Knockdown of Merm1/Wbscr22 attenuates sensitivity of H460 non-small cell lung cancer cells to SN-38 and 5-FU without alteration to p53 expression levels

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Abstract. Merm1/Wbscr22 is a novel metastasis promoter that has been shown to be involved in tumor metastasis, viability and apoptosis. To the best of our knowledge, there are currently no studies suggesting the possible correlation between the expression of Merm1/Wbscr22 in tumor cells and chemosensitivity to antitumor agents. In the present study, two human non-small cell lung cancer cell lines, H1299 and H460, were used to investigate whether Merm1/Wbscr22 affects chemosensitivity to antitumor agents, including cisplatin (CDDP), doxorubicin (ADM), paclitaxel (PTX), mitomycin (MMC), 7-Ethyl-10-hydroxycamptothecin (SN-38; the active metabolite of camptothecin) and 5-fluorouracil (5-FU). Merm1/Wbscr22 knockdown cell lines (H1299-shRNA and H460-shRNA) and negative control cell lines (H1299-NC and H460-NC) were established by stable transfection, and the efficiency of Merm1/Wbscr22 knockdown was confirmed by western blotting, immunofluorescence microscopy and quantitative polymerase chain reaction. The results demonstrated that shRNA-mediated knockdown of Merm1/Wbscr22 did not affect cell proliferation *in vitro* and *in vivo*. The H460 cells harboring wild type *p53* were markedly more sensitive to all six antitumor agents as compared with the *p53*-null H1299 cells. Downregulation of Merm1/Wbscr22 did not affect H1299 sensitivity to any of the six antitumor agents, whereas attenuated H460 sensitivity to SN-38 and 5-FU, without significant alteration in p53 at both mRNA and protein levels, was identified. The reduced H460 sensitivity to SN-38 was further confirmed *in vivo*. SN-38 demonstrated significant tumor growth inhibitory activity in both H460 and H460-NC tumor xenograft models, but only marginally suppressed the H460-shRNA xenograft tumor growth. Furthermore, CDDP (4, 10, 15 μg/ml)-resistant human non-small lung cancer cells A549 (A549-CDDPr-4, 10, 15) expressed significant amounts of Merm1/Wbscr22 protein, as compared with the parental A549 cells. In conclusion, shRNA-mediated knockdown of Merm1/Wbscr22 attenuates H460 sensitivity to SN-38 and 5-FU, suggesting Merm1/Wbscr22 is involved in chemosensitivity to SN-38 and 5-FU in H460 cells. No direct correlation between the p53 expression level and altered chemosensitivity was identified.

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Introduction

Human Merm1/Wbscr22 is located at chromosome 7q11.23. It has been identified as one of 26 genes deleted in Williams-Beuren syndrome (WBS), which is characterized by dysmorphic facial features, congenital heart and vascular disease, infantile hypercalcemi, hypertension, unique cognitive and personality profiles (1-3). Human Merm1/Wbscr22 mRNA is ubiquitously expressed in all tissues, particularly in the testis (4), heart and skeletal muscle (5) and the protein encoded by Merm1/Wbscr22 is markedly expressed in the heart, skeletal muscle and kidney (5). The protein contains a nuclear localization signal and a common S-adenosyl-L-methionine binding motif that is evolutionarily conserved in methyltransferases (4), suggesting it may function in DNA methylation (6). However, Merm1/Wbscr22 does not possess a catalytic center (Pro-Cys motif) and DNA-binding motif that is characteristic of DNA methyltransferases (7), therefore, it may be involved in the mediation of histone methylation (8). The specific cellular function of Merm1/Wbscr22 remains unknown.

Nakazawa *et al* (8) reported that Merm1/Wbscr22 is overexpressed in invasive breast cancer. Ectopic expression of Merm1/Wbscr22 in non-metastatic cells was shown to enhances metastasis formation by suppressing Zac1/p53-dependent apoptosis, but did not affect cell growth and motility. Tiedemann *et al* (9) reported that Merm1/Wbscr22 is necessary for the survival of KMS11 and 8226 multiple myeloma tumor cells. In addition, Merm1/Wbscr22 has been shown to be upregulated in both primary plasma cells and primary multiple

myeloma tumor cells, and downregulation of Merm1/Wbscr22 was shown to be more detrimental to multiple myeloma cells than A549 or HEK293 cells, implicating its function in plasma cell biology.

Although previous studies have indicated that human Merm1/Wbscr22 is involved in tumor metastasis, viability and apoptosis, there have been no reports suggesting the possible correlation between the expression of Merm1/Wbscr22 in tumor cells and chemosensitivity to antitumor agents. Chemotherapy is the most widely used approach for clinical tumor treatment, but its effectiveness is limited by the development of resistance. Various and complicated mechanisms are involved in chemoresistance, including overexpressed drug resistance-associated proteins, altered drug targets, decreased drug accumulation and escape from cell cycle checkpoints. Previous evidence has indicated that tumor angiogenesis and stem cell development are also associated with chemoresistance (10). The present study investigated whether Merm1/Wbscr22 affects the chemosensitivity of two non-small cell lung cancer cell lines, H1299 and H460, to antitumor agents which are widely used in chemotherapy, including cisplatin (CDDP), doxorubicin (ADM), paclitaxel (PTX), mitomycin (MMC), 7-Ethyl-10-hydroxycamptothecin (SN-38; the active metabolite of camptothecin) and 5-fluorouracil (5-FU). Knockdown of Merm1/Wbscr22 (H1299-shRNA and H460-shRNA) and negative control cell lines (H1299-NC and H460-NC) were produced by stable transfection. The efficiency of Merm1/Wbscr22 knockdown was confirmed by western blotting, immunofluorescence microscopy and quantitative polymerase chain reaction (qPCR). The effects of transfection on tumor cell growth *in vitro* and *in vivo* were observed. The changes in the half maximal inhibitory concentration (IC_{50}) values *in vitro* and the tumor growth inhibitory activity *in vivo* were compared between Merm1/Wbscr22 knockdown tumor cells and parental cells. The changes in p53 expression at both mRNA and protein levels were compared between Merm1/Wbscr22 knockdown cells and parental cells.

Materials and methods

Cell lines. H1299 and H460 human non-small cell lung cancer cell lines were purchased from the Shanghai Institute of Biological Sciences (Shanghai, China). The cell lines grew as monolayers in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum (Gibco, Grand Island, NY, USA) in a 10% CO_2, 90% air atmosphere.

Antitumor agents. CDDP, ADM, PTX, MMC, SN-38 and 5-FU were purchased from Sigma-Aldrich (St. Louis, MO, USA). All of the antitumor agents were dissolved as stocks and stored at -20˚C. SN-38 and PTX were dissolved at 50 and 5 mmol/l in dimethyl sulfoxide (DMSO), respectively. CDDP and ADM were dissolved at 50 and 200 μmol/l in 0.9% saline, respectively. 5-FU was dissolved at 5 mmol/l in phosphate-buffered saline (PBS). All of the agent stocks were diluted at a series of concentrations, as indicated, in serum-free DMEM immediately prior to use in the *in vitro* experiments. The final concentration of DMSO in DMEM did not exceed 0.1%. The concentration of all of the solvents in serum-free DMEM had no inhibitory effect on cell growth. For the *in vivo* studies, SN-38 was dissolved at 40 g/l in DMSO and then further diluted at 2 g/l with 0.9% saline immediately prior to use. The final concentration of DMSO in 0.9% saline was 5%. DMSO, and the same concentration in 0.9% saline was used as the solvent control.

Construction and purification of shRNA plasmids. The shRNAs were constructed in pSIREN-RetroQ vectors (Clontech Laboratories, Mountain View, CA, USA) according to the manufacturer's instructions. The shRNA sequence (8) targeting human Merm1/Wbscr22 was 5'-GCCCTGTTACCTGCTGGAT-3'; the negative control shRNA annealed oligonucleotide was provided by Clontech Laboratories. Following transformation in pSIREN-RetroQ vectors containing target or negative shRNA, JM109 cells (Promega, Madison, WI, USA) were cultured in Luria-Bertani medium with 100 μg/ml ampicillin (Sigma-Aldrich). Bacteria in the growth phase were harvested and the plasmids were purified using the Wizard PureFection plasmid DNA purification system (Promega) according to the manufacturer's instructions.

Stable transfections. H1299 and H460 cells were stably transfected with pSIREN-RetroQ vectors containing the human Merm1/Wbscr22 shRNA targeting sequence (H1299-shRNA, H460-shRNA) or the negative control shRNA (H1299-NC, H460-NC).

Stable transfections were performed using Lipofectamine™ 2000 (Gibco) according to the manufacturer's instructions. Following transfection for 24 h, the cells were detached by trypsinization and then reseeded into 6-well plates, at a density of 3,000 cells per well. The transfected cells were cultured in fresh growth medium containing 1 μg/ml of puromycin (Sigma-Aldrich) for 7-14 days until cell monoclones formed. A total of 5-10 cell monoclones was selected and further cultured in medium with 1 μg/ml puromycin for two weeks. The cellular expression of Merm1/Wbscr22 was detected by western blotting, qPCR and immunofluorescence. The established cell lines were maintained under puromycin-free conditions for at least two weeks prior to use, to avoid any effects of the puromycin.

Western blotting. Total protein was extracted from cultured cells with lysis buffer containing 2% NP-40, 0.2% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 50 mmol/l Tris (pH 8.0), 150 mmol/l NaCl and 10 mmol/l phenylmethylsulfonyl fluoride. The protein content was measured using the BCA kit (Byeotime Biotechnology, Nantong, China). Aliquots of 20 μg total protein were boiled for 3 min in loading buffer and then separated by 12% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Pall, Corp., Pensacola, FL, USA), blocked with 5% non-fat milk in Tris-buffered saline containing 0.5% Tween-20 (TBST), and then incubated with anti-human Merm1/Wbscr22 (GeneTex, Irvine, CA, USA) or anti-actin (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. Following five washes with TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Inc.). Following two washes with TBST, the labeled proteins were visualized using enhanced chemiluminescence (Santa Cruz Biotechnology, Inc., Santa

Cruz, CA, USA) on enhanced chemiluminescence films (Eastman Kodak, Co., Rochester, NY, USA)..

Immunofluorescence. The cells grown in glass-bottom dishes were fixed with 4% formaldehyde in PBS for 10 min, followed by 0.5% Triton X-100 for 20 min. Following washing, the cells were treated with 5% bovine serum albumin in PBS for 60 min and subsequently treated with monoclonal anti-human Merm1/Wbscr22 antibody (1:100 dilution) at 4˚C overnight. Following three washes, the cells were incubated with Alexa Fluor® 488-conjugated donkey anti-rabbit secondary antibody (Invitrogen Life Technologies, Grand Island, NY, USA) at a final concentration of 10 μg/ml, for 90 min. F-actin was stained using Texas Red-phalloidin (Invitrogen Life Technologies) at a final concentration of 12 units/ml and nuclear DNA was stained using DAPI for 3 min. Images were captured using a confocal laser scanning microscope (Zeiss Lsm710; Carl Zeiss AG, Oberkochen, Germany). The excitation/emission waavelengths for Texas Red, Alexa Fluor 488 and DAPI were 591 nm/608 nm, 495 nm/519 nm and 340 nm/488 nm, respectively.

qPCR. Total RNA was isolated using TRIzol™ (Invitrogen Life Technologies) according to the manufacturer's instructions, and A260/280 and A260/230 ratios were measured using the Nanodrop 2000 (Thermo Scientific, Pittsburgh, PA, USA). The integrality of the total RNA was detected by 1% gel electrophoresis. First-strand cDNA synthesis was conducted using a Reverse Transcription System (Promega) and Oligo dT (Promega). qPCR was then conducted using SYBR® Green mastermix (Roche, Basel, Switzerland) in a 7500 Fast PCR instrument (Applied Biosystems, Carlsbad, CA, USA). First, the housekeeping gene stably expressed in cell lines was selected from six candidate housekeeping genes as referenced by three programs, geNorm (http://medgen.ugent.be/~jvdesomp/genorm/), NormFinder (http://moma.dk/normfinder-software) and RefFinder (http://www.leonxie.com/referencegene.php). According to the results of these three prgrams, *Rpl32* and *Actin* were selected as the most suitable reference genes for qPCR analysis in the H1299 and H460 cells, respectively. The target mRNA was quantified using the relative standard curve method. qPCR was performed with the following primers: human *Merm1*/*Wbscr22* forward, 5'-CATTTGATGGTTGCATCAGC-3'andhuman*Merm1*/*Wbscr22* reverse, 5'-CTTGGCAGGGTTTTCAGACT-3' (8); human *Rpl32* forward, 5'-CATCTCCTTCTCGGCATCA-3' and human *Rpl32* reverse, 5'-AACCCTGTTGTCAATGCCTC-3' (11); human *Actin* forward, 5'-CATCGAGCACGGCATCGTCA-3' and human *Actin* reverse, 5'-TAGCACAGCCTGGATAGCAAC-3' (12); human *p53* forward, 5'-TAACAGTTCCTGCATGGGCGGC-3' and human *p53* reverse, 5'-AGGACAGGCACAAACACGCAC C-3' (13).

MTT assay. Cells ($2x10^3$) in the logarithmic growth phase were seeded in 100 μl of DMEM containing 10% fetal calf serum in 96-well plates overnight at 37˚C. Serial dilutions of antitumor agents in 100 μl of serum-free DMEM were then added to quadruplicate wells. The cells were incubated for an additional 72 h. The viability of the cells was determined using an MTT assay according to a method as previously described (14). The IC_{50} was calculated using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The IC_{50} values were the means of at least three independent experiments.

In vivo tumor growth inhibition assay. The animal study was approved by the Zhejiang Experimental Animal Center, (Hangzhou, Zhejiang, China) under the project number: SCXK2008-0016, and the mice were maintained in accordance with the Institute Animal Ethical Committee guidelines approved by Zhejiang Academy of Medical Sciences (Hangzhou, Zhejiang, China). BALB/c nu/nu mice (female, 5-6 weeks) were housed for seven days prior to xenograft implantation. The animals were housed in laminar air-flow cabinets under pathogen-free conditions with a 14 h light/10 h dark schedule, and fed autoclaved standard chow and water *ad libitum*. H460, H460-NC and H460-shRNA cells ($3x10^6$ cells in 200 μl of serum-free DMEM) were subcutaneously injected into the right flank of mice, respectively. After the tumor volumes (TV) reached 100 to 300 mm^3 at day 8, H460, H460-NC and H460-shRNA tumor xenograft mice were randomized into two groups (control group and treatment group) with six animals for each group. The mice in the treatment groups were treated with SN-38 by intraperitoneal (i.p) injection on a schedule of two injections at a four-day interval, at a dose of 20 mg/kg per injection, and the mice in the control groups received solvent (5% DMSO in 0.9% saline). The TV was measured every other day during the treatment period (12 days). The TV was calculated using the formula: $\pi/6$ x (length x $width^2$), where length = longest diameter and width = diameter perpendicular to length. The mean tumor volume (MTV), relative mean tumor volume (RMTV) and inhibition rate (IR) were calculated. RMTV was calculated using the formula: MTV on day n (MTV_n)/MTV on day 0 (MTV_0). The IR was calculated using the formula: (1-RMTV in treatment group/RMTV in control group) x 100.

Statistical analysis. The results of the *in vitro* experiments are presented as the means ± standard deviation. The data were analyzed using the unpaired t-test and two-tailed t-test, and a $P<0.05$ was considered to indicate a statistically significant difference. The results of the *in vivo* tumor growth inhibition assay are presented as the means ± standard error of the mean. The data of MTV in xenograft models were analyzed using the repeated-measures analysis of variance and a $P<0.05$ was considered to indicate a statistically significant difference. The data of RMTV by the end of treatment in the animal models were analyzed using the Mann-Whitney test, and a $P<0.05$ was considered to indicate a statistically significant difference. All data were analyzed with GraphPad Prism 5.0 software (GraphPad Software, Inc.).

Results

Validation of Merm1/Wbscr22 knockdown. To confirm the efficiency of Merm1/Wbscr22 knockdown in H1299 and H460 cells, western blotting, immunofluorescence and qPCR were performed. As demonstrated in Fig. 1A, Merm1/Wbscr22 protein expression in H1299-shRNA and H460-shRNA cells was significantly decreased, as compared with either the parental or negative control cells. As demonstrated in Fig. 1B, the Merm1/Wbscr22

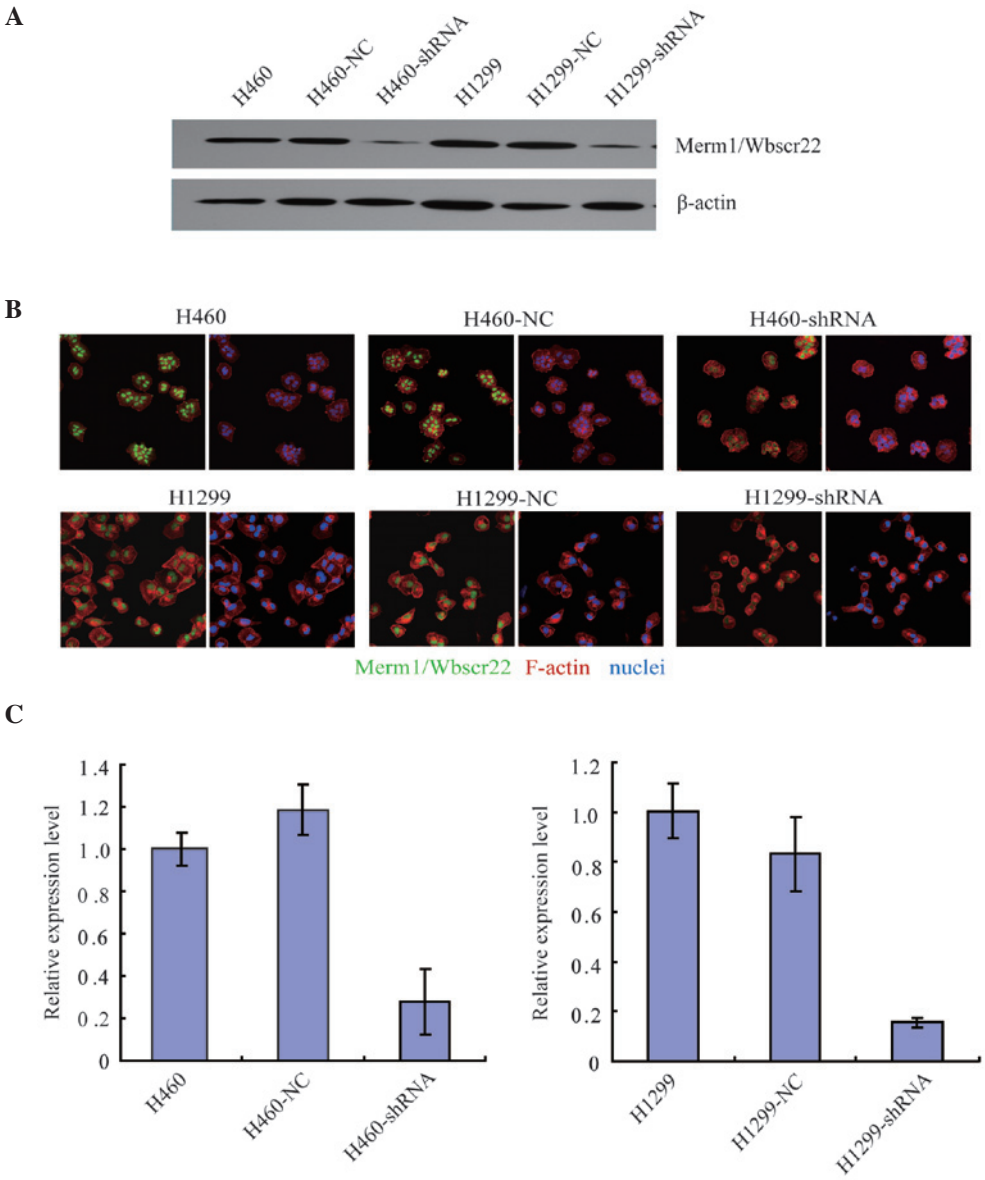

Figure 1. Analysis of Merm1/Wbscr22 protein and RNA expression following knockdown in H460 and H1299 cells. (A) Analysis of Merm1/Wbscr22 protein expression in H460 and H1299 cells by western blotting. (B) Immunofluorescence staining of Merm1/Wbscr22, nuclei and F-actin filaments in H460 and H1299 cells (x200). Merm1/Wbscr22, nuclei and F-actin filaments were visualized with Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody, DAPI and Texas Red-phalloidin, respectively. (C) The relative expression of human Merm1/Wbscr22 mRNA in H460 and H1299 cells was measured by quantitative polymerase chain reaction. The results are expressed as the means ± standard deviation. H460-shRNA and H1299-shRNA, H460 and H1299 cells transfected with human Merm1/Wbscr22 shRNA, respectively; H460-NC and H1299-NC, H460 and H1299 cells transfected with negative control shRNA, respectively.

proteins localized in nuclei were significantly lower in the H1299-shRNA and H460-shRNA cells than in either the parental or negative control cells, indicating Merm1/Wbscr22 protein was reduced significantly, consistent with the results of western blot analysis. Merm1/Wbscr22 mRNA in H1299-shRNA and H460-shRNA cells was also significantly decreased to 15 and 28% respectively, as compared with the parental cells (Fig. 1C). However, Merm1/Wbscr22 mRNA in H1299-NC and H460-NC cells was not significantly changed (P>0.05).

These results demonstrated that Merm1/Wbscr22 shRNA significantly decreased the level of Merm1/Wbscr22 mRNA in tumor cells, which consequently reduced the expression of Mem1/Wbscr22 protein, thus indicating that the cell lines were suitable for further studies.

Effects of Merm1/Wbscr22 knockdown on chemosensitivity to antitumor agents in vitro. It was first investigated whether the plasmid transfection affected the proliferation of tumor cells *in vitro*. For all of the tumor cells, including parental and transfected cells, the absorbance at 570 nm was ~0.8 (P>0.05), indicating that cell proliferation was not affected by either shRNA or vehicle, which is consistent with the literature (8).

It was subsequently examined whether vehicle transfection alone affected the IC_{50} values. The IC_{50} value for SN-38 in H1299 parental cells increased 3.5 times, as compared with the H1299-NC cells (Table IA). The IC_{50} value for MMC in H460-NC cells increased 2.8 times, as compared with the H460 parent cells (Table IB). These results indicated that the vehicle transfection affected the sensitivity to certain antitumor

agents. Therefore, the vehicle transfection-induced effects on the IC_{50} were subtracted when the effects of Merm1/Wbscr22 knockdown on IC_{50} were investigated.

In H1299 cells (Table IA), there were no significant changes in the IC_{50} values for the antitumor agents except SN-38. The IC_{50} for SN-38 was significantly lower in the H1299-shRNA and H1299-NC cells as compared with the H1299 parental cells, indicating that the vehicle transfection induced higher chemosensitivity to SN-38, rather than Merm1/Wbscr22 knockdown. Therefore, the chemosensitivity to the six tested antitumor agents in H1299 cells was not changed by knockdown of Merm1/Wbscr22.

In H460 cells (Table IB), the IC_{50} values for CDDP, ADM and PTX were marginally changed, but with no statistically significant differences. The IC_{50} values for MMC in H460-NC and H460-shRNA cells were increased, indicating it was induced by vehicle transfection. However, the IC_{50} value for 5-FU in H460-shRNA cells increased 2 times, as compared with either the H460 or H460-NC cells. Notably, the IC_{50} value for SN-38 in H460-shRNA cells increased 4 times, as compared with either the H460 or H460-NC cells. These results demonstrated the lowered chemosensitivity to 5-FU and SN-38 in H460-shRNA cells was induced by knockdown of Merm1/Wbscr22, rather than by vehicle transfection.

The expression of Merm1/Wbscr22 protein in human non-small lung cancer cells A549, cisplatin (4, 10, 15 μg/ml)-resistant A549 cells (A549-CDDPr-4, 10 and 15) was detected by western blot analysis. A549-CDDPr-4, 10 and 15 cells were established and maintained by growing A549 parental cells in the presence of 4, 10, 15 μg/ml of CDDP respectively. As demonstrated in Fig. 2, the expression of Merm1/Wbscr22 protein was minimally observed in A549 parental cells, however, the A549-CDDPr cells expressed significant amounts of Merm1/Wbscr22. The amount of protein did not increase with increasing resistance to CDDP.

Knockdown of Merm1/Wbscr22 attenuates H460 cell sensitivity to SN-38 in vivo. Due to the more significant change in IC_{50} for SN-38 than 5-FU in H460 cells, the tumor growth inhibitory activity of SN-38 in H460, H460-NC and H460-shRNA tumor xenograft models was compared. All of the nude mice bearing tumors survived during the therapy. As demonstrated in Fig. 3A, there were no significant differences for H460, H460-NC and H460-shRNA xenograft mice in the control groups with respect to the MTV ($P>0.05$), indicating neither the vehicle transfection nor knockdown of Merm1/Wbscr22 affected the proliferation of H460, H460-NC and H460-shRNA cells *in vivo*, consistent with the results *in vitro*. On day 12, the RMTV in the H460 xenograft mice was significantly smaller in the treatment group (986.73±161.95 mm^3) than the control group (1878.37±332.50 mm^3; $P=0.0476$; Fig. 3B) with an IR of 47.5%. Similarly, in H460-NC xenograft mice, the RMTV was also significantly smaller in the treatment group (481.50±22.29 mm^3), as compared with the control group (861.79±151.51 mm^3; $P=0.0476$; Fig. 3C) with IR of 44.1%. However, there was no significant difference ($P>0.05$) between the H460-shRNA control group (775.38±112.01 mm^3) and the treatment group (627.21±60.52 mm^3) with respect to the RMTV, and IR was only 19.1% (Fig. 3D).

Table I. Half maximal inhibitory concentration values for antitumor agents in (A) H1299 and (B) H460 cells.

A, Half maximal inhibitory concentration (μmol/l)

Agents	H1299	H1299-NC	H1299-shRNA
CDDP	14.665±1.462	21.120±8.167	10.100±1.505
ADM	0.355±0.163	0.303±0.114	0.312±0.076
PTX	0.0156±0.0014	0.0157±0.0046	0.0143±0.0033
MMC	11.975±1.874	10.853±2.683	11.849±3.226
5-FU	6.513±0.928	4.616±1.034	3.315±0.741
SN-38	0.265±0.019	0.075±0.031	0.076±0.009

B, Half maximal inhibitory concentration (μmol/l)

Agents	H460	H460-NC	H460-shRNA
CDDP	2.158±0.401	3.580±0.330	3.184±0.785
ADM	0.046±0.013	0.056±0.001	0.066±0.010
PTX	0.0045±0.0015	0.0065±0.0004	0.0063±0.0023
MMC	0.084±0.029	0.238±0.051	0.265±0.053
5-FU	0.809±0.050	0.889±0.101	1.565±0.298[a]
SN-38	0.012±0.002	0.015±0.002	0.053±0.002[b]

[a]$P<0.05$, [b]$P<0.01$. The cells were exposed to antitumor agents for 72 h. The results are expressed as the means ± standard deviation. The results were from at least three independent experiments in quadruplicate. The half maximal inhibitory concentration values were determined by MTT assay. H460-shRNA and H1299-shRNA, H460 and H1299 cells transfected with human Merm1/Wbscr22 shRNA, respectively; H460-NC and H1299-NC, H460 and H1299 cells transfected with negative control shRNA, respectively. CDDP, cisplatin; ADM, doxorubicin; PTX, paclitaxel; MMC, mitomycin; 5-FU, 5-fluorouracil; SN-38, 7-Ethyl-10-hydroxycamptothecin.

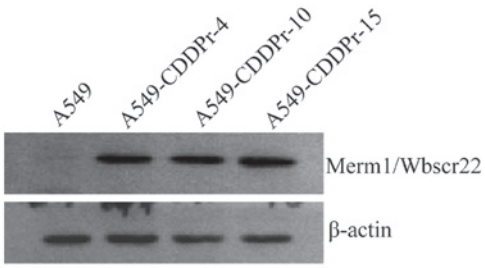

Figure 2. Western blot analysis of Merm1/Wbscr22 protein expression in A549 human non-small lung cancer cells. A549-CDDPr-4, 10, 15 cell lines were established and maintained by growing A549 parental cells in the presence of 4, 10, 15 μg/ml of CDDP, respectively. A549-CDDPr-4, 10, 15, cisplatin (4, 10, 15 μg/ml)-resistant A549 cells, respectively. CDDP, cisplatin.

p53 expression and chemosensitivity to antitumor agents in vitro. As demonstrated in Table I and Fig. 4A, the IC_{50} values for the antitumor agents in H1299 parental cells were evidently higher than in the H460 parental cells, and the ratio of $IC_{50\text{-}H1299}/IC_{50\text{-}H460}$ for CDDP, ADM, PTX, MMC, 5-FU and SN-38 was 4, 7.7, 3.5, 143, 8 and 22 respectively, indicating that H460 cells harboring wild type p53 (15) were markedly more

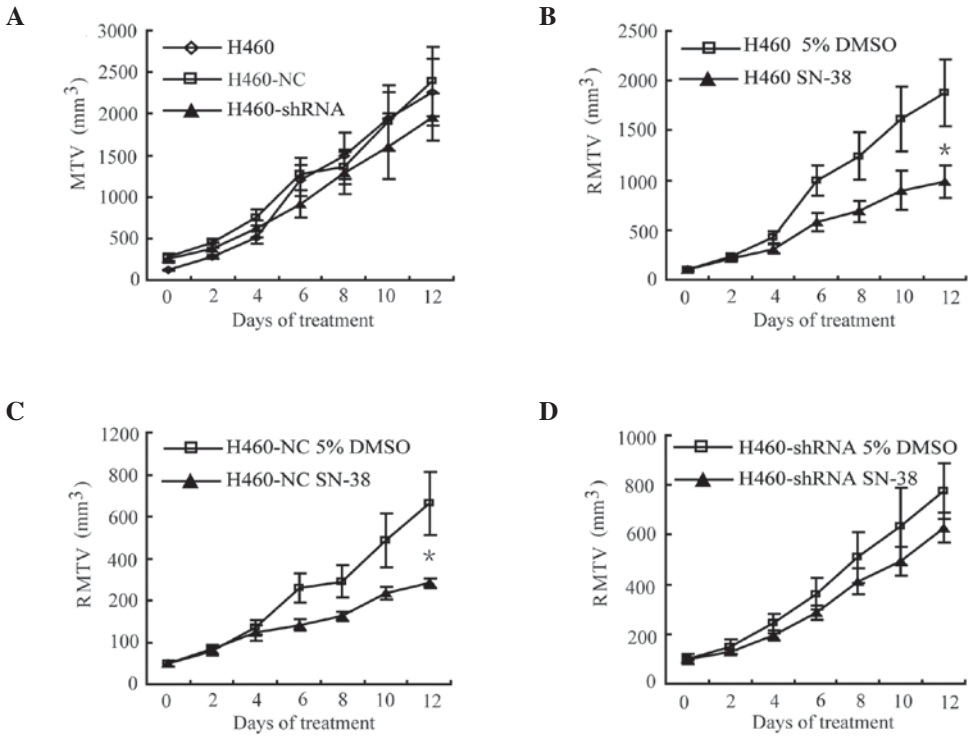

Figure 3. Knockdown of Merm1/Wbscr22 attenuates H460 sensitivity to SN-38 *in vivo*. The mice in the treatment groups were treated with SN-38 by intraperitoneal injection, on a schedule of two injections every four days at 20 mg/kg per injection. The mice in the control groups were injected with solvent (5% DMSO in 0.9% saline). (A) MTV curve of H460, H460-NC, H460-shRNA xenograft models in the control groups. (B, C and D) RMTV curve of H460, H460-NC and H460-shRNA xenograft models in the control and treatment groups. The results are expressed as the means ± standard error of the mean. *P<0.05, H460 SN-38 (treatment group) vs. H460 5% DMSO (control group), or H460-NC SN-38 (treatment group) vs. H460-NC 5% DMSO (control group). H460-shRNA and H1299-shRNA, H460 and H1299 cells transfected with human Merm1/Wbscr22 shRNA, respectively; H460-NC and H1299-NC, H460 and H1299 cells transfected with negative control shRNA, respectively. SN-38, 7-Ethyl-10-hydroxycamptothecin; MTV, mean tumor volume; RMTV, relative mean tumor volume; DMSO, dimethyl sulfoxide.

sensitive to various antitumor agents than the p53-null H1299 cells (16).

As shRNA-mediated knockdown of Merm1/Wbscr22 in the H460 cells decreased the chemosensitivity to SN-38 and 5-FU, the present study investigated whether the expression of p53 at the mRNA and protein level is associated with the chemosensitivity changes. As demonstrated in Fig. 4, neither the mRNA nor the protein level of p53 was significantly changed (P>0.05) following knockdown of Merm1/Wbscr22.

Discussion

According to a previous study (8), it was hypothesized that the knockdown of Merm1/Wbscr22 was able to sensitize tumor cells to antitumor agents. The results demonstrated that the downregulation of Merm1/Wbscr22 did not affect the sensitivity of H1299 cells to six antitumor agents, while enhanced H460 resistance to 5-FU and SN-38 *in vitro*. Furthermore, SN-38 demonstrated significant tumor growth inhibitory activity in both H460 and H460-NC tumor xenograft models, but only marginally suppressed H460-shRNA xenograft tumor growth, further indicating that downregulation of Merm1/Wbscr22 in H460 cells positively decreased the chemosensitivity to SN-38.

Merm1/Wbscr22 protein, containing a SAM-dependent MTase domain, is a putative methyltransferase (4). Methyltransferases regulate gene transcription via DNA or

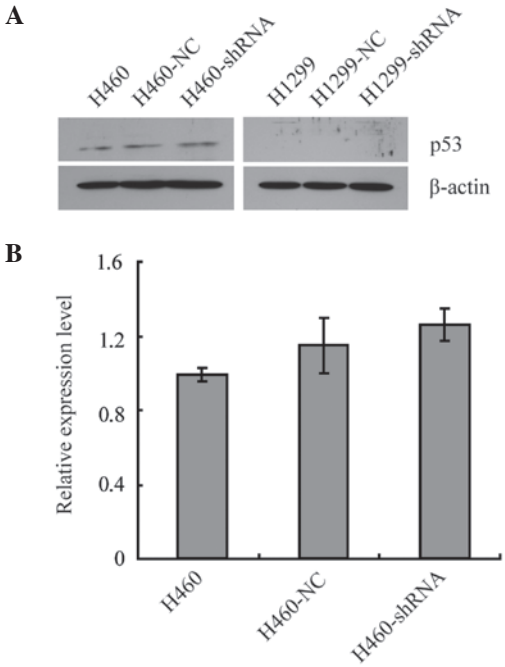

Figure 4. Analysis of p53 protein and mRNA expression levels in H460 and H1299 cells. (A) Analysis of p53 protein in H460 and H1299 cells by western blotting. (B) Relative expression of p53 mRNA in H460, H460-NC and H460-shRNA cells was measured by quantitative polymerase chain reaction. The results are expressed as the means ± standard deviation. H460-shRNA and H1299-shRNA, H460 and H1299 cells transfected by human Merm1/Wbscr22 shRNA, respectively; H460-NC and H1299-NC, H460 and H1299 cells transfected by negative control shRNA, respectively.

histone methylation activity (17,18), which has a crucial role in organism development. Its dysregulation consequentially causes gene expression changes in various diseases, including tumorigenesis (19), tumor cell metastasis (8) and others. Merm1/Wbscr22 represses the expression of Zac1/Lot1/Plagl1 by Lysine 9 methylation of the core histone H3 in the promoter region, thus promoting tumor cell metastasis (8); the loss of one copy of Merm1/Wbscr22 gene may cause methylation deficiencies in certain genes, including WBS (5). Genetic knockdown of methyltransferases results in global hypomethylation, thus causing dysregulation of specific gene expression and various biological phenomena. In the present study, the knockdown of *Merm1/Wbscr22* gene only decreased the H460 chemosensitivity to 5-FU and SN-38, suggesting its effects on chemosensitivity depend on cell types and antitumor agents. Merm1/Wbscr22 is part of a large and complex biological signaling network, where multiple factors regulate each other. The change in Merm1/Wbscr22 expression level may lead to hypomethylation and/or hypermethylation of certain genes, even at the same loci, however, the final consequences are affected by the cross-talk among multiple factors. This may explain why Merm1/Wbscr22 knockdown enhanced H460 resistance to SN-38 and 5-FU, rather than sensitized it.

By contrast, the change in Merm1/Wbscr22 expression was observed as a result rather than a cause in A549-CDDPr cells. The expression of Merm1/Wbscr22 protein in A549-CDDPr cells was notably higher as compared with A549 parental cells, but the amount of Merm1/Wbscr22 expression in A549-CDDPr cells was in a CDDP resistance degree-independent manner. Antitumor agents induce tumor cell death and/or suppress growth. However, a certain number of residual tumor cells survive from chemotherapy, which correlates with a high metastatic recurrence and poor outcome. In the present study, markedly increased Merm1/Wbscr22 protein in A549-CDDPr cells was positively associated with CDDP resistance, which may be a prognostic biomarker for chemoresistance. Further to this, blocking Merm1/Wbscr22 activity may be used as an important strategy to overcome and/or reverse chemoresistance.

Merm1/Wbscr22 promotes cancer cell metastasis by inhibiting p53-dependent apoptosis (8) and p53 has an essential role in this process. p53 is not only involved in tumor growth, cell cycle progression, apoptosis, signal transduction, ionizing radiation, cytotoxicity of antitumor agents and drug resistance development (20-24), but is also considered as an important biomarker in tumor patient prognosis (25,26). Considering the central role for p53 in multiple cellular functions, the H1299 cell line without p53 expression (16) and H460 cell line with wild-type p53 (15) were selected to investigate whether p53 is involved in Merm1/Wbscr22-mediated chemosensitivity. The results demonstrated that H1299 cells were more resistant to all six antitumor agents, as compared with the H460 cells, similar to previous results (21-24). The loss of p53 function in H1299 cells leads to more resistant to antitumor agents. In addition, H1299 sensitivity to six antitumor agents was unchanged by the knockdown of Merm1/Wbscr22, which may be also explained by the absence of p53.

5-FU induces apoptosis in gastric cancer cells harboring the wild-type p53 gene, but not in gastric cancer cells with the p53 mutation or deletion (27). Transfection with wild-type p53 gene partly reverses the resistance to 5-FU in Bel7402/5-FU cells (28) and LoVo/5-FU cells (29). However, in UMSCC12 and UMSCC11A laryngeal carcinoma, 5-FU-induced apoptosis and G1/S cell cycle phase arrest are not dependent on p53 (30). The pharmacological effects of SN-38 appear to be correlated with the status of p53 in some cell lines (31-37), but not others (37-40), with p53 knockdown shown to be more advantageous to the cytotoxicity of SN-38 in glioblastoma cells (41). Taken together, p53 does not always mediate the pharmacological effects of SN-38 and 5-FU, but rather it depends on the cell types and the treatment strategy. The results of the present study demonstrated that H460-shRNA cells were more resistant to 5-FU and SN-38 without being accompanied by a significant alteration in p53 mRNA and protein expression, as compared with H460 and H460-NC cells, indicating no direct correlation between p53 expression level and the alteration of chemosensitivity. However, the alteration in chemosensitivity to SN-38 and 5-FU may be due to p53 inactivation induced by knockdown of Merm1/Wbscr22, consequently, disrupting the p53-mediated signaling pathway. Furthermore, the p53-independent signaling pathway may be involved in this process.

In conclusion, the results of the present study demonstrated that shRNA-mediated knockdown of Merm1/Wbscr22 attenuates H460 sensitivity to SN-38 and 5-FU. This suggests that Merm1/Wbscr22 is involved in the chemosensitivity to SN-38 and 5-FU in H460 cells, and that there is no direct correlation between the p53 expression level and the alteration in chemosensitivity.

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