Down-Regulation of MutS Homolog 2 (MSH2) Expression by Curcumin Enhances Cytotoxicity Induced by Gemcitabine in Human Lung Adenocarcinoma Cells

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Abstract

Gemcitabine (2′,2′-difluorodeoxycytidine) is a difluorinated analog of deoxycytidine. It is used clinically to treat patients with non-small-cell lung cancer (NSCLC). Curcumin is a yellow pigment derived from the rhizome of Curcuma longa, and has been proven to have antioxidant and antitumor properties. Human MutS homolog 2 (MSH2) is a key DNA mismatch repair protein that plays an important role in maintaining genomic stability. Depletion of MSH2 from cells can reverse resistance to certain DNA-damaging agents. In this study, exposure of human lung adenocarcinoma A549 and H1975 cells to gemcitabine increased protein phosphorylation of MKK3/6 and p38 MAPK in a time- and dose-dependent manner; this was accompanied by increased expression of MSH2 mRNA and protein. Gemcitabine-induced cytotoxicity was significantly enhanced by MSH2 siRNA transfection or inactivation of p38 MAPK by SB203580 or p38 MAPK siRNA transfection. However, overexpression of MSH2 cDNA reduced gemcitabine-induced cytotoxicity. Furthermore, curcumin enhanced gemcitabine-induced cytotoxicity via inactivation of MKK3/6/p38 MAPK and downregulation of MSH2. Enforced expression of constitutively active MKK6 rescued cell viability and restored MSH2 protein levels that were suppressed by curcumin and gemcitabine. Suppression of MSH2 expression and a combination with curcumin may be considered as potential therapeutic modalities for gemcitabine-resistant NSCLC cells.

Keywords: Gemcitabine; Curcumin; MSH2; p38 MAPK; Non-small cell lung cancer

Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer death in the world [1], and the majority of patients with NSCLC present with advanced metastatic stages [2]. The cytotoxic chemotherapies can improve the median overall survival of NSCLC patients [3]. Gemcitabine (2′, 2′-difluorodeoxycytidine, dFdC) has been clinically utilized for NSCLC patients [4]. It’s cytotoxic effect requires intracellular transport and activation [5,6]. When gemcitabine introduced into the cells by membrane transporters, it undergoes intracellular phosphorylation and yields gemcitabine diphosphate (dFdCDP) and triphosphate (dFdCTP), which can be incorporated into DNA and RNA [5,7]. A previous study has shown that gemcitabine can be used in combination with platinum drugs to treat advanced-stage NSCLC, and can act as a single agent for the treatment of pancreas adenocarcinoma [8]. The anti-proliferative activity of gemcitabine in pancreatic cancer results from p38 mitogen-activated protein kinase (MAPK) activation, which mediates cell apoptosis [9]. Moreover, down-regulation of gemcitabine-induced extracellular signal-regulated kinase 1/2 (ERK1/2) in hepatocellular and cholangiocellular carcinomas enhances cell death [10]. However, the molecular mechanisms leading to the lesser effectiveness of gemcitabine in lung adenocarcinoma cells than in squamous carcinoma cells in terms of growth inhibition and cytotoxicity are poorly understood and remain to be elucidated.

Human MutS homolog 2 (MSH2), a crucial element in the DNA mismatch repair (MMR) system, maintains genetic stability and avoids gene mutation in cells [11]. The mismatch recognition activity in MMR is carried out by either MutSα (MSH2-MSH6) or MutSβ (MSH2-MSH3). Deletion or mutation of MSH2 can cause genomic instability [12]. In addition, loss of MSH2 expression in tumors from patients with advanced NSCLC led to higher rates of response to oxaliplatin-based chemotherapy [13]. Moreover, both the p38 MAPK and c-Jun N-terminal kinase (JNK) pathways have been confirmed to mediate the ectopic expression of MSH2 under oxidative stress in renal carcinoma cells [14]. We previously found that down-regulation of the MKK3/6–p38 MAPK signal, with the subsequent reduction of MSH2 expression, enhanced the cytotoxic effect of pemetrexed in human lung squamous cells [15]. Whether gemcitabine can affect MSH2 expression in NSCLC is still unknown, and the role of p38 MAPK in MSH2 expression, regulating gemcitabine-induced cytotoxicity in NSCLC, has not been elucidated yet.

Curcumin (diferuloylmethane) is a phytopolyphenol pigment isolated from the plant Curcuma longa (turmeric) and has a variety of pharmacologic properties, including anti-inflammatory, antioxidant and anticancer properties [16,17]. A previous study showed that curcumin inhibited cisplatin-induced p38 MAPK activation and enhanced cisplatin-induced cytotoxicity in human lung cancer cells [18]; therefore, this study was designed to evaluate the role of MSH2 in cell survival in human lung adenocarcinoma cells when they were treated with curcumin.

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exposed to gemcitabine and curcumin. We hypothesized that the p38 MAPK signaling pathway was responsible for MSH2 expression and gemcitabine resistance, and that a combination of gemcitabine and curcumin may enhance gemcitabine-mediated cytotoxicity by decreasing the activation of p38 MAPK and the expression of MSH2 in human lung adenocarcinoma cell lines.

Materials and Methods

Cell lines and reagents

Human lung bronchioalveolar carcinoma A549 cells (CCL-185) and lung adenocarcinoma H1975 cells (CRL-5908) were obtained from the American Type Culture Collection (Manassas, VA). Gemcitabine was obtained from Lilly (Fegersheim, France). Curcumin, cycloheximide and actinomycin D were purchased from Sigma-Aldrich (St Louis, MO, USA). SB202190, MG132, and lactacystin were purchased from Calbiochem-Novabiochem (San Diego, CA, USA).

Western blot analysis

After different treatments, equal amounts of proteins from each set of experiments were subjected to Western blot analysis. The relative protein blot intensities were determined using a computer densitometer equipped with the ImageQuant analysis program (Amerham Biosciences).

Quantitative real-time polymerase chain reaction (PCR)

Polymerase chain reactions (PCRs) were performed using an ABI Prism 7900HT, according to the manufacturer’s instructions. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems). The designated primers were: for MSH2 forward primer, 5'-AAGGCGGATTGCCATTG-3'; MSH2 reverse primer, 5'-CTTGGCAAGCTGCAAAAGC-3'; GAPDH forward primer, 5'-CATGAGAAGTATGACAACAGCCT-3'; GAPDH reverse primer, 5'-AGTCCCTTCCACGATACCAAGT-3'. The thermal cycling conditions were as follows: 10 min denaturation at 95°C, 40 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s. For each sample, data was normalized to the housekeeping gene GAPDH.

Transfection of small interfering RNA and expression plasmids into NSCLC cells

Exponentially growing human lung cancer cells (106) were plated for 18 h, and then the MKK6E or MSH2 cDNA expression vectors were transfected into A549 or H1975 cells using Lipofectamine 2000 (Invitrogen). Cells were transfected with siRNA duplexes (100 nM) by using Lipofectamine 2000 (Invitrogen) for 24 h. Cell viability analysis

Cells were cultured at 5000 per well in 96-well tissue culture plates. To assess cell viability, drugs were added after plating. At the end of the culture period, 20 µL of MTS solution (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) was added, the cells were incubated for a further 2 h, and the absorbance was measured at 490 nm using an ELISA plate reader (Biorad Technologies, Hercules, CA). Cell viability was calculated as follows: cell viability (\%)=(OD of drug-treated sample/OD of untreated sample) × 100. The values of cell viability were calculated from the mean amount of 3 independent experiments.

Determination of cell death

After treatment, unattached and attached cells were collected and stained using trypan blue solution (Sigma-Aldrich, St Louis, MO, USA). The stain was excluded from living cells but could penetrate dead cells. The proportion of dead cells was determined by counting the cells stained with trypan blue using a hemocytometer.

Statistical analysis

For each protocol, 3 or 4 independent experiments were performed. Results were expressed as mean ± standard error of the mean (SEM). Statistical calculations were performed using SigmaPlot 2000 software (Systat Software, San Jose, CA). Differences in measured variables between the experimental and control groups were assessed using an unpaired t test. *P<0.05 was considered statistically significant.

Results

MSH2 mRNA and protein levels were increased after gemcitabine exposure

First, we examined the effects of gemcitabine on signal molecules in 2 human lung adenocarcinoma cell lines, A549 and H1975. As shown in Figure 1A, gemcitabine treatment increased MSH2 protein levels, and this was accompanied by the activation of MKK3/6-p38 MAPK during different exposure times. In addition, these 2 NSCLC cell lines were treated with various concentrations of gemcitabine (2.5–20 µg/ml) for 24 h, resulting in up-regulation of the protein levels of MSH2 in a dose-dependent manner (Figure 1B). To confirm the transcriptional regulation of MSH2 in the gemcitabine-treated A549 and H1975 cells, mRNA levels were examined using real-time PCR. In Figure 1C and 1D, gemcitabine increased MSH2 mRNA expression in a time- and dose-dependent manner.

Gemcitabine increased MSH2 mRNA and protein expression through up-regulation of p38 MAPK activity

To examine whether the up-regulation of MSH2 expression by gemcitabine was a result of p38 MAPK signal activation, cells were pretreated with SB202190 (a p38 MAPK inhibitor) or transfected with specific p38 MAPK siRNA. In Figure 2A-2D, inactivation of p38 MAPK by the pharmacological inhibitor or the specific siRNA decreased the gemcitabine-induced MSH2 mRNA and protein levels. Furthermore, A549 or H1975 cells were transiently transfected with a plasmid carrying MKK6E, a constitutively active form of MKK6. Compared to transfection with the control vector-pcDNA3, transfection with MKK6E increased the p38 MAPK phosphorylation and MSH2 mRNA and protein levels at A549 or H1975 cells (Figure 2E and 2F).

Blockage of p38 MAPK activation decreased the MSH2 mRNA and protein stability induced by gemcitabine

Next, we examined the possible mechanisms for post-transcriptional regulation of MSH2 transcripts under gemcitabine treatment. To evaluate the stability of MSH2 mRNA in gemcitabine-exposed A549 or H1975 cells, the cells were treated with gemcitabine for 12 h; actinomycin D was then added to block de novo RNA synthesis and the levels of existing MSH2 mRNA were measured using real-time PCR at 4, 8, and 12 h after treatment. After actinomycin D co-exposure, higher levels of MSH2 mRNA were observed with gemcitabine treatment than in the untreated cells (Figure 3A). Then, cycloheximide (an inhibitor of de novo protein synthesis) was added to gemcitabine treatment for 4, 8, and 12 h, and the remaining MSH2 protein was analyzed by Western blot. In Figure 3B, MSH2 protein levels were progressively reduced with time in the presence of cycloheximide. However, gemcitabine treatment significantly decreased MSH2 degradation after cycloheximide treatment, compared to the untreated cells. Moreover, more MSH2 protein remained after gemcitabine treatment, compared
Figure 1: Time course- and dose-dependent MSH2 induction by gemcitabine. (A) A549 or H1975 cells were treated with gemcitabine (20 µg/mL) or DMSO in complete media for the indicated times. (B) cells were exposed to various concentrations of gemcitabine (2.5–20 µg/mL) for 24 h. Cells were then extracted for the determination of MSH2, phospho-MKK3/6, phospho-p38 MAPK, actin, MKK3 and p38 MAPK protein levels by Western analysis. (C and D) after treatment as in (A) and (B), total RNA was isolated and subjected to real-time PCR for MSH2 mRNA.

Figure 2: Inactivation of p38 MAPK decreased MSH2 mRNA and protein levels in gemcitabine-exposed NSCLC cells. (A and B) NSCLC cells were exposed to gemcitabine (10, 20 µg/mL) and SB202190 (10 µM) for 24 h. The results (mean ± SEM) were from 3 independent experiments. **p < 0.01 using Student’s t-test for comparison between the cells treated with gemcitabine–DMSO and those treated with a gemcitabine–SB202190 combination. (C and D) A549 or H1975 cells were transfected with siRNA duplexes (200 nM) specific to p38 MAPK or scrambled (control) in complete medium for 24 h prior to treatment with gemcitabine (20 µg/mL) in complete medium for 24 h. The average knockdown efficiency of p38 MAPK was 86.02% and 72.43% in A549 and H1975 cells, respectively. The results (mean ± SEM) were from 3 independent experiments. **p<0.01, *p<0.05 using Student’s t-test for comparison of cells transfected with pcDNA3 and those transfected with MKK6E vector. After treatment, the cell extracts were examined via real-time PCR (A, C, E) and Western blot (B, D, F) for determination of MSH2 mRNA and protein levels, respectively.
to the untreated cells (Figure 3B). Only ~45% of MSH2 remained in the H1975 cells treated with cycloheximide for 12 h; however, ~84% of MSH2 remained in cells co-treated with gemcitabine and cycloheximide, indicating that gemcitabine remarkably increased the stability of the MSH2 protein (Figure 3B). Moreover, SB202190 had a significantly decreased effect on gemcitabine-elicited MSH2 mRNA and protein stability in both A549 and H1975 cells (Figure 3A and 3B). These results indicated that gemcitabine increased MSH2 mRNA and protein levels by augmentation of mRNA and protein stability through p38 MAPK activation in gemcitabine-treated NSCLC cells.

**MSH2 expression was involved in gemcitabine-mediated cell growth inhibition and cytotoxicity**

To determine whether MSH2 participated in the gemcitabine-elicited inhibition of cell growth and cytotoxicity, we knocked down MSH2 using a specific siRNA duplex (si-MSH2 RNA). As shown in Figure 4A and 4B, si-MSH2 RNA effectively suppressed the MSH2 mRNA and protein expressions induced by various concentrations of gemcitabine. Of interest, the suppression of MSH2 protein expression by si-MSH2 RNA markedly increased the sensitivity of the cells to gemcitabine, compared to the si-control RNA-transfected cells (Figure 4C and 4D). In addition, the blockage of MSH2 expression significantly enhanced the antiproliferative effect brought about by gemcitabine (Figure 4E). Then, the cells were transfected with MSH2 cDNA vector and incubated with gemcitabine, and cell viability was analyzed using the MTS assay. The induction of cytotoxicity and growth inhibition by gemcitabine was significantly reduced following the overexpression of MSH2 in A549 and H1975 cells (Figure 4F and 4G). Therefore, MSH2 protected human lung adenocarcinoma cells from the cytotoxicity induced by gemcitabine.

Next, we investigated the roles of p38 MAPK activation induced by gemcitabine in the gemcitabine-elicited cytotoxicity in lung cancer
Induction of p38 MAPK phosphorylation and MSH2 expression by gemcitabine can be prevented by curcumin

A previous study showed that curcumin inhibited cisplatin-induced p38 MAPK activation and enhanced cisplatin-induced cytotoxicity in human lung cancer cells [18]; therefore, we tested whether curcumin could enhance the cytotoxicity of gemcitabine via modulating p38 MAPK activation and MSH2 expression. As shown in Figure 5A, under gemcitabine treatment, the protein levels of MSH2 and phospho-p38

Figure 4: Knockdown of MSH2 expression by si-RNA transfection enhanced the cytotoxicity induced by gemcitabine. (A and B) A549 or H1975 cells were transfected with siRNA duplexes (200 nM) specific to MSH2 or scrambled (control) in complete medium for 24 h prior to treatment with gemcitabine (10 and 20 µg/mL) in complete medium for 24 h; the cell extract was examined by real-time PCR (A) and Western blot (B) for determination of MSH2 mRNA and protein levels, respectively. (C and D) After the above-mentioned treatment, cytotoxicity was determined by MTS assay and trypan blue dye exclusion assay. (E) After the cells were transfected with si-MSH2 or si-scrambled RNA, the cells were treated with gemcitabine (5 µg/mL) for 24, 48, and 72 h, after which living cells were determined by MTS assay. The results (mean ± SEM) were from 3 independent experiments. **p<0.01 using Student's t-test for comparison between the cells treated with gemcitabine in si-MSH2 RNA and the si-scrambled RNA-transfected cells. (F) After the cells were transfected with pcDNA3.1 or MSH2 cDNA expression vector, they were treated with gemcitabine (5 µg/mL) for 24, 48, and 72 h, and cytotoxicity was determined by MTS assay. **p<0.01 using Student's t-test for comparison of cells treated with gemcitabine in MSH2 cDNA and pcDNA3.1 vector-transfected cells. Inhibition of p38 MAPK activation enhanced the cytotoxicity induced by gemcitabine. (H) Left panel, A549 or H1975 cells were transfected with siRNA duplexes (200 nM) specific to p38 MAPK or scrambled (control) in complete medium for 24 h prior to treatment with gemcitabine (20 µg/mL) in complete medium for 24 h. Right panel, A549 or H1975 cells were pretreated with SB202190 (2.5, 5 and 10 µM) for 1 h and then co-treated with gemcitabine (5 µg/mL) for 24 h. Cytotoxicity was determined by MTS assay. **p<0.01 using Student's t-test for comparison of cells pretreated with and without SB202190 in gemcitabine exposed cells. (I) Cells were treated with gemcitabine (5 µg/mL) and/or SB202190 (2.5 and 5 µM) for 1–3 days, after which living cells were determined by trypan blue dye exclusion assay. **p<0.01 using Student's t-test for comparison of cells treated with gemcitabine alone and those with a gemcitabine and SB202190 combination.
MAPK were inhibited by curcumin. In addition, when compared to the untreated cells, treatment with curcumin or curcumin plus gemcitabine down-regulated the levels of MSH2 mRNA (Figure 5B). We also examined whether degradation of MSH2 by curcumin and gemcitabine co-treatment occurred through ubiquitin-26S proteasome-mediated proteolysis of MSH2. As shown in (Figure 5C), both MG132 and lactacystin (both common 26S proteasomal inhibitors) rescued the MSH2 protein levels decreased by curcumin and gemcitabine. Next, we determined whether the inactivation of p38 MAPK was involved in the downregulation of MSH2 protein expression after treatment with gemcitabine plus curcumin, and the constitutively active form of MKK6 (MKK6E) vectors was transfected into human lung cancer cells. MKK6E rescued the levels of phospho-p38 MAPK and MSH2 expression inhibited by treatment with curcumin and gemcitabine (Figure 5D and 5E). However, pretreatment with SB202190 further decreased MSH2 mRNA and protein levels in curcumin and gemcitabine co-treated A549 and H1975 cells (Figure 5F and 5G). Taken together, curcumin could down-regulate gemcitabine-induced p38 MAPK phosphorylation and MSH2 expression in A549 and H1975 cells.

Combined treatment with curcumin enhances gemcitabine-induced cytotoxicity in NSCLC cells

We next examined whether curcumin could enhance the cytotoxicity of gemcitabine in A549 and H1975 cells. In the MTS assay, curcumin enhanced the cytotoxicity of cells treated with gemcitabine (Figure 6A). Consistent with these observations, cell death was higher in cells that were co-treated with the 2 drugs than in those that were treated with gemcitabine or curcumin alone, as determined using the trypan blue exclusion assay (Figure 6B). Moreover, curcumin significantly enhanced the antiproliferative effects induced by gemcitabine (Figure 6C).

Curcumin enhances gemcitabine-induced cytotoxicity via the inactivation of p38 MAPK and down-regulation of MSH2 expression

We determined whether activation of p38 MAPK and overexpression of MSH2 could protect the cells from cell death induced by gemcitabine and curcumin. A549 and H1975 cells were transfected with MKK6E or MSH2 cDNA vectors to evaluate the viability of cells treated with curcumin and/or gemcitabine. Transfection of MKK6E or
MSH2 cDNA rescued the cells from the cell death induced by curcumin and gemcitabine in A549 and H1975 cells (Figure 6D). Inactivation of p38 MAPK activity by SB202190 could further enhance curcumin and gemcitabine-induced cytotoxicity (Figure 6E). Together, the activation of p38 MAPK and the expression of MSH2 protein and mRNA were suppressed by curcumin, leading to the enhancement of gemcitabine-induced cytotoxicity.

Discussion

In this study, we first found that gemcitabine induced the expression of MSH2 via the activation of p38 MAPK. Knockdown of MSH2 expression enhanced gemcitabine-induced cytotoxicity in human lung adenocarcinoma cells. In a previous study, the inactivation of ERK1/2 enhanced cell death in gemcitabine-treated hepatocellular and cholangiocellular carcinoma cells [10]. In addition, nuclear factor κB (NF-κB) [19] and bcl-2 [20] were associated with gemcitabine resistance in pancreatic carcinoma cell lines. Low levels of BRCA1 were correlated with increased survival in NSCLC patients treated with gemcitabine plus cisplatin [21,22]. In our previous study, down-regulation of Rad51 expression enhanced the cytotoxicity of gemcitabine in NSCLC cell lines [23]. In this study, the overexpression of MSH2 enhanced the viability of lung cancer cells treated with gemcitabine, and down-regulation of MSH2 expression enhanced gemcitabine-induced cytotoxicity and cell growth arrest.

A previous study indicated that phosphorylation of p38 MAPK induced by vascular endothelial growth factor (VEGF) was...


