-dependent apoptotic pathway. Restoration of doxorubicin-induced cytotoxicity as well as the reactivation of MMR gene MSH2 by DNA-demethylating agent 5-Aza-2dC further suggests the epigenetic basis for oxidative stress–induced drug resistance. To our knowledge, this is the first report suggesting the role of an epigenetic mechanism in oxidative stress–induced doxorubicin resistance and potential application of epigenetic therapy to restore doxorubicin-induced cytotoxicity in kidney cancer cells.

Kidney is a well known target organ for adverse effects of oxidative stress that lead to many pathologic changes, including cancer (Forbes et al., 2008; Mahalingaiah et al., 2015). Normal kidney cells produce low levels of ROS that get detoxified by naturally occurring antioxidants. However, excessive levels of ROS produced by pro-oxidants generated either endogenously through cellular metabolism or through various exogenous sources cause oxidative injury to kidney cells leading to kidney diseases. This is further supported by the evidence of higher levels of oxidative stress in RCC patients (Hori et al., 2007; Ganesamoni et al., 2012), in renal injury related to obesity (Quigley et al., 2009), and in an experimental model of renal carcinogenesis (Gago-Dominguez et al., 2002). Increased oxidative stress and activation of oxidative stress–induced DNA damage repair pathway in kidney cells of diabetic patients have been reported (Forbes et al., 2008; Kashihara et al., 2010; Caramori et al., 2015). Additionally, several chemotherapeutic agents, including doxorubicin itself, also generate ROS and thereby increase the oxidative stress burden (Ferlini et al., 1999; Pervaiz and Clement, 2004; Sullivan and Graham, 2008). Although the adverse effects of oxidative stress in malignant transformation of kidney cells are known (Mahalingaiah et al., 2015), the influence of oxidative stress on the sensitivity/efficacy of doxorubicin-induced cytotoxicity in cancer cells is not well understood. It is in this context that the present study evaluated sensitivity to doxorubicin in kidney cancer cells adapted to chronic oxidative stress. The findings of this study suggest that chronic exposure to low levels of oxidative stress leads to increased sensitivity to doxorubicin-induced cytotoxicity in Caki-1 cells. Similar results showing the effects of oxidative stress on the cellular sensitivity to several chemotherapeutic drugs, such as cisplatin, 5-fluorouracil, cyclophosphamide, and vincristine, have previously been reported (Spitz et al., 1993; Hwang et al., 2007; Tome et al., 2012). Therefore, the findings of this study together with the previous reports suggest that the cancer cells adapted to oxidative stress have increased tolerance for chemotherapeutic drugs.

Various mechanisms, such as enhanced cell survival (Zaffaroni and Daidone, 2002), inhibition of apoptotic potential (Scheltema et al., 2001), increased repair and/or tolerance of DNA damage (Salehan and Morse, 2013), and altered drug metabolism and transport (Gottesman, 2002; Gottesman et al., 2002; Raguz and Yagüe, 2008), have previously been proposed for chemotherapeutic resistance in cancer cells. Therefore, to further identify the molecular mechanism of the decreased sensitivity to doxorubicin in kidney cancer cells that were adapted to chronic oxidative stress, the expression of genes involved in drug transport, cell survival, and DNA damage–induced apoptosis were analyzed. The results of this study suggest that chronic oxidative stress–induced aberrations in the expression of critical genes, such as increased expression of cell survival and antiapoptotic protein Survivin, drug transporters such as MDR1 and BCRP, and decreased expression of doxorubicin target TOP2A, as well as the DNA damage–response protein MSH2, have the potential to
contribute in decrease sensitivity to doxorubicin in kidney cancer cells. Similar findings showing reduced sensitivity to chemotherapeutic agents associated with the decreased expression of TOP2A and MSH2, and increased expression of anti-apoptotic genes, drug efflux transporter, and antioxidant genes have been reported in various cancers (Konopleva et al., 2002; Burgess et al., 2008; Kaplan and Gunduz, 2012; AbuHammad and Zihlif, 2013).

Persistent exposure to ROS (chronic oxidative stress) renders cancer cells physiologically adapted to ROS, thus facilitating increased survival (Mahalingaiah and Singh, 2014) through upregulation of Survivin, which abolishes the G2/M checkpoint and allows cells to progress through mitosis (Li et al., 1998). Owing to its antiapoptotic function, Survivin inhibits radiotherapy- and chemotherapy-induced cell death, thus resulting in tumor progression and drug resistance (Zaffaroni and Daidone, 2002; Schlette et al., 2004; Rödel et al., 2005; Fuessel et al., 2006). These previous reports and present findings suggest that cancer cells adapted to a microenvironment of oxidative stress can develop intrinsic resistance to chemotherapeutic drugs, possibly through alterations in genes involved in cell survival and drug transport. Additionally, the data of this study from cell cycle analysis revealed that compared with control Caki-1 cells, the Caki-1LA cells with low-dose chronic oxidative stress exposure not only had increased S-phase population but also had decreased apoptotic Pre-G1 population. It is well documented that ROS-induced cellular effects depend on the concentration and duration of exposure (Valko et al., 2006; Okoh et al., 2013).

In contrast to high-dose ROS-induced apoptosis, the low-dose ROS acts as a mitogenic signaling molecule (Felty et al., 2005) and this could be the explanation for the observed increase in S-phase population in Caki-1LA cells along with the decrease in apoptotic Pre-G1 population. Therefore, the acquisition of increased proliferative potential resulting from low-dose oxidative stress could also be a factor contributing to decreased sensitivity to doxorubicin and cisplatin in Caki-1LA cells.

Loss of apoptotic potential is another mechanism for the drug resistance and MMR-mediated apoptosis, which is an important pathway among the various mechanisms for apoptosis. Several genes involved in the MMR pathway have dual roles in apoptosis as well as DNA repair. For example, mismatch repair protein MSH2 is involved in DNA damage–induced apoptosis by recruiting ataxia telangiectasia- and Rad3-related protein (ATR) to DNA damage sites for its activities in apoptosis (Pabla et al., 2011). Experimental evidence from MSH2-deficient mice also suggest that the MMR system has dual functions of avoiding mutagenesis and inducing apoptosis to inhibit oxidative stress–induced tumorigenesis (Piao et al., 2013). The data of this study revealed that chronic oxidative stress causes loss of MSH2 expression in kidney cancer cells and, interestingly, these cells were also resistant to doxorubicin-induced cytotoxicity. Furthermore, the data from this study supports the necessity of the MMR system not only for repair of oxidative DNA damage but also for DNA-damage-dependent apoptotic signaling. Additionally, an increased tolerance to doxorubicin-induced cytotoxicity in cells with decreased expression of MSH2 during adaption to...
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Oxidative stress was observed in this study. In line with this finding, a recent study also suggests that treatment of MSH2-deficient cells with cisplatin, a chemotherapeutic agent, did not activate the ATR pathway and downstream signaling for DNA damage response and apoptosis (Pabla et al., 2011). Loss of MMR pathway in developing apoptotic resistance against a variety of chemotherapeutic and methylating agents has been reported (Fink et al., 1998; Stojic et al., 2004; O’Brien and Brown, 2006; Hewish et al., 2010; Guillotin and Martin, 2014). Cisplatin is another class of chemotherapeutic drug, and loss of MMR has also been known to reduce the sensitivity to cisplatin-induced cytotoxicity (Drummond et al., 1996). Therefore, in this study, to further confirm the loss of MMR as a causal factor of reduced sensitivity to chemotherapeutic drug, Caki-1 LA cells were also treated with cisplatin. Interestingly, the result of reduced sensitivity to cisplatin in Caki-1 LA cells provided additional evidence for the loss of MMR as a causal factor of reduced sensitivity to chemotherapeutic drug in Caki-1 LA cells. Therefore, previous reports and the findings of this study together suggest that the MMR system is needed for chemotherapeutic drug–induced DNA damage response and apoptosis.

The next logical question in our study was, “How does the oxidative stress alter genes that confer increased resistance to cell death, as well as the increased resistance to doxorubicin-induced cytotoxicity?” Though the genetic mechanism is well established for regulation of gene expression, recent study overwhelmingly implicates epigenetic mechanisms in gene-expression changes (Dawson and Kouzarides, 2012). Therefore to further understand the role of epigenetic mechanisms in decreased expression of MMR genes in oxidative stress–adapted kidney cancer cells and consequently the inactivation of MMR-dependent apoptotic potential, the cells were first treated with DNA-demethylating agent 5-Aza-2dC and then cotreated with doxorubicin. The result of this study revealed that 5-Aza-2dC treatment restored not only the expression of MMR genes but also the sensitivity to doxorubicin-induced cytotoxicity in kidney cancer cells. This suggests that oxidative stress causes DNA hypermethylation–mediated silencing of MMR genes, which ultimately contributes to the loss of MMR-dependent apoptotic potential and leads to resistance to doxorubicin cytotoxicity. The result of pyrosequencing analysis further confirmed DNA hypermethylation of one among six CpG sites from MSH2 promoter region in Caki-1 LA cells compared with Caki-1. Since we evaluated only a small part of the MSH2 promoter region, hypermethylation of more CpG sites from remaining regions of MSH2 promoter cannot be ruled out. In addition, it is also possible that decreased MSH2 observed in our study may be a result of DNA hypermethylation–mediated silencing of transcription factors that regulate MSH2 expression. Further studies are needed to investigate these additional causes for decreased expression of MSH2 in cells exposed to chronic oxidative stress.

Previous studies have shown that genome-wide demethylation resestitifies the tumor cells, such as lung cancer cells (Gomyo et al., 2004), melanoma cells (Kaminski et al., 2004), and breast cancer cells (Hurtubise and Momparler, 2004), to the cytotoxic effects of chemotherapeutics (Missiaglia et al., 2005). DNA-demethylating agent 5-Aza-2dC has shown additive effect with doxorubicin, as well as synergistic effect with paclitaxel, in various cancer cells, including Caki-1 kidney cancer cells (Shang et al., 2007). However, the target genes for demethylation and their involvement, as well as the mechanistic basis for demethylation-mediated resensitization to chemotherapy, were not clear in these previous reports. Therefore, the present study has not only confirmed that demethylation sensitizes cancer cells to chemotherapy but has also provided a mechanistic basis for resensitization to chemotherapy through reactivation of MMR–mediated apoptosis.

The data of Western blot analysis in this study revealed downregulation of HDAC1 and increase in the level of histone 3 acetylation (acetyl H3Lys9 and acetyl H3Lys9/14) in cells adapted to chronic oxidative stress. The gene expression data also revealed 4- and 2.4-fold increases in the expression of drug transporter MDR1 gene in Caki-1 LA and Caki-1 HA cells, respectively. Likewise, a previous report showed increased expression of MDR1, and increased levels of lysine 9-acetylated histone H3, both globally at genome-wide as well as locally at the promoter of MDR1 in doxorubicin-resistant MCF-7 breast cancer cells (Toth et al., 2012). Therefore, on the basis of this previous report and the finding of this study, it is logical to conclude that chronic oxidative stress–induced aberrations in histone acetylation also play an important role in acquisition of resistance to doxorubicin in cells adapted to oxidative stress.

In summary, the findings of this study for the first time revealed that kidney cancer cells adapted to chronic oxidative stress can develop chemotherapeutic resistance, at least in part, through the epigenetic inactivation of MMR-dependent apoptotic potential and through increased expression of drug transporter MDR1, which may cause increased drug efflux.

Authorship Contributions
Participated in research design: Ponnusamy, Mahalingaiah, Singh.
Conducted experiments: Ponnusamy, Mahalingaiah.
Performed data analysis: Ponnusamy, Mahalingaiah, Singh.
Performed new reagents or analytic tools: Singh.
Wrote or contributed to the writing of the manuscript: Ponnusamy, Mahalingaiah, Singh.

References


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