p53 dominant-negative mutant R273H promotes invasion and migration of human endometrial cancer HHUA cells

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Running title: DN p53 mutant promotes tumor invasion

Abstract

Dominant negative (DN) mutations of tumor suppressor p53 (*TP53*) are clinically associated with cancer progression and metastasis of endometrial malignancy. To investigate the DN effect on tumor migration and invasion, we generated cells that stably co-expressed wild-type (wt) and R273H DN mutant *TP53* (273H cells), and wt and R213Q recessive mutant *TP53* (213Q cells), by transfection in endometrial cancer cells HHUA that expressed wt p53. R273H, but not R213Q, repressed wt p53-stimulated transcription of *p21*, *Bax* and *MDM2*. 273H cells also showed markedly increased in vitro invasion and migration potentials, and displayed reduced *Maspin*、*PAI-1* and *KAI1* mRNA expressions as compared with 213Q and wt cells. The induction of wt p53 function by use of Adriamycin resulted in the inhibition of the invasion/migration capacity in association with the up-regulation of p53 target genes to a far greater degree in 213Q and wt cells than in 273H cells. R273H expression in p53-null cancer cell SK-OV-3 and Saos-2 did not significantly affect cell invasion and migration activities. Taken together, these results suggest that transdominance of R273H mutant over wt p53 rather than a gain-of-function promotes tumor metastasis by increasing invasion and migration in HHUA cells.

Keywords: endometrial cancer; dominant negative p53 mutation; migration; invasion; adenocarcinoma

Introduction

Endometrial cancer is the most frequent gynecologic malignancy in the US and other Western nations [1]. Asian nations such as China, Japan, and Korea have an incidence that is 4-5 times lower than in Western industrialized nations [2]. The incidence of endometrial cancer in Asian countries, however, has been increasing markedly in recent years [3]. Patients with advanced-stage endometrial cancer usually exhibit a poor prognosis, even after radical resection combined with radiotherapy or chemotherapy. These poor outcomes are due mainly to the progression and metastasis of the disease after the standard surgical treatment. Clearly, a better understanding of the molecular mechanisms underlying the progression of endometrial cancer is needed to control the disease.

The p53 tumor suppressor gene (*TP53*), the most frequently mutated gene in human cancers, is a crucial transcription factor that mediates cellular responses to various kinds of stress such as DNA damage, hypoxia, and aberrant growth signals. It induces the expression of genes, which lead to cell cycle arrest, apoptosis, and DNA repair [4]. In addition, several lines of evidence have suggested the functional linkage between *TP53* and cell invasion and motility. For example, the overexpression of *TP53* significantly reduced the invasion and migration of vascular smooth muscle cells (SMC) [5]. The deletion of

TP53 led to actin cytoskeleton reorganization and a significant increase in cell motility in mouse embryonic fibroblast cells [6]. Moreover, p53 has been shown to bind directly to and activate the promoters of *Maspin*, *PAI-1* and *KAI1*, which are involved in the inhibition of tumor invasion and migration [7, 8, 9]. These findings indicate a possible role of wt *TP53* in the negative regulation of cell invasion and migration through the activation of *Maspin*, *PAI-1* and *KAI1*.

TP53 mutations are found in more than half of human tumors. Approximately 90% of these are missense mutations in the central DNA-binding core domain responsible for the sequence-specific binding of wt p53 protein to target genes. These mutations lead to single amino acid changes that influence the sequence-specific binding or the conformation of the mutant protein, abrogating its ability to induce the transcription of target genes (loss of function). Some mutations, however, can reduce the ability of the wt p53 to bind to the p53-responsive element in its target genes, and can lead to the inactivation of wt p53 [10] or can hetero-oligomerize with wt p53 and inhibit the transactivation of p53-responsive elements in a dominant-negative fashion [11]. Furthermore, some mutations can exhibit oncogenic properties attributable to a gain of function. In our previous study, mutant TP53 was found in 24 out of 92 (26.1%) endometrial cancers, of which 14 cases showed

dominant-negative activity that was associated with advanced stages, non-endometrioid-type tumors and grade 3 tumors. The presence of a DN mutant p53 was related to poor outcomes as the most important, independent prognostic factor for patients with stage III/IV endometrial cancer [12], suggesting that DN mutant *TP53* may play an important role in the progression of endometrial cancer.

In this study, we generated cells that stably co-expressed wt p53 and R273H DN mutant p53 (273H cells), and cells that co-expressed wt p53 and R213Q recessive mutant p53 (213Q cells), and compared them with parent cells expressing wt p53 in order to assess the effect of dominant negative p53 mutant on tumor cell invasiveness. We show that R273H, but not R213Q, can markedly enhance the cellular invasion and migration potentials, in association with the repression of wt p53-driven transcriptional activation of p53 target genes. Expression of R273H did not significantly influence invasion and migration in p53-null ovarian cancer cell line SK-OV-3 and p53-null osteosarcoma cell line Saos-2. Thus, our results provide the first demonstration that a mutant p53 enhances the invasive characteristics of cancer cells through transdominant repression of wt p53 activities.

Materials and Methods

Cell culture and chemicals

The endometrial carcinoma cell line HHUA, ovarian cancer cell line SK-OV-3 and osteosarcoma cell line Saos-2 cell were grown in Ham's F12 medium (Kohjin Bio, Saitama, Japan) supplemented with heat-inactivated 20% fetal bovine serum (Cambrex Bio Science, Walkersville, MD). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Adriamycin (ADR) was purchased from Sigma (Steinhein, Germany), and used in DMSO (less than 0.1% at final concentration). The p53 rescue agent CP-31398 (gift from Pfizer Inc., Groton, CT) was stored at -20 °C and dissolved immediately before each experiment to a concentration of 1 mg/ml in DMSO. Prior to the experiments, non-cytotoxic dose ranges of CP-31398 and ADR were determined using an MTT assay for the parent and transfectant cells described below.

Plasmid construction and stable transfection

cDNA of R273H (CGT > CAT) mutant *TP53* mRNA derived from a human cancer was PCR-subcloned with degenerated primers, which gave *Nhe*I and *Xho*I sites at the both ends. The cDNA was transferred to pcDNA3.1/Hygro mammalian expression vector (Invitrogen, Carlsbad, CA) to give pcDNA-R273H. The *Afe*I-cut part of pcDNA-R273H

(468 bp corresponding codons 180 to 336 of *TP53*) was replaced with that of R213Q (CGA > CAA) mutant *TP53* cDNA to give pcDNA-R213Q. HHUA cells at 80% confluency were transfected with pcDNA-R273H, pcDNA-R213Q or empty pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA) using Lipofectamine PLUS Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The selection of stably transfected clones was achieved using medium containing 250 units/ml of Hygromycin for HHUA, 50 units/ml of Hygromycin for SK-OV-3 and 10 units/ml of Hygromycin for Saos-2, respectively. Isolated resistant clones of HHUA cells were checked for the expression of mutant *TP53* by the yeast functional assay described below. Selected clones of HHUA cells were expanded into 273H-, 213Q- and mock (empty vector)-transfectants, respectively, which were used for additional experiments.

Yeast p53 functional assay

The p53 yeast functional assay was performed as described previously [13]. Briefly, RNA samples were reverse synthesized at 37°C for 1 h with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA) and 25 pM *p53*-specific primer RT-1. cDNA was amplified by PCR with Pfu Turbo DNA polymerase (Stratagene,

La Jolla, CA) and the P3 and P4 primers on a Thermal Cycler Model 2400 (Perkin-Elmer, Chiba, Japan) using the following conditions: 95°C for 1 min, then 35 cycles of 95°C for 40 sec, 65°C for 70 sec and 78°C for 90 sec, followed by 78°C for 2 min. Satisfactory amplification was confirmed by examining the PCR product in a 1% agarose gel. Crude PCR product and a linearized p53-expression vector pSS16 were co-transfected into the yeast reporter strain yIG397 and plated and grown for 48 h in a 30°C humidified chamber. Experiments were repeated twice. More than 200 colonies were examined on each culture plate and the average percentage of red colonies was determined. In this assay system, more than 16% red colonies were judged as positive for p53 mutation [14].

Drug treatment

After overnight incubation in Ham's F12 medium supplemented with 20% FBS, cells at 70% confluency were treated with CP-31398 at a final concentration of 0, 1.0, 1.5 or 2.0 μg/ml for 24, 48, or 72 h, and were used in invasion/migration assay, luciferase assay or RNA extraction. For the induction of wt p53 activity with ADR, cells at 70% of confluency were treated with ADR at a final concentration of 0, 0.25, or 0.5 μg/ml for 3 h. Cells were then washed twice with PBS and maintained in Ham's F12 medium with 20% FBS for

another 3 h, and served for invasion/migration assay, luciferase assay, RNA extraction, and MTT assay.

MTT assay

For measurements of cell proliferation rates, 1×10^3 cells/100 µl medium were plated into 96-well plates. For induction of ADR, cells were grown in 96-well plates until 70% of confluency and treated with ADR. After 24, 48 or 72 h incubation, 10 µl of MTT solution (Cell counting kit-8, Dojindo, Kumamoto, Japan) was added into each well, and plates were incubated for 4 h at 37°C, and 450 nm UV absorbance of each sample was measured in a microplate reader. Assay was done in triplicate wells, and each experiment was repeated three times.

In vitro Matrigel invasion assay

Matrigel invasion assay was performed using a 24-well invasion chamber system (BD Biosciences, Bedford, MA) with Matrigel membrane (8.0-µm pore), as described in our previous report [15]. Briefly, each 750 µl of Ham's F12 medium supplemented with 20% FBS and 10 µg/ml of bovine fibronectin (chemoattractant) was placed in the lower

compartment of the chamber. In the prewarmed and rehydrated upper compartment, 2×10^4 cells in 500 µl of Ham's F12 medium supplemented with 20% FBS were added, and the cells were allowed to migrate through the intermediate membrane for 48 h at 37°C. Membranes were then fixed with 10% neutral-buffered formalin and stained in 5% Giemsa solution. The cells attached to the lower side of the membrane were counted in 10 high-powered (×200) fields under a microscope. Assays were done in triplicate for each experiment, and each experiment was repeated three times.

In vitro cell migration assay

This migration assay was a modification of the assay described previously [16], which measured cell migration through an 8.0-µm pored membrane (BD Biosciences, Bedford, MA). In the lower chamber, 600 µl of Ham's F12 medium containing 20% FBS and 10 µg/ml of bovine fibronectin was placed. After being treated with CP-31398, ADR or DMSO (vehicle) as indicated above, 2×10^4 cells in 100 µl of Ham's F12 medium supplemented with 20% FBS were placed in the upper chamber. After 6 h-incubation, the number of migrated cells (lower side of the membrane) was counted as described above. Assays were done in triplicate for each experiment, and each experiment was repeated three

times.

Quantitative PCR analysis (qPCR)

First-strand cDNA was synthesized from 1 µg of total RNA by using ExScript RT reagent kits (Takara, Otsu, Japan) and random hexamer primers. Quantitative PCR was performed using real-time SYBR Green PCR technology and an ABI PR 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). The primer used were 5'-CCTTCATCTTCACATTTGGTTTC-3' (forward), 5'-GATTCACTGCTACTGCTTCTTTC-3'(reverse) for *MDM2*; 5'- CCAGTCACATTGCCATCACTCT -3' (forward), 5'-GCCACAGTGGACTCTGAGATGA-3' (reverse) for *PAI-1*; 5'-ATGAACATGGAGGCCACGTTCT-3' (forward), 5'-TGTGGACTCATCCTCCACATCC-3' (reverse) for *Maspin*; 5'-TGGTCCTGTCCATCTGCTTGT-3' (forward), 5'-AGCAGAGGAACCACAGAACAG-3' (reverse) for *KAI1*; 5'-TTGCCGACAGGATGCAGAA-3' (forward), 5'-GCCGATCCACACGGAGTACT-3' (reverse) for beta-actin. Primers for *p21* and *Bax* were previously reported [17]. Results were calculated after standardization on beta-actin mRNA content.

Luciferase assay

CP-31398-, ADR- or DMSO-treated cells were trypsinized and plated into 12-well plates then incubated at 37°C overnight. Cells at 80-90% of confluency were rinsed with serum-free medium and transfected with 1 μg of the following luciferase (firefly) plasmids: p21 reporter plasmid, PG13 Py luc (gift of Dr. Bert Vogelstein), Bax reporter plasmid, pMO23 and MDM2 reporter plasmid, PGL2h mdm2-Hx-luc (gift of Dr. Moshe Oren), along with 1 ng of pRL-CMV *Renilla* control luciferase plasmid (Promega, Madison, WI) by use of Lipofectamine reagent. After 24 h of incubation, the cells were lysed and subjected to a chemiluminescence reaction using a dual-luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions. Relative luciferase activity was expressed as the firefly luciferase activity normalized by the *Renilla* luciferase activity measured in a luminometer (Bio-Rad, Hercules, CA).

Western blot analysis

Whole cellular protein was obtained with M-Per Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). The aliquots were separated on SDS-PAGE (10%) and transferred to nitrocellulose membranes. Antigen-antibody complexes were detected ECL

blotting analysis system (Amersham Biosicences Inc.). The following primary antibodies were used: p53 (DO-1, Santa Cruz Biotechnology, Inc.) and G3PDH (sc-25778, Santa Cruz Biotechnology, Inc.).

Results

Expression of wt and mutant TP53 in the HHUA cells and derived transfectants

Western blot analysis detected the expression of p53 in mock (clone 2) cells, and much higher levels of p53 expression in 273H (clone 2) and 213Q (clone 2) cells (Fig. 1a). We then examined the functional status of p53 in HHUA cells (untransfected parent) using a yeast functional assay performed in a yeast strain (yIG397) containing an integrated plasmid with a p53-responsive promoter driving *ADE2* expression. In this assay, a strain containing wt p53 activates *ADE2* transcription and forms a white colony. In contrast, a strain containing non-functional mutant p53 fails to activate *ADE2* transcription and forms a red colony. If an amplified cDNA contains both wt and mutant *TP53*, this assay gives a mixed population of white and red yeast colonies, the ratio being that of the wild type to mutant *TP53* cDNA. However, even cDNA containing only wt p53 usually provides less than 20% red colonies (named background), which mainly result from PCR-induced point mutation

[18], RNA degradation [19] or an alternative splicing of intron 9 [20]. Untransfected HHUA cells gave 14% red colonies, confirming that only wt p53 is expressed in untransfected HHUA cells (Fig. 1b) [3].

To determine the possible roles of DN mutant *TP53* on endometrial cancer invasion and migration, we transfected HHUA cells with pcDNA-R273H vector expressing a DN mutant *TP53* R273H, which was shown to be strong in repressing the wild-type p53 activity [21], or with pcDNA-R213Q vector expressing a recessive mutant *TP53* R213Q, which was proven to be unable to repress the wt p53 activity [22], or with an empty pcDNA3.1 vector, to establish 273H, 213Q and mock cells, respectively. We began to examine the background in mock cells. As expected, mock (clone 2 and 4) cells produced wt p53 and gave 13% (clone 2) and 14% (clone 4) red colonies. In contrast, 273H (clone 2) and 213Q (clone 2) expressed p53 mutations, and yielded 59% and 56% red colonies, respectively. This ensured that approximately equivalent amounts of wt p53 and mutant p53 were expressed in both 273H and 213Q cells (Figure 1b). Therefore, we used 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells throughout our study, and also used 273H clones (1 and 8) that expressed higher levels of p53 mutants (64 and 69%, respectively) as indicated (Fig. 1c).

R273H, but not R213Q, can repress wt p53-stimulated transcription of p21, Bax and MDM2

To determine whether DN mutant *TP53* R273H can repress the wt p53-responsive transcription, we investigated mRNA expression of wt p53 target genes (*p21*, *Bax* and *MDM2*) in 273H, 213Q and mock cells using quantitative RT-PCR after treatment with DMSO (vehicle) or CP-31398, a low-molecular weight pharmacological compound that can rescue certain mutant p53 to wt p53 conformation and activity [23] and can also stabilize wild-type p53 [24]. As shown in Fig. 2a, without CP-31398 treatment, 273H cells showed reduced levels of *p21*, *Bax* and *MDM2* mRNA expressions as compared with 213Q and mock cells. However, 48 h treatment with CP-31398 reversed these suppressions in a dose-dependent manner. We also noted a slight increase in *p21*, *Bax* and *MDM2* expressions in either 213Q or mock cells in response to increased CP-31398 concentrations (1.0-2.0 μg/ml).

To determine whether decreased *p21*, *Bax* and *MDM2* expressions in 273H cells were due to the repression of promoter activity, we transfected 273H, 213Q and mock cells with the luciferase reporter plasmids to examine the transcriptional activity of *p21* or *Bax* or

MDM2 in these three cells. Consistent with the results from RT-PCR, the promoter activities of *p21*, *Bax* and *MDM2* in 273H cells were significantly diminished compared with those in 213Q and mock cells without CP-31398 treatment. On the other hand, CP-31398 treatment (1.0-2.0 μg/ml) triggered a dose-dependent increase in transcription from *p21*, *Bax* and *MDM2* promoters and resulted in a significant increase of relative luciferase values in 273H cells. In agreement with the results from RT-PCR, CP-31398 treatment (1.0-2.0 μg/ml) slightly increased the promoter activity of these three genes in 213Q and mock cells (Fig. 2b), suggesting that the DN mutant p53 R273H, but not the recessive mutant p53 R213Q, can efficiently repress wt p53-stimulated transcription for *p21*, *Bax* and *MDM2*, and consequently demonstrated the dominant negative effect of R273H in the endometrial cancer cell HHUA.

R273H stimulates proliferation rate in HHUA cells

Using an MTT assay, we determined the proliferation rate of 273H, 213Q and mock cells incubated for 24, 48 or 72 h. The cell proliferation rate did not differ significantly between 273H and 213Q/mock cells after 24 and 48 h ($P > 0.05$). It was, however, significantly increased in 273H cells after 72 h of this assay when compared with 213Q and

mock cells ($P < 0.05$) (Fig. 3). Therefore, we incubated each HHUA-derived transfectant for 48 h in the following invasion assay.

R273H promotes invasion and migration in HHUA cells

To evaluate whether DN mutant *TP53* R273H contributes to the invasive behavior of HHUA cells, we examined the invasive potential of 273H, 213Q and mock cells using an *in vitro* Matrigel-coated invasion assay. In contrast to 213Q and mock cells, which showed low penetration of the Matrigel-coated membrane, 273H cells showed a high level of penetration after 48-h incubation. The number of 273H cells that passed through the Matrigel-coated membrane was three times larger than the number of 213Q and mock cells ($P < 0.05$) (Fig. 4a, top). To evaluate the role of R273H expression on the motility properties of HHUA cells, we also compared the cell motility using an *in vitro* cell migration assay. After 6 h-incubation, 273H cells migrated more actively by approximately 30% than 213Q and mock cells ($P < 0.05$) (Fig. 4a, bottom). This enhanced invasion and migration seemed to be associated with the increases of the mutant R273H expression level, but they were independent of the increase in R213Q expression as assessed by the yeast assay.

To confirm that this enhanced invasion and migration was specific to p53 DN mutant R273H but was not seen in the recessive mutant R213Q, we treated each HHUA clone with CP-31398 to rescue the functional activity of R273H. Pretreatment with p53 rescue agent CP-31398 at 1.0-2.0 μg/ml up-regulated the p53 protein levels in all these three endometrial cell lines (Fig. 4c), and significantly inhibited the increased invasion and migration in 273H cells in a dose-dependent manner but inhibited invasion and migration in 213Q and mock cells only slightly (Fig. 4b, top and bottom). In particular, pretreatment with CP-31398 at 2.0 μg/ml reversed cell invasion and migration to levels approximately equivalent to those found in untreated 213Q or mock cells, suggesting that this elevated invasion and migration in HHUA cells was dependent on the p53 DN mutant R273H (Fig. 4d, middle column).

To extend these findings to additional endometrial cells, *in vitro* Matrigel invasion assay and migration assay were also performed for another wt p53-expressing endometrial cancer cell HOUA-I [3] transfected with pcDNA-R273H, pcDNA-R213Q or pcDNA3.1 plasmids. As in the results for HHUA, HOUA-I cells transfected by pcDNA-R273H exhibited increased invasion and migration compared with HOUA-I cells transfected by pcDNA-R213Q or pcDNA3.1 vector (data not shown). Taken together, these results

suggest that DN mutant *TP53* R273H, but not recessive mutant *TP53* R213Q, can enhance *in vitro* cell invasive and migratory capability in the endometrial cancer cell HHUA.

R273H reduces Maspin, PAI-1 and KAI1 expressions in HHUA cells

To explore whether the enhanced invasion and migration potential of 273H cells was associated with a reduction of the wt p53 target genes *Maspin*, *PAI-1* and *KAI1*, which are known to be important in inhibiting both tumor invasion and migration, we performed quantitative RT-PCR analyses to examine the mRNA expressions of *Maspin, PAI-1* and *KAI1* in 273H, 213Q and mock cells. Without CP-31398 treatment, 273H cells showed a significant reduction in *Maspin, PAI-1* and *KAI1* expression when compared with 213Q and mock cells. The rescue of 273H by CP-31398 reversed these changes in a dose-dependent manner, indicating that R273H expression in HHUA cells reduced the expression of *Maspin, PAI-1* and *KAI1*. Interestingly, slight increases in *Maspin, PAI-1* and *KAI1* expressions were also detected in 213Q and mock cells when they were treated with 1.0-2.0 μg/ml of CP-31398 (Fig. 5).

R273H works in a dominant negative fashion, but not through gain-of-function effect, to

promote invasion and migration in endometrial cancer cell HHUA

Besides the dominant negative effect, certain mutant p53s contribute to tumor development and progression that does not depend on wt p53 (gain-of-function). These activities range from enhanced resistance to anticancer drugs, enhanced proliferation in culture or increased tumor invasiveness [25]. To investigate the possible involvement of gain-of-function that might explain the up-regulated tumor invasion and migration as well as down-regulated *Maspin*, *PAI-1* and *KAI1* expressions in 273H cells, we treated 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells for 3 h with ADR, which causes DNA damage in order to induce the wt p53 expression. The expression level of p53 protein in either of R273H, R213Q and mock cells was efficiently induced in a dose-dependent manner upon exposure to ADR (Fig. 6b). We then investigated the effects of ADR treatment on cell invasion and migration. The wt p53 induction by ADR (0.25 μg/ml) significantly inhibited tumor invasion and migration by approximately 14% in 273H cells. On the other hand, the cell invasion and migration was significantly repressed by approximately 75% in 213Q and mock cells (Fig. 4c right column and Fig. 6a). The addition of ADR (0.5 μg/ml) completely blocked the invasion and migration in either of 273H, 213Q and mock cells, which implies the possibility that the loss of wt p53 function

resulting from the DN effect due to R273H p53 contributes to the invasive process in endometrial cancer.

For the effect of ADR treatment on *Maspin*, *PAI-1* and *KAI1* expression, we performed quantitative RT-PCR and detected a dramatic, ADR-dependent induction of *Maspin*, *PAI-1* and *KAI1* mRNA levels in 213Q or mock cells. In contrast, the corresponding increase in the expression was weak in 273H cells (Fig. 6c), suggesting that DN mutant *TP53* R273H can effectively repress wt p53-responsive transcription of *Maspin*, *PAI-1* and *KAI1*, and can possibly promote cell invasion and migration in the endometrial cancer cell HHUA.

To evaluate the effect of ADR treatment on cell survival, we treated 273H, 213Q and mock cells with increasing doses of ADR (0, 0.25, or 0.5 μg/ml) for 3 h, further incubated them for 48 h, and evaluated cell viability with an MTT assay. ADR treatment caused a mild decrease in viability in these three types of cells (Fig. 7a). The lack of correlation between cellular viability and migration/invasion capacity, however, strongly suggests that the major part of the observed ADR effect cannot be explained solely by the difference in cell survival. We then examined the effects of ADR treatment on *p21*, *Bax* and *MDM2* expressions with quantitative RT-PCR. ADR treatment induced a marked, dose-dependent

increase in *p21*, *Bax* and *MDM2* mRNA expression in 213Q or mock cells, in contrast to the mild increase in 273H cells (Fig. 7b). This finding further confirmed the dominant negative effect of R273H, and also suggested that mRNA expression levels in *p21*, *Bax* and *MDM2* may not significantly affect cell viability in HHUA cells when exposed to ADR at the concentrations used in this study.

To gain further insight into the possible gain-of-function by R273H allele, we transfected the p53-null ovarian cancer cell line SK-OV-3 and osteosarcoma cell line Saos-2 with either pcDNA-R273H or empty pcDNA3.1 vector. After the Hygromycin selection, we generated SK-OV-3 stable cell lines (SK-OV-3-R273H and SK-OV-3-mock cells) and Saos-2 stable cell lines (Saos-2-R273H and Saos-2-mock cells). Using Western blot analysis, we observed that SK-OV-3-mock cells and Saos-2-mock cells did not express p53, while mutant p53-overexpressing SK-OV-3-R273H cells and Saos-2-R273H cells expressed high levels of p53 (Fig 8a). After incubation for 24 h, there was no significant difference in invasion to Matrigel between SK-OV-3-R273H/Saos-2-R273H cells and SK-OV-3-mock/Saos-2-mock cells, respectively (Fig 8b). Similarly, we noticed that there was no significant difference in migration activities between SK-OV-3/Saos-2-R273H cells and SK-OV-3/Saos-2-mock cells, respectively (Fig 8c). Quantitative RT-PCRs revealed that

expression of R273H did not cause significant changes in *Maspin, PAI-1* and *KAI1* mRNA levels in SK-OV-3 and Saos-2 cells (Fig. 8d). These results supported the notion that R273H does not show gain-of-function properties involved in promotion of invasion and migration in p53-null human cancer cells.

Discussion

In our previous study [12], we demonstrated a strong link between DN mutant *TP53* and the progression of endometrial cancer. In the present work, we focused on the precise role of DN mutant *TP53* R273H in the cell invasion and migration of the endometrial cancer cell HHUA, and showed for the first time that DN mutant *TP53* R273H, a *TP53* missense mutation commonly found in endometrial cancer, strongly induces the invasive and migratory activities of the endometrial cancer cell HHUA. This induction of activities was associated with the down-regulation of *Maspin, PAI-1* and *KAI1* expressions. Furthermore, the p53 rescue agent, CP-31398, could abolish the above-mentioned effects of R273H. We also showed that these results were due mainly to the transdominance of R273H mutant over wt p53 rather than to a gain-of-function.

Previous study showed that an excess of mutant p53 over wt p53 is required to exert

the dominant negative effect [26]. However, we found that although mutant p53 R273H and wt p53 were co-expressed at an approximately equivalent level, R273H strongly repressed wt p53-stimulated transcription of *p21*, *Bax* and *MDM2* at the mRNA expression and promoter activity level, and promoted cell proliferation rates in the endometrial cancer cell HHUA. This finding is consistent with the result that when R273H and wt p53 were co-expressed at an equivalent level, R273H definitely decreased the capacity of wt p53 to induce either cell cycle arrest, apoptosis, or a negative regulator of wt p53 [19]. More importantly, we showed that the expression of R273H, but not of the recessive mutation R213Q, leads to increased invasion and migration in the endometrial cancer cell HHUA. These differences in invasion and migration were not due to differences in cell proliferation, as these three cells showed comparable proliferation rates during the 48 h-incubation we used. Consistent with these results, the rescue of the p53 mutation function by treatment with CP-31398 significantly reduced this increased invasion and migration in 273H cells, suggesting a possible molecular mechanism for the observed high invasiveness of endometrial cancers with DN mutant *TP53* R273H.

Metastasis is a complex process that requires a number of specific steps such as degradation of the extracellular matrix (ECM) by proteolytic enzymes as well as increased

cell motility. In our study, DN mutant *TP53* R273H, but not R213Q, reduced *Maspin*, *PAI-1* and *KAI1* mRNA expressions in the endometrial cancer cell HHUA. All three of these genes were shown to negatively modulate tumor invasion and migration. PAI-1 can reduce invasiveness in breast and ovarian cancer cells [27, 28]. Maspin, a tumor-suppressing serpin (serine protease inhibitor) has been shown to suppress tumor cell invasion [29] and cell motility [30]. KAI1 is a known suppressor of tumor invasion and metastasis [31, 32]. Taking these facts into consideration, the dominant negative inhibition of wt p53-responsive *Maspin*, *PAI-1* and *KAI1* expressions may favor two critical steps known to be important in tumor metastasis and progression (invasion and migration).

The mechanism by which R273H induces the invasive properties of endometrial cancer cells might also involve gain-of-function, where R273H plays a role as an oncogene, independent of the inactivation of wt p53 [33]. On the other hand, the dominant negative effect that is dependent on the inactivation of wt p53 function could be overcome by surplus activation of wt p53 by use of a DNA-damaging agent such as Adriamycin (ADR) [34]. In this context, to determine whether gain-of-function was involved in the enhanced invasion and migration in 273H cells, we tested the effect of the activation of wt p53 function by ADR treatment. The wt p53 activation could induce only a moderate reduction of cell

invasion/migration capacity in 273H cells, in contrast to the drastic suppression seen in 213Q and mock cells. These changes seen in the 273H, 213Q, and mock cells correlated with the degrees of increase of *Maspin*, *PAI-1* and *KAI1* mRNA expressions after ADR treatment, but not with the changes in cell viability as assessed in the MTT assay. Previous report has suggested the inhibitory effects of Adriamycin on tumor aggression [35]. The precise mechanisms responsible for this process are not fully understood. However, recent study has shown that Adriamycin at non-cytotoxic concentrations reduced the invasive behavior of human melanoma cell by suppressing collagenase-1 expression [36]. In addition, Adriamycin at low concentrations lower than those used to suppress cell growth, can inhibit invasion and migration of mouse melanoma cell by modulating cell adhesion [37]. All these findings suggested that Adriamycin-induced inhibition of cell invasion and migration was not solely due to the ability of Adriamycin to cause DNA damage, which triggers p53 protein elevations [38]. We observed that pretreatment of 273H cells with 0.5 μg/ml of Adriamycin resulted in a more complete block of cell invasion and migration and less increased relative mRNA expression of *Maspin*, *PAI-1* and *KAI1* when compared to pretreatment of 273H cells with 2.0 μg/ml of CP-31398. Thus, our results supported the possibility that Adriamycin may decrease tumor cell invasion and migration via *TP53*-

independent mechanisms. To further investigate potential gain-of-function properties of R273H for enhanced tumor invasion, we transfected pcDNA-R273H vector into p53-null cancer cell line SK-OV-3 and Saos-2, and found that expression of R273H in these two cell lines did not significantly affect the invasion/migration activities and *Maspin*, *PAI-1* and *KAI1* mRNA expressions. Our findings were consistent with previous reports that unlike other DN p53 mutations, R273H is unable to transform primary rat embryo fibroblast cells when transfected with activated *ras* oncogene [39], and also not enough to drive tumor development in transgenic mice [40]. All of these functional characteristics may reflect the facts that R273H retains the conformation of p53 nearly identical to the conformation of wt p53 [41] and binds p53 DNA recognition sequences to behave as a transcriptional factor similar to wt p53 [42]. Taken together, our findings suggested that the invasive phenotype in HHUA endometrial cancer cells was mostly given by a balance between the transdominant canceling effect of R273H and wt p53 transcriptional activity rather than a gain-of-function effect induced solely by R273H mutant p53. This may, at least in part, afford a possible molecular basis by which DN mutant p53 promotes tumor progression in patients with the advanced stages and nonendometrioid subtype of endometrial cancer [12].

The mechanisms leading to cell cycle arrest or apoptosis in response to

Adriamycin-induced DNA damage are not well understood, but there is recent evidence showing that in human cancer cells, p21 exhibits inhibitory effects on wt p53-dependent apoptosis induced by low concentrations of ADR [43]. In this study, ADR treatment at quite low doses dramatically elevated *p21* and *Bax* mRNA expression in 213Q/mock cells, but this did not significantly influence their cell viability; thus, it is reasonable to speculate that p21 may block the wt p53-mediated loss of cell viability in 213Q/mock cells with a low degree of DNA damage. Interestingly, we noticed that even 213Q and mock cells showed a slight decrease in cell invasion and migration, accompanied by a slight increase in the transcriptional activities or mRNA expressions of wt p53 target genes after CP-31398 treatment. Thus CP-31398 seems to not only rescue R273H function, but also to stabilize wt p53 as shown here and in other studies [23]. Thus, it appears to be a promising agent for inhibiting the p53 DN mutation-induced invasive and migratory activities of endometrial cancer cells.

In conclusion, our data highlight that DN mutant *TP53* R273H acts in a dominant negative fashion to promote both the invasion and migration potential of the endometrial cancer cell HHUA. The inhibition of DN p53 mutants may offer an interesting strategy for cancer therapy.

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Legends to figures

Figure 1

Expression of wt and mutant *TP53* in the HHUA cells and derived transfectants.

(**a**) Expression of p53 protein in 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells. Whole-cell lysates were subjected to western blot analysis with anti-p53 and anti-G3PDH antibody as described in Materials and Methods.

(**b**) Yeast p53 functional assay in 273H (clone 2), 213Q (clone 2), mock (clone 2) cells and untransfected HHUA cells. This assay shows expressed mutant *TP53* as red colonies almost exactly corresponding to the original cellular *TP53* mRNA content.

(**c**) The percentage of red clones in total yeast clones for 273H (clone 1, 2 and 8), 213Q (clone 2 and 4) and mock (clone 2 and 4) cells and untransfected HHUA cells were shown.

Figure 2

R273H, but not R213Q, can repress wt p53-stimulated transcription of *p21*, *Bax* and *MDM2*.

(**a**) 273H, 213Q and mock cells were treated with or without CP-31398 for 48 h, and the levels of *p21*, *Bax* and *MDM2* mRNA was analyzed by qPCR normalized to beta-actin as internal control. Each bar represents the mean ± S.E. of three independent experiments.

(**b**) Luciferase assay for the promoter activities of *p21*, *Bax*, and *Mdm2* genes under rescue of p53 function by CP-31398. 273H, 213Q and mock cells were treated with graded concentrations of CP-31398 for 48 h, transfected with 1 μg of PG13 Py luc, pMO23, or PGL2h mdm2-Hx-luc luciferase plasmids, along with 1 ng of pRL-CMV *Renilla* control luciferase vector. After 24 h-incubation, relative luciferase activity was measured as described in Materials and Methods. Data represent the mean ± S.E. of three independent experiments in triplicate wells.

Figure 3

R273H promotes proliferation rate in HHUA cells.

Cells were grown in 96-well plates until 30% of confluency. MTT assay was done after 24, or 48, or 72 h. Each experiment was done in triplicates. Data represent the mean ± S.E. from three separate experiments. NS: not significant versus 213Q or mock cells. *, $P <$ 0.05 versus 213Q or mock cells.

Figure 4

R273H promotes invasion and migration in HHUA cells. The p53 rescue agent CP-31398

abrogates this promotion in a dose-dependent manner.

(**a**) 273H (clone 1, 2 and 8), 213Q (clone 2 and 4) and mock (clone 2 and 4) cells were seeded onto a Matrigel-coated invasion chamber and incubated for 48 h, or seeded onto a Boyden chamber and incubated for 6 h, respectively. The number of invading cells (upper panel) or migrating cells (lower panel) was determined by counting Giemsa-stained cells in the lower side of the membrane. The table underneath shows expression levels of mutant *TP53* R273H or R213Q in the corresponding clones. Data represent the mean ± S.E. of three independent experiments. *, $P < 0.05$ versus 213Q or mock cells.

(**b**) CP-31398 treatments significantly inhibited the increased invasion and migration in 273H cells. 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells were treated with or without CP-31398 for 48 h, and assayed for invasion (*upper panel*) and migration (*lower panel*) as described above. Data represent the means ± S.E. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ as compared to 273H cells treated without CP-31398.

(**c**) Western blot analysis using anti-p53 antibody (DO-1) was performed on proteins extracted from 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells treated with or without CP-31398 for 48 h. G3PDH was used as a loading control.

(**d**) 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells were treated with vehicle

(DMSO, left column), CP-31398 at 2.0 μg/ml (middle column), or ADR at 0.25 μg/ml (right column) as described in Materials and Methods, then subjected to Matrigel invasion assay and migration assay. Representative photomicrographs of invading and migrating cells (original magnification ×100) were shown.

Figure 5

R273H repress *Maspin*, *PAI-1* and *KAI1* mRNA expressions in HHUA cells. The p53 rescue agent CP-31398 abrogates this repression in a dose-dependent manner.

273H, 213Q and mock cells were treated with or without CP-31398 for 48 h, and the levels of *Maspin*, *PAI-1* and *KAI* mRNA were analyzed by qPCR normalized to beta-actin as internal control. Each bar represents the mean ± S.E. of three independent experiments.

Figure 6

R273H acts in a dominant negative fashion to promote invasion and migration in HHUA cells.

(**a**) 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells were treated with or without ADR for 3 h, and assayed for invasion (*left panel*) and migration (*right panel*) as described

in Materials and Methods. Data represent the means ± S.E. of three independent experiments. * $P < 0.01$ as compared to 273H, 213Q and mock cells treated without ADR.

(**b**) 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells were treated with or without ADR for 3 h, then cellular protein was extracted and Western blot analysis was performed to analyze p53 and G3PDH (control) expression.

(**c**) Effect of ADR treatment on *Maspin*, *PAI-1* and *KAI1* mRNA expressions. 273H (clone 2), 213Q (clone 2) and mock (clone 2) were treated with or without ADR for 3 h, and the levels of *Maspin*, *PAI-1* and *KAI* mRNA were analyzed by qPCR normalized to beta-actin as internal control. Each bar represents the mean ± S.E. of three independent experiments.

Figure 7

The effect of ADR-induced wt p53 activation on cell survival.

(**a**) 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells were treated with or without ADR for 3 h, further incubated for 48 h, and assayed for cell viability. Data represent the means ± S.E. of three independent experiments in triplicate wells. NS, not significant ($P > 0.05$) versus 213Q or mock cells.

(**b**) 273H (clone 2), 213Q (clone 2) and mock (clone 2) cells were treated ADR as described

above, and the levels of *p21*, *Bax* and *MDM2* mRNA was analyzed by qPCR normalized to beta-actin as internal control. Each bar represents the mean ± S.E. of three independent experiments.

Figure 8

R273H did not significantly affect the invasion and migration in SK-OV-3 and Saos-2 cells.

(**a**) Stable expression of p53 mutation R273H in SK-OV-3-R273H and Saos-2-R273H cells. Whole-cell lysates were subjected to western blot analysis with anti-p53 and anti-G3PDH antibody as described in Materials and Methods.

(**b**) SK-OV-3 and Saos-2-derived transfectant cells were seeded onto a Matrigel-coated invasion chamber and incubated for 24 h. The number of invading cells was determined by counting Giemsa-stained cells in the lower side of the membrane. Data represent the mean ± S.E. of three independent experiments. NS: not significant.

(**c**) SK-OV-3 and Saos-2-derived transfectant cells were seeded onto a Boyden chamber and incubated for 6 h. The number of migrating cells was determined by counting Giemsa-stained cells in the lower side of the membrane. Data represent the mean ± S.E. of three independent experiments. NS: not significant.

(**d**) Effect of R273H expression on *Maspin*, *PAI-1* and *KAI1* mRNA expressions. Total RNA was extracted from SK-OV-3 and Saos-2-derived transfectant cells and the levels of *Maspin*, *PAI-1* and *KAI* mRNA were analyzed by qPCR normalized to beta-actin as internal control. Each bar represents the mean ± S.E. of three independent experiments. NS: not significant.