Downregulation of *MUTYH* contributes to cisplatin-resistance of esophageal squamous cell carcinoma cells by promoting Twist-mediated EMT

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Abstract. Acquired resistance to cisplatin (CDDP) in esophageal squamous cell carcinoma (ESCC) remains a major challenge in cancer therapy. Although progress has been made in identifying the mechanisms responsible for resistance to CDDP, the underlying mechanisms of resistance in ESCC are still not entirely understood. In the present study, a CDDP-resistant ESCC cell line EC109/CDDP was established by culturing parental EC109 cells in increasing concentrations of CDDP, and it was demonstrated that MutY homolog (*MUTYH*), a critical base excision repair gene, was significantly downregulated in the resistant EC109/CDDP cells compared with that noted in the parental cells. Ectopic expression of *MUTYH* by transient transfection of pcDNA3.1-*MUTYH* plasmid significantly enhanced the CDDP-mediated inhibitory effect on resistant cell proliferation and induction of apoptosis, while silencing of *MUTYH* by transiently transfecting *MUTYH*-targeted siRNA in parental cells led to decreased sensitivity to CDDP as demonstrated by MTT assay, suggesting the crucial involvement of *MUTYH* in CDDP resistance. Further experiments demonstrated that the CDDP-resistant cells went through epithelial-mesenchymal transition (EMT) driven by its master regulator Twist, and *MUTYH* overexpression significantly reduced the Twist expression level and reversed the phenotype of EMT as detected by western blot analysis and RT-qPCR assays, suggesting that downregulation of *MUTYH* contributed to the Twist-mediated EMT. Moreover, it was observed that the effect of *MUTYH* on Twist was also associated with its degradation in addition to transcription. *MUTYH* acted as a positive regulator of reactive oxygen species (ROS) that showed a low level in resistant cells via flow cytometry assay, as demonstrated by increased ROS production in response to *MUTYH* overexpression. Reduced ROS by using *N*-acetylcysteine led to a decrease in proteasome activity and sequentially inhibited the degradation of Twist. In conclusion, the present data demonstrated that EMT activation mediated by *MUTYH* downregulation, by both enhancing Twist transcription and blocking its degradation, is one of the mechanisms for acquisition of CDDP resistance in ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC), the predominant histological subtype of esophageal carcinoma, is one of the most refractory cancer types with a high mortality rate in the world (1,2). Although various treatment regimens have been adopted to improve curability, the overall 5-year survival rate for patients with ESCC over the past 30 years is still poor at ~20% (2). At present, surgical treatment combined with radiotherapy and chemotherapy is the main clinical therapeutic method of ESCC, and cisplatin (CDDP) is still used as one of the pivotal chemotherapeutic drugs in the front-line therapeutic regimen, playing an irreplaceable role in prevention of recurrence and metastasis of ESCC (3). However, satisfactory chemotherapeutic effects have rarely been achieved, as CDDP-based therapies always just obtain initial chemotherapeutic success and eventually induce CDDP resistance in ESCC (4,5).

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It has been proposed that several molecular mechanisms can drive chemoresistance to CDDP, which may be unique to different types of cancer (4,5). Prominent CDDP-resistance mechanisms involve various biological regulatory processes, including blockade of DNA damage response signal, activation of DNA repair pathways, epithelial-mesenchymal transition (EMT), thiols and metallothionein-mediated detoxification, and efflux of CDDP from cancer cells (4). Despite the progress in identifying mechanisms responsible for resistance to CDDP, the underlying mechanisms of resistance in ESCC are not entirely understood and the clinical treatment of CDDP-resistant ESCC remains a critical obstacle. Therefore, the present study aimed to further elucidate mechanisms behind CDDP resistance in ESCC and novel biomarkers, which may be used to predict prognosis and chemosensitivity.

Accumulating evidence has suggested that CDDP-induced nuclear DNA damage alone is not enough to illustrate its high-level of effectiveness or cytotoxicity (6,7). Several recent studies have demonstrated that, independent of CDDP-DNA adduct generation, the mechanism of CDDP-stimulated production of reactive oxygen species (ROS) and subsequent oxidative damage of the DNA base also have a crucial involvement in CDDP cytotoxicity (7-9). One of the best characterized oxidative DNA lesions is 7,8-dihydro-8-oxoguanine, which can induce G:C to T:A transversion mutations. The mutations are recognized and repaired by mutY homolog (*MUTYH*) (10,11). *MUTYH* is a key component of the system of base excision repair (BER), which is able to remove the oxidized bases paired erroneously, thereby ensuring the maintenance of DNA integrity (10,11). It has been confirmed by experimental evidence that *MUTYH* has an important role in various cancer types; it was reported that modification of the tissue expression of *MUTYH* is associated with the elevated risk of development of various cancer types, such as colorectal cancer, lung cancer, esophageal carcinoma, thyroid cancer and head and neck cancer (12-17). Mutations in *MUTYH* reduce BER effectiveness and predisposition to cancer (17,18). Recent research also suggests that patients with colorectal cancer have lower *MUTYH* expression in cancer tissues compared with normal tissues (19). Meanwhile, the *MUTYH* gene was found to present reduced expression in more advanced stages of colorectal cancer, and lower *MUTYH* expression may contribute to worse patient prognosis (12). In the present study, the association of *MUTYH* with CDDP resistance in ESCC cells was analyzed. The present data demonstrated that downregulation of *MUTYH* induced the EMT process by regulating both the transcription and degradation of Twist, which eventually contributed to resistance to CDDP.

Materials and methods

Establishment of a CDDP-resistant ESCC cell line. The human ESCC cell line EC109 was obtained from The Cell Bank of the Chinese Academy of Sciences and was maintained in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences). The CDDP-resistant cell line EC109/CDDP was selected by culturing EC109 cells in increasing concentrations of CDDP (Selleck Chemicals) starting from 0.1 μM to a final concentration of 5.0 μM. The cells were maintained in a humidified incubator with 5% CO_2 at 37˚C.

Cell viability and cell death analysis. The inhibitory effect of CDDP on cell viability in EC109 and EC109/CDDP cells was determined by an MTT (Sigma-Aldrich; Merck KGaA) colorimetric assay using a Thermo Fisher Multiskan microplate reader (Thermo Fisher Scientific, Inc.).

Apoptosis induced by CDDP in EC109 and EC109/CDDP cells was tested using an Annexin V-FITC/Propidium Iodide Apoptosis Detection kit, according to the manufacturer's protocol (BD Biosciences). A flow cytometer (FACSCaliber; BD Biosciences) was used for fluorescence quantification.

Proteasome activity assay. The proteasome substrates Suc-LLVY-AMC (N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin), Bz-LLE-AMC (benzyloxycarbonyl-L-leucyl-leucyl-glutamyl-methylcoumarylamide) and Bz-VGR-AMC (Bz-Val-Gly-Arg-7-amino-4-methylcoumarin) from Enzo Life Sciences, Inc., were used to detect activities of chymotrypsin-like (ChT-L), trypsin-like and peptidyl-glutamyl peptide-hydrolyzing (PGPH) of proteasomes in cells, respectively. Whole cell lysates were extracted with lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40 and 2 mM DTT], and incubated at 37˚C for 40 min with the substrates in the assay buffer. Proteasome activities were determined by measuring fluorescent activities of the substrates using a Mithras LB-940 reader (Berthold Technologies).

Transwell Matrigel invasion assay. Cell invasion was measured by a Transwell Matrigel invasion assay using Growth Factor Reduced Matrigel Invasion Chambers from BD Biosciences. In total, $0.5x10^6$ cells were resuspended in RPMI-1640 medium supplemented with 1% FBS serum into the upper chamber with Matrigel matrix added (BD Biosciences). RPMI-1640 medium supplemented with 10% serum, serving as a chemoattractant, was added to the bottom well. After 48 or 72 h, cells that migrated to the bottom chamber were stained with Giemsa (Bejing Solarbio Science & Technology Co., Ltd.) and were imaged under a bright-field microscope (Nikon Corp.; Magnification, x200). The stained cells were counted using ImageJ software (National Institutes of Health).

Wound healing assay. A wound healing assay was performed to examine cell migration. When cells grew to approximately 95% confluence in 6-well plates, a scratch (wound) was introduced using a pipette tip. Then, the cells were washed three times with PBS to remove detached cells and cultured continually for 24 or 48 h. The wound was imaged under a bright-field microscope (Nikon Corp.; magnification, x100) at 0, 24 and 48 h. The wound gaps were digitally quantified using ImageJ software (National Institutes of Health). The equation for the 'relative wound area' is: Relative wound area=wound area of different group/wound area of EC109 cells at 0 h.

ROS measurement. The production of ROS was monitored using the fluorescent dye hydroethidine 2',7'-dichlorodihydrofluorescein diacetate [H(2)DCFDA; Sigma-Aldrich; Merck KGaA]. After treatment as indicated, cells were incubated with

Table I. Primers for the RT-qPCR analysis.

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')
MUTYH	TGCCACGTACAGCAGAGAC	CAAAGGCGATAGAGGCAATGG
CDH1	ATTTTTCCCTCGACACCCGAT	TCCCAGGCGTAGACCAAGA
CDH2	TTTGATGGAGGTCTCCTAACACC	ACGTTTAACACGTTGGAAATGTG
VIM	AGTCCACTGAGTACCGGAGAC	CATTTCACGCATCTGGCGTTC
TWIST1	GCCGGAGACCTAGATGTCATT	TTTTAGTTATCCAGCTCCAGAGTC
SNAI2	GCGCTCCTTCCTGGTCAAGA	CGCCCAGGCTCACATATTCC
SNAI1	AGCGAGCTGCAGGACTCTAA	ATCTCCGGAGGTGGGATGG
MMP7	GAGTGAGCTACAGTGGGAACA	CTATGACGCGGGAGTTTAACAT
MMP9	TTCCAAACCTTTGAGGGCGA	GCAAAGGCGTCGTCAATCAC
CLDN1	CTGTCATTGGGGGTGCGATA	GACTGGGGTCATAGGGTCAT
GAPDH	TGGTCACCAGGGCTGCTT	AGCTTCCCGTTCTCAGCCTT

MUTYH, MutY homolog; *MMP7*, matrix metallopeptidase 7; *MMP9*, matrix metallopeptidase 9; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

10 μM H(2)DCFDA at 37˚C for 30 min. Then, the cells were washed with PBS and subjected to ROS measurement by flow cytometry. In the experiments, to test the scavenging effect of the antioxidant *N*-acetylcysteine (NAC; Sigma-Aldrich; Merck KGaA) on ROS generation, cells were treated with 2 mM NAC for 24 h before ROS measurement.

Western blot assay. A western blot assay was performed, as previously described (20). Whole cell lysates were prepared with RIPA buffer, according to the manufacturer's protocol (Beyotime Institute of Biotechnology). Proteins were quantified using a bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). Blots were incubated with primary antibodies against MUTYH (cat. no. 19650-1-AP; rabbit polyclonal antibody; dilution 1:500), E-cadherin (cat. no. 60335-1-lg; mouse monoclonal antibody; dilution 1:2,000), vimentin (cat. no. 60330-1-lg; mouse monoclonal antibody; dilution 1:2,000), Twist (cat. no. 25465-1-AP; rabbit polyclonal antibody; dilution 1:500) and ubiquitin (cat. no. 10201-1-AP; rabbit polyclonal antibody; dilution 1:500) (all from ProteinTech Group, Inc.) overnight at 4˚C, followed by incubation with appropriate peroxidase-conjugated secondary antibodies (anti-mouse IgG (H+L) peroxidase-labeled polyclonal antibody, cat. no. 074-1806; dilution 1:5,000; anti-rabbit IgG (H+L) peroxidase-labeled polyclonal antibody; cat. no. 074-1506; dilution 1:5,000; purchased from KPL, Inc.; SeraCare Life Sciences, Inc.). GAPDH (cat. no. sc-47724; mouse monoclonal antibody; dilution 1:3,000; Santa Cruz Biotechnology, Inc.) served as a protein loading control. Immunocomplexes were visualized using chemiluminescence (EMD Millipore) and exposed to X-ray film.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was prepared using an RNAiso Plus Kit (Takara Bio, Inc.). cDNA was synthesized using a PrimeScript RT Reagent Kit (Takara Bio, Inc.) and RT-qPCR was carried out using the Eppendorf RT-qPCR System (Mastercycler ep realplex; Eppendorf). The PCR reaction conditions for all assays were as follows: 95˚C for 30 sec, followed by 40 cycles of amplifcation (95˚C for 5 sec, 58˚C for 30 sec and 72˚C for 30 sec). Changes in the mRNA levels were normalized to the level of GAPDH and calculated using the $2^{-\Delta\Delta Cq}$ method (21). The primer sequences are summarized in Table I.

Transient transfection of plasmids and small interfering RNAs (siRNAs). The pcDNA3.1-*MUTYH* plasmid was constructed to induce overexpression of *MUTYH* (Shanghai Integrated Biotech Solutions Co., Ltd.). Empty vector pcDNA3.1 served as a control. Knockdown of *MUTYH* was performed by transiently transfecting siRNA duplex oligonucleotides targeting *MUTYH* (BioSune). The *MUTYH* siRNA sequences were as follows: Sense, 5'-GGAGGCAGAAGCAUGCUAATT-3' and antisense, 5'-UUAGCAUGCUUCUGCCUCCTT-3'. The scramble siRNA sequences were as follows: Sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Transfection of the overexpression plasmid or siRNA was performed using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Statistical analysis. Data are presented as the mean ± SD and were analyzed using GraphPad Prism software (GraphPad Software, Inc.). The statistical significance of mean differences between the treated and control groups was determined using a two-tailed Student's unpaired t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Evaluation of the CDDP resistance of the established CDDP-resistant ESCC cell line. The aim of the present study was to test the sensitivity of the established EC109/CDDP cells and the parental EC109 cells to CDDP. As shown in Fig. 1A, EC109/CDDP cells showed markedly higher cell viability than EC109 after 48 h treatment with CDDP at concentrations of 2.5-100 μM and the concentrations that induced cellular death at 50% (IC_{50}) were calculated. EC109/CDDP cells were resistant to CDDP, with a relatively

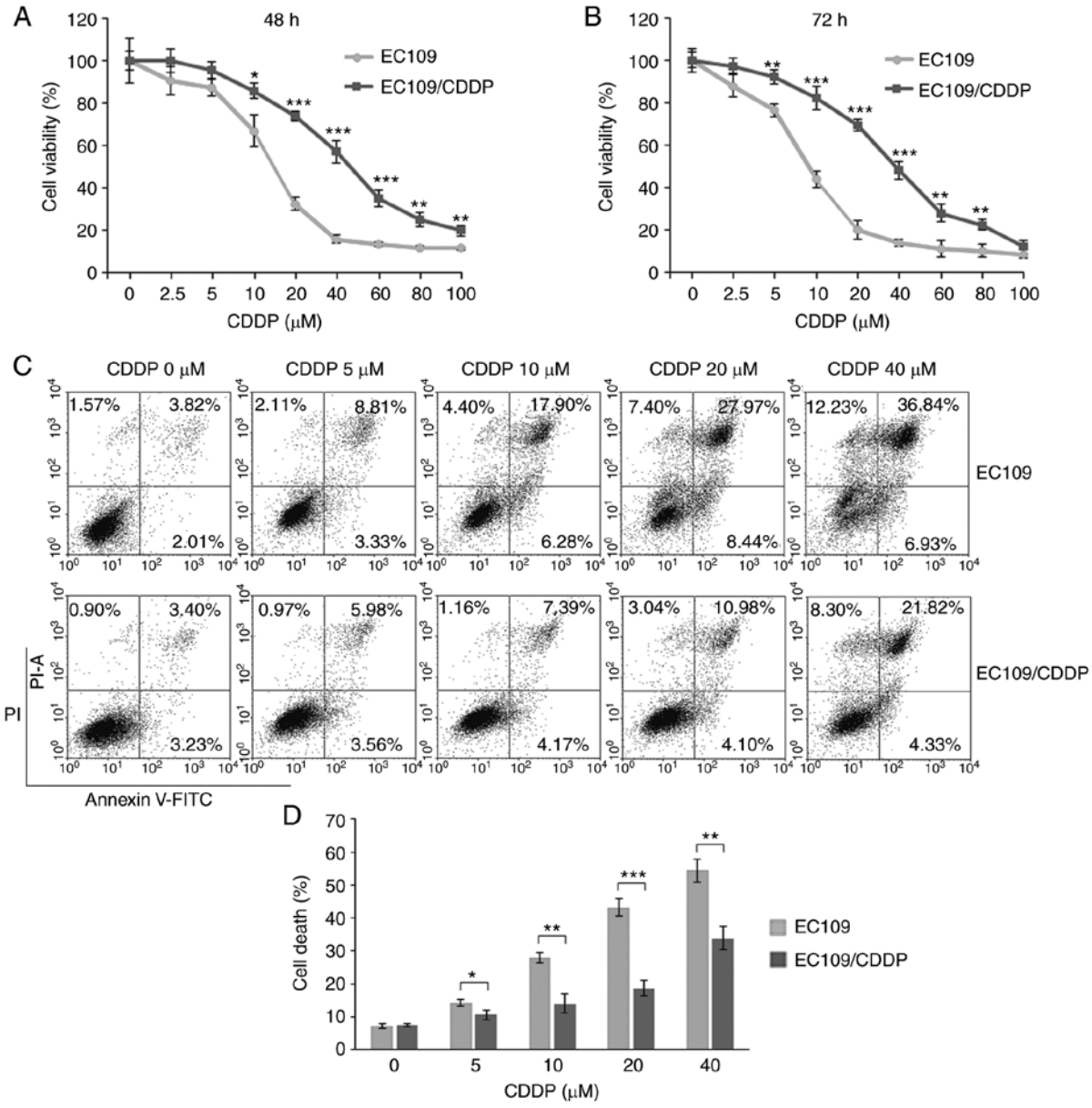

Figure 1. Evaluation for CDDP-resistance of the established resistant cell model EC109/CDDP. (A and B) Cell viability in response to CDDP was determined by the MTT assay after 48 (A) or 72 h (B) of treatment in the CDDP-resistant EC109/CDDP cells and parental EC109 cells. (C and D) CDDP-induced cell death was detected by Annexin V/PI staining and flow cytometric analysis, and the statistical analysis is shown. In A, B and D, the results are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. the EC109 cells treated with the corresponding concentrations of CDDP, respectively. CDDP, cisplatin.

marginally increased IC_{50} value at ~50 μM compared with ~10 μM in EC109 cells. The resistant index (RI) of EC109/CDDP cells for CDDP was ~5 at 48 h. Fig. 1B demonstrates that the CDDP-resistance of EC109/CDDP cells was more prominent at 72 h of CDDP incubation, with an RI at ~7. Flow cytometry was performed to detect the apoptotic cells exposed to CDDP in EC109/CDDP cells. The present results demonstrated that CDDP at 5, 10, 20 and 40 μM caused significant increases in the proportion of apoptotic EC109 cells, ~14.25, 28.58, 43.81 and 56.00%, respectively. By comparison, the apoptotic EC109/CDDP cell fractions were 10.51, 12.72, 18.12 and 34.45% respectively, showing a significant reduction (Fig. 1C and D). Therefore, the present data demonstrated that EC109/CDDP cells were resistant to CDDP when compared with parental EC109 cells.

Downregulation of MUTYH contributes to CDDP resistance in ESCC cells. In the process of identifying potential targets involved in CDDP resistance, *MUTYH*, the crucial gene for excision repair of oxidized bases, was noted for its pronounced downregulation in the resistant cells. As shown in Fig. 2A, RT-qPCR analysis demonstrated that the mRNA expression of *MUTYH* decreased ~5-fold in resistant EC109/CDDP cells compared with that noted in the parental cells. Similar to the mRNA level, the resistant cells presented a significant decrease in the corresponding protein level of MUTYH (Fig. 2B).

To further investigate the effect of *MUTYH* in CDDP resistance, MUTYH was either overexpressed with a *MUTYH* expression plasmid in resistant cells or ablated using siRNA in parental cells, and the cell proliferation in response to

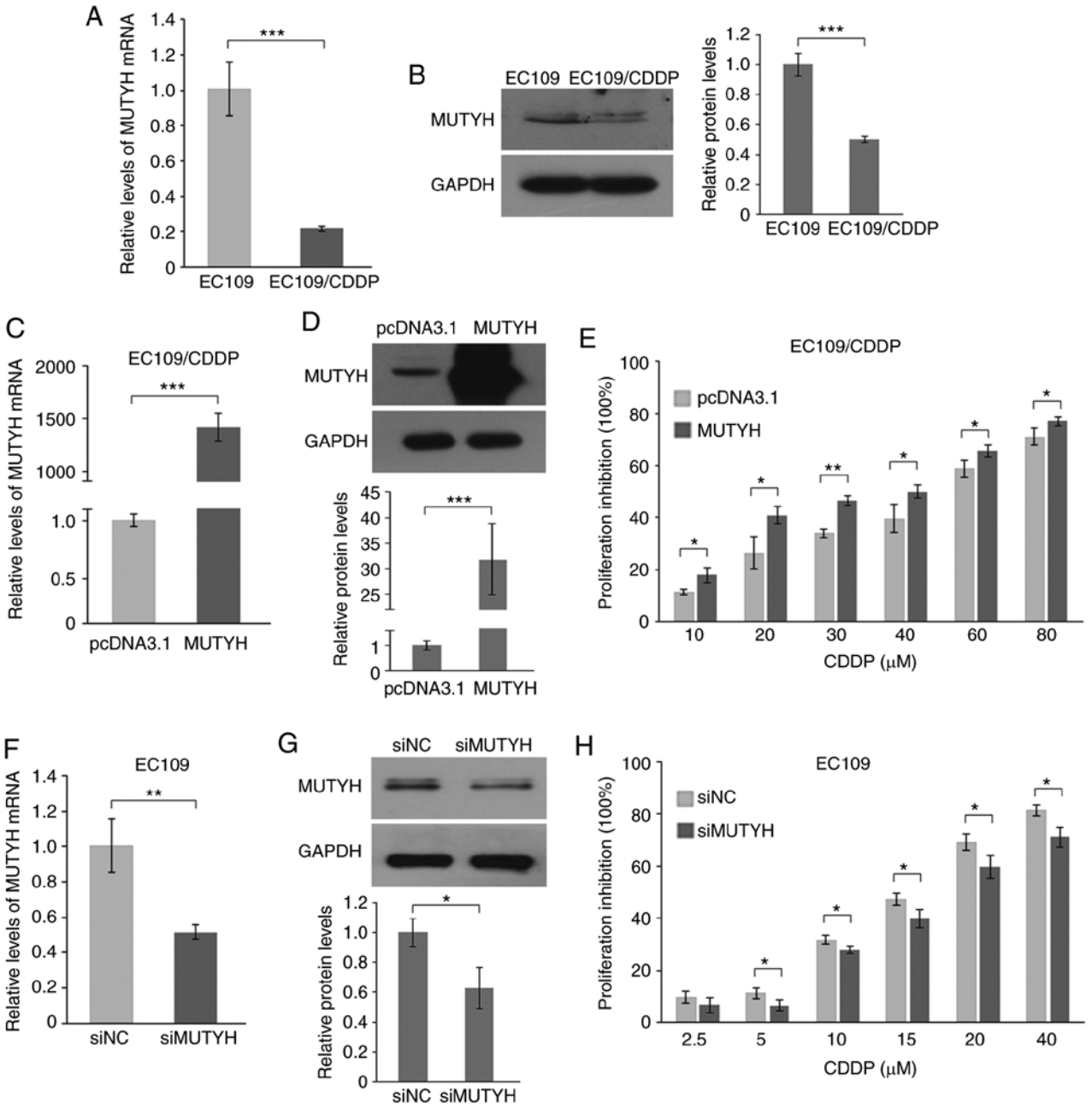

Figure 2. Association of *MUTYH* downregulation and CDDP resistance. (A) RT-qPCR analysis of mRNA expression of *MUTYH* in EC109 and EC109/CDDP cells. (B) Protein expression of *MUTYH* in EC109 and EC109/CDDP cells as detected by western blot assay. GAPDH served as the loading control. Protein amount was quantified by densitometry of X-ray film. In A and B, the results are the mean ± SD of three independent experiments. ***P<0.001 vs. the EC109 cells, respectively. (C-E) *MUTYH* overexpression increased the sensitivity of EC109/CDDP cells to CDDP. After 48 h transfection of the *MUTYH* expression vector, the overexpression efficiency was examined using RT-qPCR (C) and western blot analysis. (D) Protein amount was quantified by densitometry of X-ray film. Inhibition of proliferation was assessed by the MTT assay. (E) Cells transfected with empty vector pcDNA3.1 served as the negative control. Results are the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. the EC109/CDDP cells transfected with pcDNA3.1, respectively. (F-H) Silencing of *MUTYH* expression by siRNA (siMUTYH) decreased cytotoxicity induced by CDDP in EC109 cells. Cells were transfected with siRNA of *MUTYH* (siMUTYH) or with a negative control siRNA (siNC). The silencing efficiency was examined using RT-qPCR (F) and western blot analysis. (G) Protein amount was quantified by densitometry of X-ray film. Inhibition of proliferation was assessed by the MTT assay. (H) Results are the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. the EC109 cells transfected with siNC, respectively. MTUYH, MutY homolog; CDDP, cisplatin.

CDDP was determined. The present results demonstrated that the *MUTYH* expression level was significantly enhanced by transfecting cells with a *MUTYH* expression plasmid (Fig. 2C and D), and ectopic expression of MUTYH significantly re-sensitized resistant cells to CDDP treatment at concentrations of 10-80 μM for 48 h (Fig. 2E). Conversely, it was observed that the *MUTYH*-targeted siRNA reduced the expression of *MUTYH* from the basal level in sensitive EC109 cells (Fig. 2F and G), and the result of the cell viability assay demonstrated that knockdown of *MUTYH* in sensitive EC109 cells resulted in an attenuated response to CDDP (Fig. 2H). Therefore, these data demonstrated that downregulation of *MUTYH*, at least in part, contributes to the CDDP-resistant phenotype.

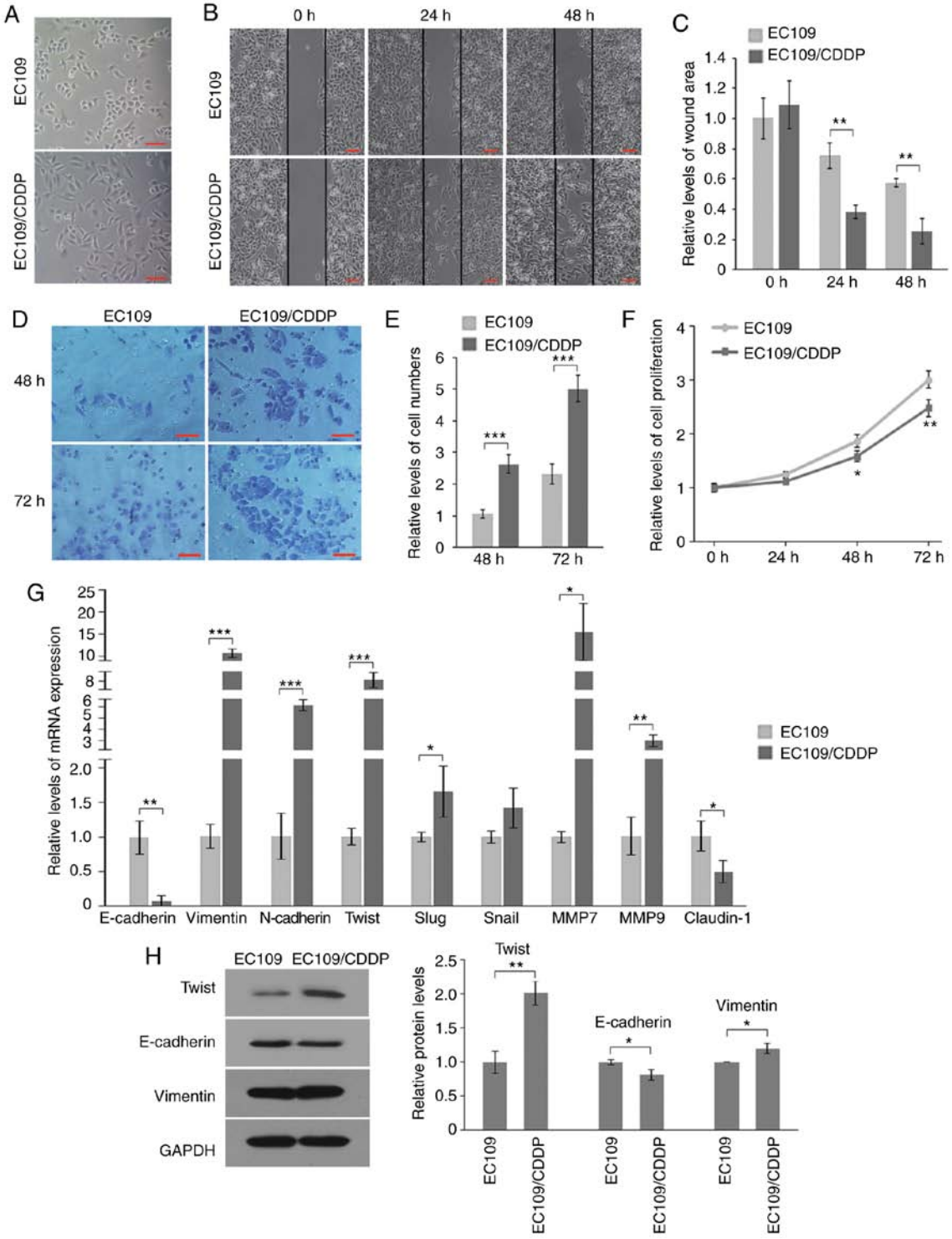

Figure 3. Resistant cells display an EMT phenotype. (A) Morphology of EC109 and EC109/CDDP cells (scale bar, 100 μm). (B and C) Representative images (B) and quantification analysis (C) of the wound-healing assay of EC109 and EC109/CDDP cells (scale bar, 100 μm). (D and E) Representative images (D) and quantification analysis (E) of invasive behavior of EC109 and EC109/CDDP cells (scale bar, 100 μm). (F) Cell proliferation of EC109 and EC109/CDDP cells was determined by the MTT assay after 24, 48 or 72 h. (G and H) RT-qPCR (G) and western blotting (H) analysis of mRNA and protein levels of various EMT-associated markers, respectively. Protein quantification for Twist, E-cadherin and vimentin was shown. In C, E-H: *P<0.05, **P<0.01 and ***P<0.001 vs. the EC109 cells, respectively. EMT, epithelial-mesenchymal transition; CDDP, cisplatin.

CDDP-resistant ESCC cells exhibit an EMT-like phenotypic change. Based on the significant effect of *MUTYH* on CDDP-resistance, the signaling mechanisms underlying *MUTYH*-mediated CDDP resistance in resistant cells were investigating. By comparing the differential phenotype between the resistant and parental cells, it was observed that EMT was markedly enhanced in resistant cells, which may be associated with *MUTYH* downregulation. EMT plays an important role in various cancer-associated biological events, which can contribute to invasion, metastasis as well as drug resistance of cancer (22,23). As shown in Fig. 3A, in contrast to parental cells, EC109/CDDP cells displayed morphological

characteristics of EMT, as observed by the transition from cobblestone-like cells into slender spindle-shaped cells with increased detachment.

Enhanced capacities of migration and invasion are common along with resistance acquisition in cancer cells. Wound healing assays showed that EC109/CDDP cells displayed significantly higher migratory potential, as demonstrated by more rapid and complete wound healing than parental cells (Fig. 3B). Wound areas in parental cells were ~2- and ~2.5-fold at 24 and 48 h, respectively, as large as those in resistant cells (Fig. 3C). Additionally, Transwell invasion analysis revealed that the invasiveness through Matrigel was also significantly increased in the resistant cells, 2-3-fold compared with that noted in the parental cells (Fig. 3D and E). To eliminate the interference of cell proliferation on migration and invasion, proliferation rates of the two cell lines were also compared. The present results showed that the proliferation rate of resistant cells decreased ~13, ~28 and ~52% at 24, 48 and 72 h, respectively, compared with the parental cells (Fig. 3F). Therefore, the enhanced wound healing and invasiveness through Matrigel of the EC109/CDDP cell line were not caused by proliferation, which further demonstrated its increased migratory and invasive capacities.

Moreover, the expressions of various genes associated with migration and invasion in both cells were detected. The results of RT-qPCR showed that epithelial marker E-cadherin (encoded by *CDH1* gene) was significantly downregulated (~14-fold) while mesenchymal markers N-cadherin (encoded by *CDH2* gene) and vimentin (encoded by *VIM* gene) were significantly upregulated (~10-fold and ~6-fold, respectively) in the resistant cells compared with levels in the parental cells (Fig. 3G). Notably, Twist (encoded by *TWIST1* gene), a key transcription factor of EMT that transcriptionally regulates the expression of E-cadherin, N-cadherin and vimentin, was significantly enhanced in the EC109/CDDP cells, ~8-fold increase in resistant cells compared with parental cells (Fig. 3G). In contrast, the upregulation of Slug (encoded by *SNAI2* gene) and Snail (encoded by *SNAI1* gene), other transcription factors of EMT, was only ~1.6-fold and 1.4-fold, respectively (Fig. 3G), much lower than Twist (~8-fold). Expression changes in Twist, E-cadherin and vimentin were further validated; the protein levels were similar, as determined by western blotting (Fig. 3H). In addition, matrix metalloproteinase (MMP)7 and MMP9 (encoded by *MMP7* and *MMP9* genes, respectively), two core MMPs, are positively correlated with invasion and were also markedly upregulated in CDDP-resistant cells (Fig. 3G). Expression of Claudin1 (encoded by *CLDN1* gene), another important epithelial-related factor, was also markedly decreased in CDDP-resistant cells (Fig. 3G). Taken together, these data demonstrated an EMT phenotypic conversion in CDDP-resistant EC109/CDDP cells, which was largely in a Twist-dependent manner.

Downregulation of MUTYH contributes to EMT via enhancement of Twist in resistant ESCC cells. Subsequently, it was further assessed whether there was an association between *MUTYH* downregulation and induction of EMT in resistant cells. The functional involvement of *MUTYH* in EMT was studied by overexpressing *MUTYH* in resistant cells. As shown in Fig. 4A, RT-qPCR results showed that ectopic expression of *MUTYH* prominently suppressed the mRNA level of Twist in EC109/CDDP cells by ~5-fold, suggesting a role of *MUTYH* in the transcriptional regulation of Twist. Correspondingly, cells transfected with the *MUTYH* expression plasmid demonstrated a significant induction of E-cadherin (~1.4-fold) and Claudin-1 (~2.5-fold), and a pronounced suppression of N-cadherin (~5-fold), vimentin (~5-fold), MMP7 (~3-fold) and MMP9 (~17-fold). Similar to the mRNA levels, restoration of *MUTYH* expression in resistant cells significantly elevated the protein level of E-cadherin and caused a reduction in the protein expression of vimentin and Twist to different levels (Fig. 4B).

Furthermore, it was analyzed whether knockdown of *MUTYH* led to induction of EMT in the parental cells. As shown in Fig. 4C, following *MUTYH* knockdown, the parental cells presented an EMT phenotypic conversion as demonstrated by induction of Twist, N-cadherin, vimentin, MMP7 and MMP9, as well as the inhibition of E-cadherin and Claudin-1. Concordantly, the expression changes of these genes in response to depletion of *MUTYH* were further confirmed by their protein levels, as observed in Fig. 4D. Therefore, these data suggested that downregulation of *MUTYH* contributed to induction of EMT by enhancing Twist and subsequently regulating its downstream targets, including E-cadherin, N-cadherin and vimentin in CDDP-resistant EC109/CDDP cells.

Downregulation of MUTYH blocks Twist degradation via reduced ROS and proteasome activity in resistant cells. Due to the essential role of Twist in EMT mediated by downregulation of *MUTYH*, we aimed to ascertain whether *MUTYH* affects the degradation of Twist in resistant cells. As *MUTYH* is associated with ROS and previous studies have found that ROS exert a regulatory effect on proteasome activity (24,25), the present study aimed to investigate whether *MUTYH* affects the proteasomal degradation of Twist, which also contributed to an increase in Twist in EC109/CDDP cells. The results in Fig. 5A and B demonstrated lower levels of ROS in resistant cells compared with parental cells. Additionally, a lower level of proteasome activity was identified, as demonstrated by reduced ChT-L and PGPH activities (Fig. 5C) as well as increased ubiquitinated proteins (Fig. 5D) in EC109/CDDP cells, suggesting that a blockade of proteasomal degradation of Twist occurred in resistant cells.

The effect of *MUTYH* on ROS and proteasome activity was additionally investigated. As shown in Fig. 6A and B, ectopic expression of *MUTYH* in resistant cells led to an increase in the level of ROS. Furthermore, as a consequence of *MUTYH* overexpression, the resistant cells demonstrated downregulation of ubiquitinated proteins, suggesting that *MUTYH* suppression attenuated proteasomal degradation of proteins, including Twist (Fig. 6C).

To determine whether *MUTYH* is involved in EMT via modulation of ROS, NAC, an antioxidant agent, was used. As shown in Fig. 6D, NAC significantly reduced the amount of ROS induced by overexpression of *MUTYH*. Furthermore, following the blocking of ROS generation by NAC, decreases in ubiquitinated proteins (Fig. 6E) and Twist (Fig. 6F) as well as changes of EMT markers (Fig. 6F) in response to overexpression of *MUTYH*, were predominantly rescued under conditions where ROS was blocked by NAC in EC109/CDDP cells. Correspondingly, ectopic expression of *MUTYH* led to

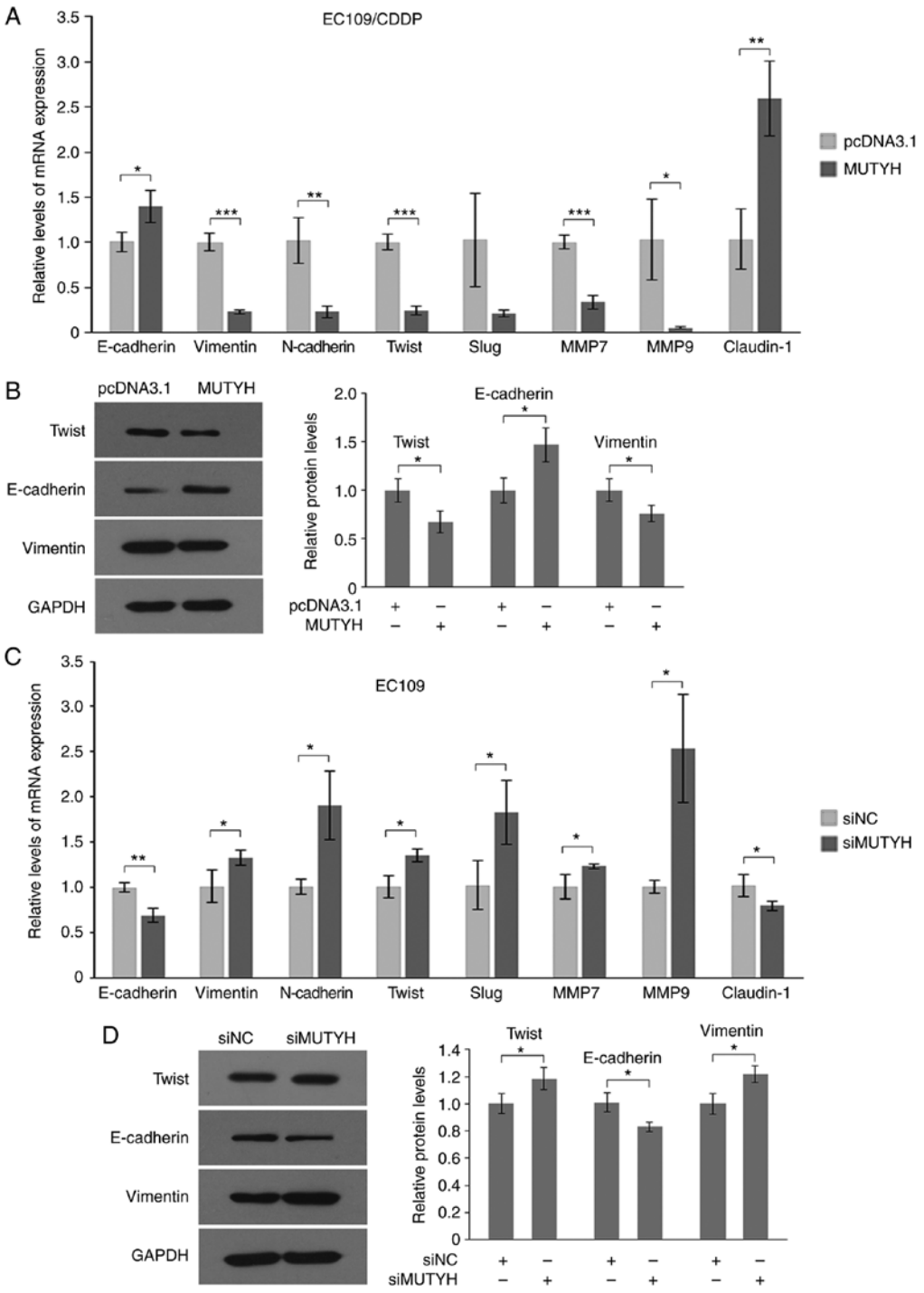

Figure 4. *MUTYH* downregulation contributes to promotion of Twist expression and EMT. (A and B) Effect of *MUTYH* overexpression (*MUTYH*) on Twist and other EMT-associated markers in EC109/CDDP cells. RT-qPCR (A) and western blot analysis (B) of mRNA and protein levels, respectively. Protein quantification for Twist, E-cadherin and vimentin is shown. Results are the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. the EC109/CDDP cells transfected with pcDNA3.1, respectively. (C and D) Effect of *MUTYH* knockdown on Twist and other EMT-associated markers in EC109 cells. RT-qPCR (C) and western blot analysis (D) of mRNA and protein levels, respectively. Protein quantification for Twist, E-cadherin and vimentin is shown. Results are the mean ± SD of three independent experiments. *P<0.05 and **P<0.01 vs. the EC109 cells transfected with siNC, respectively. MTUYH, MutY homolog; CDDP, cisplatin; siMUTYH, *MUTYH* siRNA; siNC, negative control siRNA.

fewer morphological characteristics of EMT in resistant cells while this reversing effect was markedly abolished when scavenging of ROS occurred from the addition of NAC (Fig. 6G). Therefore, the present data demonstrated that downregulation of *MUTYH* was able to reduce the degradation of Twist through ROS, which could function together with the increase in Twist transcription to accelerate EMT.

Discussion

MutY homolog (*MTUYH*) was initially thought to play a prominent role in the BER system of oxidized DNA bases in response to reactive oxygen species (ROS). Notably, recent studies have identified that *MTUYH* is associated with tumorigenesis or development, including in esophageal

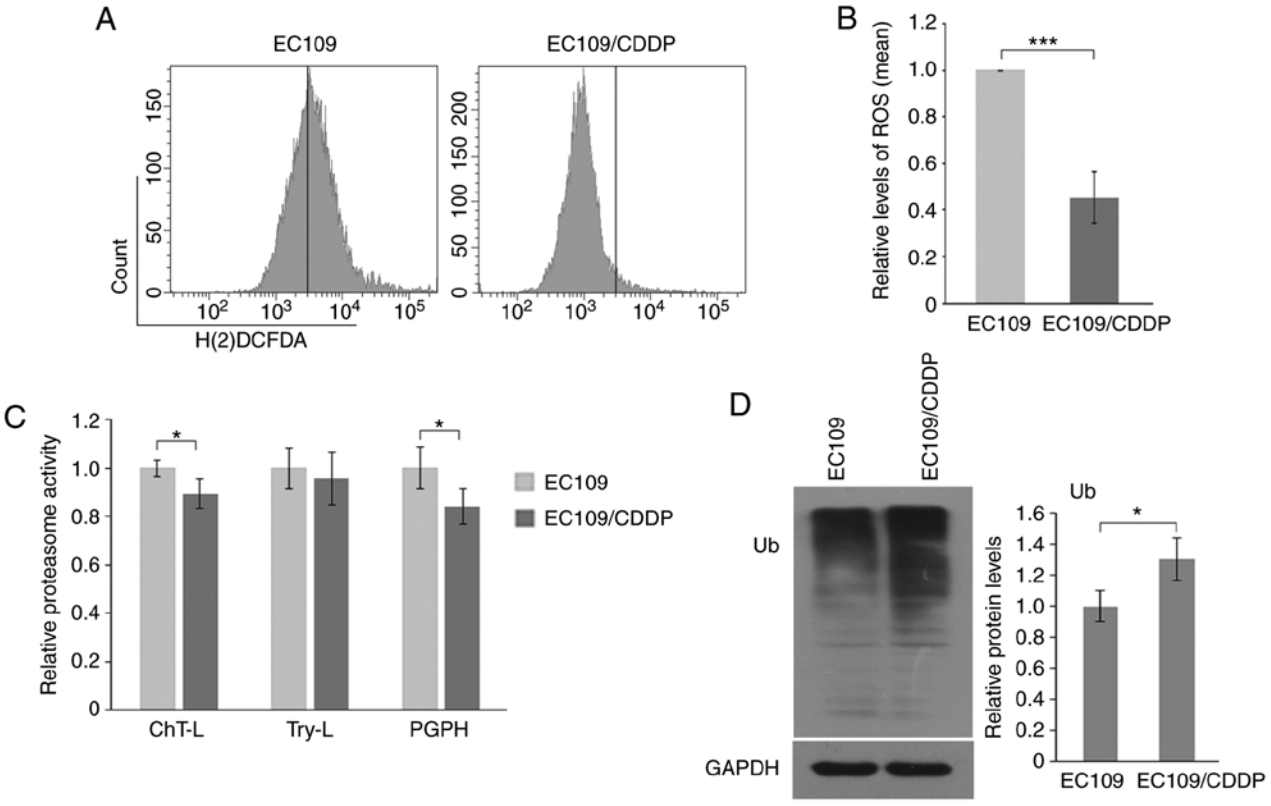

Figure 5. Resistant EC109/CDDP cells show a reduced ROS level and proteasome activity. (A) The ROS generation of EC109 and EC109/CDDP cells was detected by flow cytometry. (B) Statistical analysis of the relative ratio of ROS formation. (C) Proteasome activities in whole cell lysates were measured using fluorescent substrates. (D) Western blotting analysis of polyubiquitinated proteins in EC109 and EC109/CDDP cells. GAPDH served as the loading control. Protein amount was quantified by densitometry of X-ray film. In B-D, the results are the mean ± SD of three independent experiments. *P<0.05 and ***P<0.001 vs. the EC109 cells, respectively. CDDP, cisplatin; ROS, reactive oxygen species.

carcinoma. In the present study, the exact role of MTUYH in the cisplatin (CDDP) resistance of esophageal squamous cell carcinoma (ESCC) was further investigated. Based on the establishment of a CDDP-resistant cell model EC109/CDDP, *MUTYH* was initially identified to be downregulated in resistant ESCC cells. It was identified for the first time, to the best of our knowledge, that downregulation of *MUTYH* contributed to CDDP resistance of ESCC. Further experiments revealed that downregulation of *MUTYH* stimulated EMT by enhancing both mRNA and protein levels of Twist in CDDP-resistant EC109/CDDP cells; moreover, downregulation of *MUTYH* contributed to low ROS levels and subsequently inhibited proteasome activity, which in turn led to suppression of Twist degradation. The present data suggested that, as a consequence of these events, a low level of *MUTYH* might, at least partially, confer Twist-mediated EMT and drug resistance.

Although CDDP alone or in combination with other chemotherapeutic drugs is recommended as an effective front-line strategy to treat advanced ESCC, occurrence of resistance to CDDP is the main challenge in chemotherapy. The CDDP-resistance mechanisms are multi-factorial and may be unique to different types of cancer (4,5). Further understanding concerning the CDDP-resistance mechanisms at a molecular level remains a critical goal for cancer therapy. In the present study, a CDDP-resistant ESCC cell line EC109/CDDP was established from parental EC109 cells. The CDDP-resistant cell line was characterized with decreased sensitivity to CDDP and increased EMT phenotypes compared with parental cells. Notably, a significant downregulation of *MUTYH* was observed in resistant cells. The causal association between *MUTYH* downregulation and CDDP-resistance acquisition was demonstrated by transfecting an expression vector or RNA interference. Overexpression of *MUTYH* restored CDDP sensitivity, while *MUTYH* inhibition made cells resistant to CDDP. To the best of our knowledge, this is the first study to demonstrate that downregulation of *MUTYH* contributed to acquisition of CDDP-resistance in ESCC.

Epithelial-mesenchymal transition (EMT) has been now widely recognized as a vital process that contributes to cancer progression and drug resistance (22,23). It has been identified that EMT is executed by a relatively small number of master regulators, mainly including the transcription factors Twist, Snail and Slug (22,23). Accumulating evidence has demonstrated that EMT activation confers multi-drug-resistance to cancer cells (22,23,26). EMT-induced multidrug resistance involves various mechanisms, including a lower level of proliferation, increased expression of anti-apoptotic proteins as well as ATP-binding cassette transporters (23,26,27). In the present study, EC109/CDDP cells underwent EMT, as characterized by the changes in cell morphology and the expression of EMT markers, displaying upregulation of two mesenchymal markers, N-cadherin and Vimentin, and reduction of an epithelial marker E-cadherin. Notably, the master regulator of EMT, Twist, also presented a marked increase in expression in CDDP-resistant cells, demonstrating that the

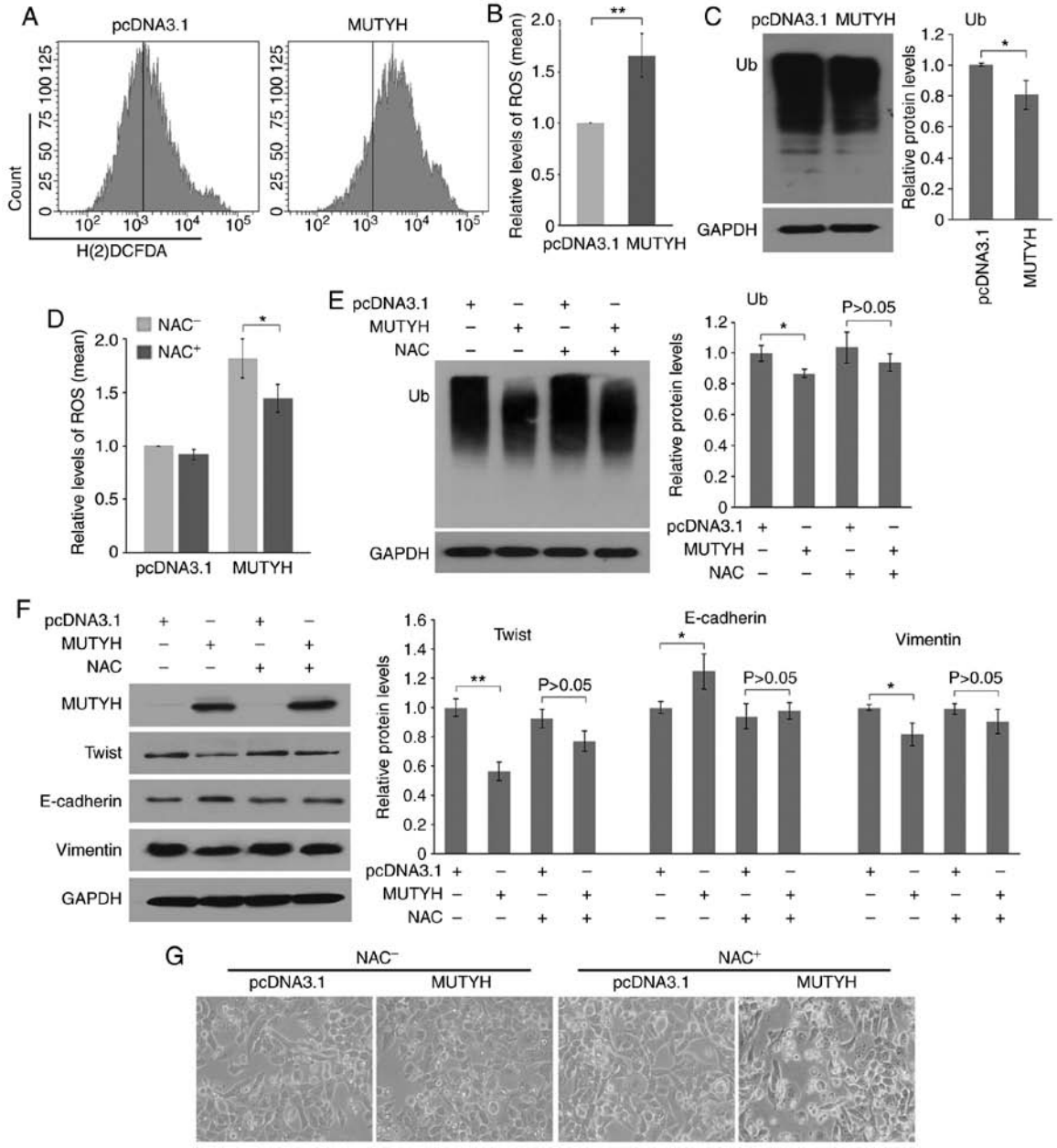

Figure 6. Downregulation of *MUTYH* attenuates Twist degradation via ROS in resistant cells. (A and B) *MUTYH* overexpression (*MUTYH*) elevated ROS generation in EC109/CDDP cells as detected by flow cytometry. (C) Western blot analysis of polyubiquitinated proteins (Ub) in response to *MUTYH* overexpression in EC109/CDDP cells. GAPDH served as the loading control. Protein quantification for Ub is shown. In B and C, the results are the mean ± SD of three independent experiments. *P<0.05, **P<0.01 vs. the EC109/CDDP cells transfected with pcDNA3.1, respectively. (D) The antioxidant NAC reduced ROS generation induced by *MUTYH* overexpression in EC109/CDDP cells. Results are the mean ± SD of three independent experiments. *P<0.05 vs. the NAC-untreated (NAC^{-}) EC109/CDDP cells transfected with *MUTYH*. (E) Addition of NAC significantly rescued downregulation of polyubiquitinated proteins induced by *MUTYH* overexpression in EC109/CDDP cells. (F and G) Impact of NAC on EMT under conditions where *MUTYH* was overexpressed in resistant cells. Western blot analysis of Twist, E-cadherin and vimentin. (F) Morphologic analysis. (G) In E and F, protein quantification for Ub, Twist, E-cadherin and vimentin is shown. Results are the mean ± SD of three independent experiments. *P<0.05, **P<0.01 vs. the EC109/CDDP cells transfected with pcDNA3.1, respectively. P>0.05 vs. the EC109/CDDP cells simultaneously treated with NAC and pcDNA3.1 transfection. *MTUYH*, MutY homolog; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; CDDP, cisplatin; EMT, epithelial-mesenchymal transition.

occurrence of EMT in EC109/CDDP cells was largely in a Twist-dependent manner. Based on these events mentioned, it was hypothesized that downregulation of *MUTYH* was associated with Twist-mediated EMT and in turn conferred CDDP resistance to ESCC. In support of this hypothesis, *MUTYH* suppression enhanced the Twist level and stimulated cells to acquire a higher potential for EMT in parental cells; conversely, restoring *MUTYH* in CDDP-resistant cells reduced the Twist level and inhibited the EMT process.

Moreover, in addition to transcriptionally regulating Twist, it was also observed that *MUTYH* affected the degradation of Twist. Twist is a labile protein, rapidly polyubiquitinated and degraded via the ubiquitin-proteasome pathway (UPP) (28). UPP is regarded as the primary cytosolic proteolytic machinery for protein degradation (29,30). In the process of UPP, multiple ubiquitin molecules firstly tag substrate proteins, which are then degraded by the 26S proteasome. Therefore, the UPP is a major pathway responsible for the protein quality control

mechanism (29,30). Changes in UPP capacity of degrading proteins can occur in response to cellular environmental insults, including oxidative stress (24,25). It has been demonstrated that exposure to various forms of oxidative stress leads to increased intracellular protein degradation, which results from the elevation of the rate of ubiquitin conjugation by increasing substrate availability and enhancement of activities of ubiquitin-conjugating enzymes (24,25,31), thereby elevating ubiquitination and degradation capacities. In the present study, it was determined that compared with parental cells, resistant E109/CDDP cells presented a lower level of ROS and reduced activity of UPP, which led to the hypothesis that *MUTYH* modulates ROS and UPP. The present data demonstrated that *MUTYH* downregulation contributed to reduced ROS and UPP activity, thereby leading to an inhibition of Twist degradation. The addition of an antioxidant agent NAC significantly abolished the effect of *MUTYH* on UPP and the EMT phenotype, further validating this hypothesis. Nevertheless, the underlying mechanisms of these signaling pathways require further investigation.

In summary, the present study provided novel insight into the role of *MUTYH* in the acquisition of resistance to CDDP in ESCC cells and suggested a novel mechanism in that downregulation of *MUTYH* conferred EMT-mediated drug resistance via an increased level of Twist. Regulation of the signaling response may provide a practical therapeutic strategy to strengthen the effect of CDDP-based chemotherapy in ESCC cells.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

CP, YL and YG designed the research. YG, YL, YJ, SW, DG, LZ and ZL performed the experiments. FK, NL and SW contributed new reagents and analytic tools in conducting the experiments. YG and YJ analyzed the data. YG and YL wrote the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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