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Downregulation of histone deacetylase 1 by microRNA-520h contributes to the chemotherapeutic effect of doxorubicin

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ABSTRACT

Doxorubicin induces DNA damage to exert its anti-cancer function. Histone deacetylase 1 (HDAC1) can protect the genome from DNA damage. We found that doxorubicin specifically downregulates HDAC1 protein expression and identified HDAC1 as a target of miR-520h, which was upregulated by doxorubicin. Doxorubicin-induced cell death was impaired by exogenous HDAC1 or by miR-520h inhibitor. Moreover, HDAC1 reduced the level of γH2AX by preventing the interaction of doxorubicin with DNA. In summary, doxorubicin downregulates HDAC1 protein expression, by inducing the expression of HDAC1-targeting miR-520h, to exacerbate DNA–doxorubicin interaction. The upregulation of HDAC1 protein may contribute to drug resistance of human cancer cells and targeting HDAC1 is a promising strategy to increase the clinical efficacy of DNA damage-inducing chemotherapeutic drugs.

1. Introduction

Gastric cancer remains one of the leading causes of cancer-related mortality. Doxorubicin-containing systemic chemotherapy is the preferential treatment option for gastric cancer [1]. As an anthracycline anticancer drug, doxorubicin exerts its anti-cancer action due to its interaction into double strand DNA and its role of DNA topoisomerase II inhibitor. DNA double strand breaks are induced by DNA–doxorubicin interaction, consequently accelerate cancer cell death. The structures of the DNA-anthracycline complexes have been revealed by X-ray diffraction method [2]. The anthraquinone ring of doxorubicin is sandwiched between two neighboring base pairs, following a formation of an extra space between these two base pairs [3]. Recently doxorubicin was found to bind DNA minor groove to disturb double strand structure of DNA [4,5]. The out-binding pattern offers a feasible explanation for the intricate dynamics observed in the doxorubicin–DNA studies. Despite its wide clinical application, acquired or intrinsic resistance and severe cardiac toxicity during chemotherapy often leads to the failure of doxorubicin-based treatments. Substantial efforts have been made to overcome drug resistance or improve side effects of doxorubicin.

Histone deacetylase (HDAC) family proteins modulate chromatin condensation and transcriptional repression [6]. Besides, histone acetylation is important to DNA double strand break repair [7]. HDAC1- and 2-depleted cells were hypersensitive to DNA-damage [8]. All of these findings indicate a potential relevance of HDACs to the DNA damage induction and anti-cancer effect of doxorubicin. Indeed, Phase I/II clinical studies on combined HDACi (histone deacetylase inhibitor) and doxorubicin have found potential superiority on effectiveness in the treatment of various cancer such as mesothelioma [9], non-Hodgkin's lymphoma [10], breast cancer [11] and other advanced solid tumors [12,13]. However, how HDACs influence DNA damage induction and anti-cancer effect of doxorubicin remained unclarified.

In this report, we found that HDAC1 protein was down-regulated by doxorubicin in gastric cancer cells. However, doxorubicin affects neither protein stability nor mRNA level of HDAC1. We further defined HDAC1 as a target of miR-520h which was markedly upregulated after doxorubicin treatment. The viability inhibitory effect of doxorubicin was impaired in the presence of exogenous HDAC1 expression or when doxorubicin-induced HDAC1 protein down-regulation was blocked by miR-520h inhibitors. Moreover, HDAC1 reduced DNA damage by attenuating the interaction of doxorubicin with double strand DNA. Therefore, doxorubicin exacerbates DNA damage by epigenetically down-regulating HDAC1 protein expression.

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2. Results

2.1. Doxorubicin down-regulates HDAC1 protein expression in gastric cancer cells

As expected, doxorubicin inhibited the viability of human gastric cancer cell lines MKN45 and MKN28 in a dose-dependent manner (Fig. 1A and B). Next, we wonder whether doxorubicin has any effects on the expression of HDACs. Interestingly, HDAC1 protein expression was remarkably reduced after 4 μM doxorubicin treatment for 8 h (Fig. 1C). However, the expression of other HDACs such as HDAC2, 3 and 8 were not affected by doxorubicin (Fig. 1C). Furthermore, we found that doxorubicin-induced HDAC1 protein down-regulation started at 4–6 h after 4 μM doxorubicin treatment (Fig. 1D and E), suggesting that doxorubicin probably did not affect the stability of HDAC1 protein.

2.2. Doxorubicin did not reduce HDAC1 protein stability or decrease its mRNA level

Indeed, treatment with 10 μM or 20 μM MG132 for 9 h, a widely used inhibitor of ubiquitin–proteasome system, cannot rescue the down-regulation of HDAC1 protein caused by doxorubicin in MKN45 and MKN28 cells (Fig. 2A). In addition to ubiquitin–proteasome system, autophagy–lysosome system was another important pathway of protein degradation. However, the treatment of 50 nM autophagy inhibitor chloroquine (CQ) for 24 h has no influence on the down-regulation of HDAC1 protein caused by doxorubicin (Fig. 2B). To further ascertain whether the HDAC1 is down-regulated at post-translational level, Flag-tagged HDAC1 expressing plasmid was transfected into MKN45 cells. The levels of exogenous Flag-HDAC1 protein expression were not affected by 4 μM doxorubicin (Fig. 2C), suggesting that doxorubicin did not induce the degradation of HDAC1 protein.

Since the level of mRNA could also influence protein expression, real time RT-PCR was performed to detect HDAC1 mRNA expression in MKN45 and MKN28 cells before and after doxorubicin treatment. Interestingly, HDAC1 mRNA was not down-regulated after the same doxorubicin treatment (Fig. 2D). These results indicated that doxorubicin did not inhibit HDAC1 expression at the transcriptional level.

2.3. Doxorubicin down-regulates HDAC1 protein by inducing miR-520h expression

MicroRNA (miRNA) could affect gene expression by regulating mRNA translation efficiency, thus regulating gene expression at a post-transcriptional but pre-translational level. Therefore, we wonder whether doxorubicin affects HDAC1 through miRNA. Several prediction tools such as Targetscan and PicTar were used to search miRNAs potential regulating HDAC1. Among these miRNAs, only miR-520h was dramatically upregulated after 4 μM doxorubicin treatment in MKN45 cells so that we focused on miR-520h in the following studies (Fig. 3A). First, we confirmed its up-regulation in both MKN45 and MKN28 cells upon doxorubicin treatment (Fig. 3B). In addition, 50 nM miR-520h inhibitor rescued doxorubicin-induced down-regulation of HDAC1 in both MKN45 and MKN28 cells (Fig. 3C). Furthermore, 10 nM and 20 nM miR-520h mimics down-regulated HDAC1 expression in both cell lines (Fig. 3D). These results demonstrated that doxorubicin affects HDAC1 expression through upregulation of miR-520h. The dual luciferase reporter system was utilized to further clarify whether HDAC1 is the direct target of miR-520h. The 3′-UTR of the HDAC1 gene that contains a complementary binding site for miR-520h was

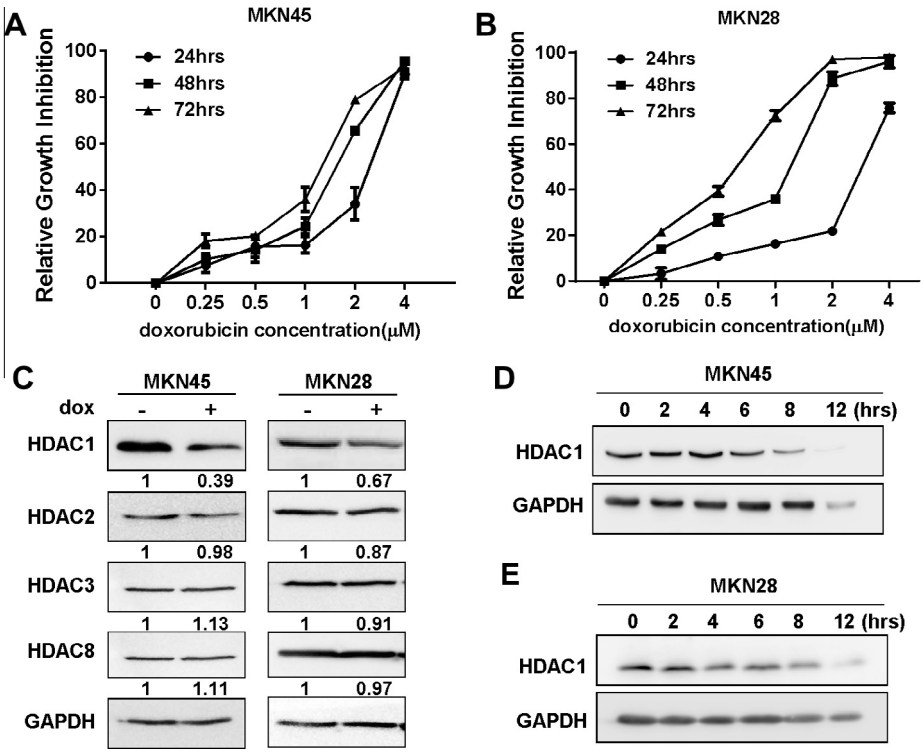

Fig. 1. HDAC1 expression was down-regulated in gastric cancer cells after doxorubicin treatment. The viability inhibitory effect of doxorubicin on human gastric cancer cells MKN45 (A) and MKN28 cells (B) were determined by MTS assay. (C) The expression of other HDACs (HDACs 1, 2, 3, and 8) in MKN45 cells and MKN28 cells cultured with 4 μM doxorubicin for 8 h were analyzed by Western blot analysis. The numbers under the blot indicate its relative laser densitometric quantification. HDAC1 expression in MKN45 cells (D) and MKN28 cells (E) treated with 4 μM doxorubicin were analyzed by Western blot analysis. All experiments were repeated three times.

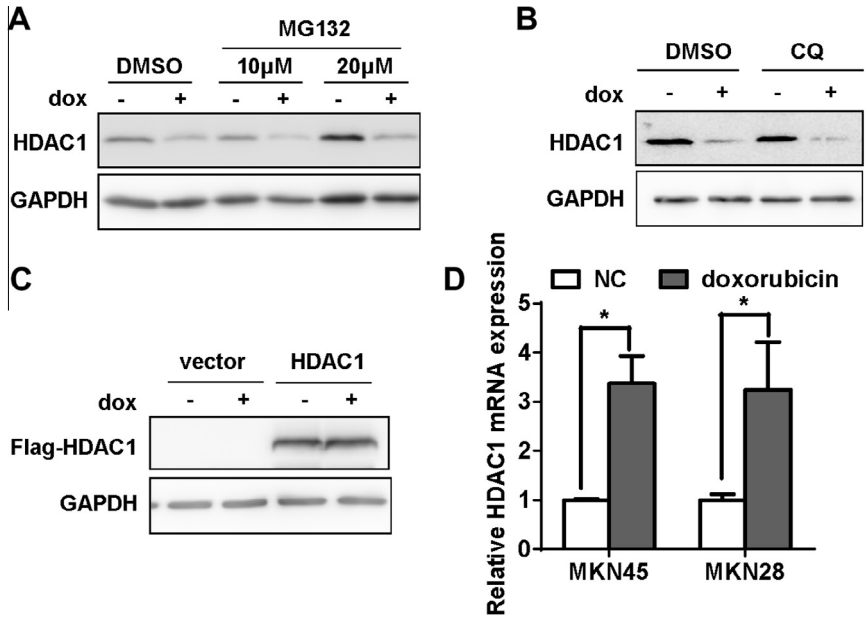

Fig. 2. Expression of HDAC1 is not down-regulated at post-translational level or transcriptional level in human gastric cancer cells after doxorubicin treatment. HDAC1 expression in MKN45 cells preincubated with 10 μM or 20 μM MG132 for 9 h (A) or 50 nM CQ for 24 h (B) before doxorubicin treatment was determined by Western blot analysis. (C) The expression of exogenous HDAC1, by transfected with Flag-HDAC1 expressing plasmid in MKN45 cells, after doxorubicin treatment was analyzed by Western blotting. (D) HDAC1 mRNA level in MKN45 cells and MKN28 cells cultured with 4 μM doxorubicin for 8 h was analyzed by real-time RT-PCR. All experiments were repeated three times.

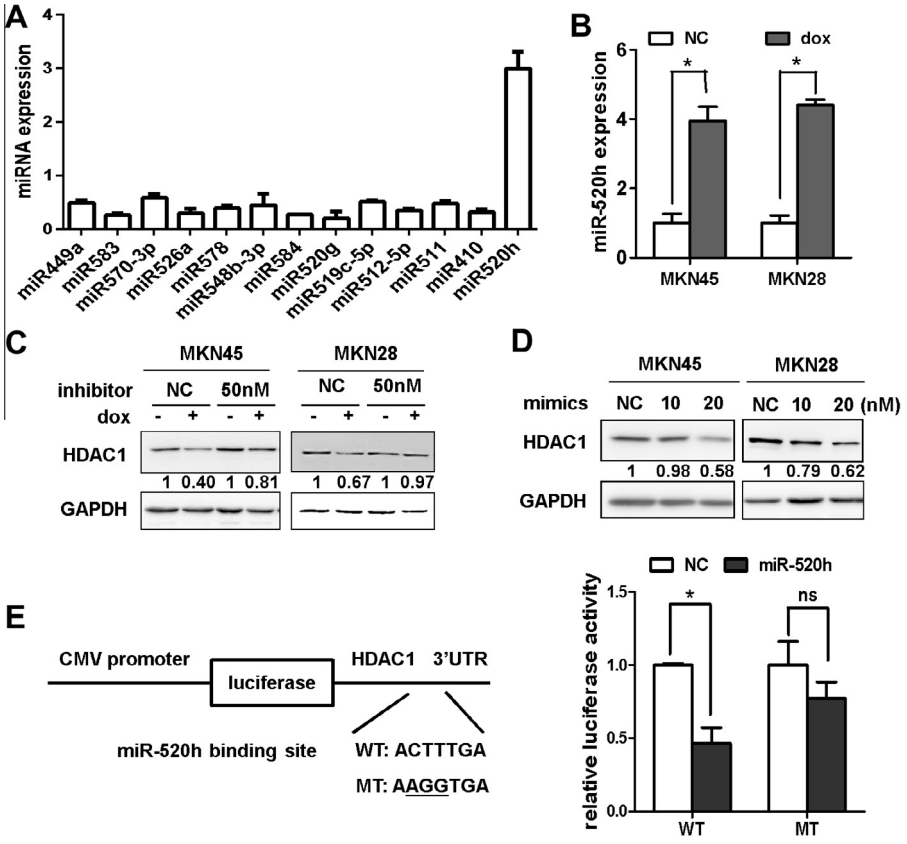

Fig. 3. Down-regulation of HDAC1 induced by doxorubicin was mediated by miR-520h. (A) Potential miRNAs selected by target-prediction tools were screened by real-time RT-PCR analysis of their expression in MKN45 cells after doxorubicin treatment as above mentioned. (B) MiR-520h expression in MKN45 and MKN28 cells after 4 μM doxorubicin treatment for 8 h was analyzed by real-time RT-PCR. (C) Western blot analysis of HDAC1 expression in MKN45 cells and MKN28 cells preincubated with 50 nM miR-520h inhibitor 48 h before doxorubicin treatment. (D) Western blot analysis of HDAC1 expression in MKN45 cells and MKN28 cells treated with 10 nM or 20 nM miR-520h mimics for 8 h. (E) The effect of 20 nM miR-520h mimics on the expression of the luciferase reporter under control of a HDAC1 3′ untranslated region (3′ UTR) was determined by luciferase activity assay. All experiments were repeated three times. The asterisk indicates statistical significance ($P < 0.05$) while ns indicates no statistical significance ($P > 0.05$).

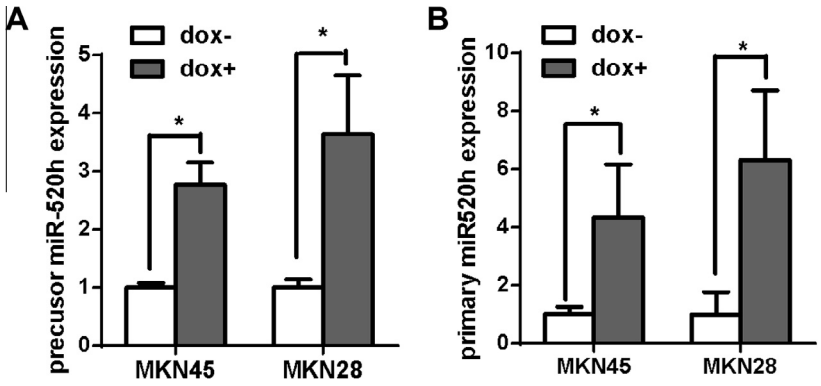

Fig. 4. Doxorubicin up-regulated expression of precursor miR-520h and primary miR-520h. (A) Precursor miR-520h expression in MKN45 and MKN28 cells after 4 μM doxorubicin treatment for 8 h was analyzed by real-time RT-PCR. U6 snRNA served as loading control. B, Primary miR-520h expression in MKN45 and MKN28 cells after 4 μM doxorubicin treatment for 8 h was analyzed by real-time RT-PCR. GAPDH served as loading control. All experiments were repeated three times. The asterisk indicates statistical significance ($P < 0.05$) while ns indicates no statistical significance ($P > 0.05$).

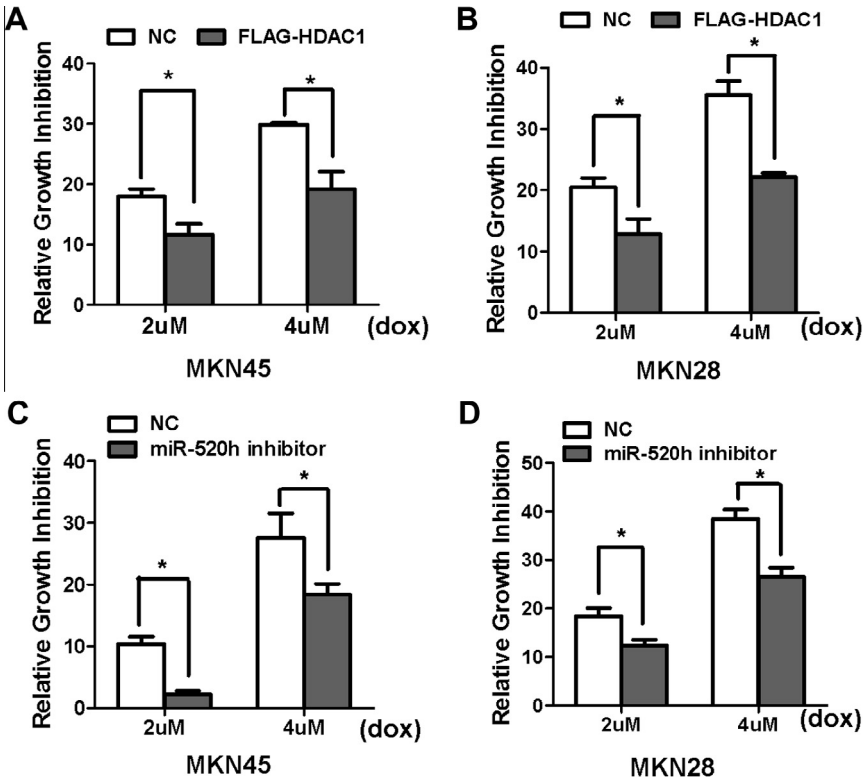

Fig. 5. Up-regulation of HDAC1 enhanced drug resistance to doxorubicin. The viability inhibitory effect of doxorubicin on MKN45 (A and C) and MKN28 (B and D) cells before and after exogenous HDAC1 expression (A and B) or 50 nM miR-520 inhibitor treatment (C and D) were determined by MTS assay. All experiments were repeated for three times. The asterisk indicates statistical significance ($P < 0.05$) while ns indicates no statistical significance ($P > 0.05$).

cloned into the luciferase construct pMIR-REPORT Luciferase vector (Fig. 3E, left panel). A mutant plasmid that contains point mutations at 3 nucleotides within the miR-520h binding site was also constructed. The expression of luciferase driven by the wild-type HDAC1 3′-UTR was significantly down-regulated by 20 nM miR-520h mimics ($P < 0.05$) (Fig. 3E, right panel). In contrast, miR-520h mimics had no effects on the expression of luciferase with mutated HDAC1 3′-UTR ($P > 0.05$). This result indicated that HDAC1 is directly targeted by miR-520h.

2.4. Doxorubicin up-regulates expression of precursor miR-520h and primary miR-520h

The upregulation of microRNAs could be attributed to enhanced gene transcription or increased efficacy of microRNA maturation from primary microRNA transcripts. Therefore, we examined the precursor and primary miR-520h expression after 4 μM doxorubicin treatment. Real-time PCR revealed that doxorubicin caused an increase of precursor miR-520h similar to mature miR-520h (Fig. 4A). Similarly, primary miR-520h was also upregulated after doxorubicin treatment (Fig. 4B), indicating that doxorubicin upregulated miR-520h expression by promoting the transcription rather than the maturation of miR-520h.

2.5. HDAC1 reduces the sensitivity of cancer cells to doxorubicin

Next, we tried to elucidate the relevance of HDAC1 protein down-regulation to doxorubicin-induced anti-cancer effects in gastric cancer cells. The effect of exogenous HDAC1 protein expression on the viability inhibition induced by doxorubicin was

determined by MTS assay in MKN45 and MKN28 cells. HDAC1 was up-regulated (confirmed by Western blot analysis, data not shown) by transfection with HDAC1 expressing plasmid for 48 h. As shown in Fig. 4A and B, up-regulation of HDAC1 attenuated viability inhibition induced by doxorubicin. Furthermore, miR-520h inhibitor, similar to exogenous HDAC1, impaired doxorubicin-induced viability inhibition (Fig. 5C and D). These results indicated that changes in HDAC1 expression are relevant to the anti-cancer effect of doxorubicin.

2.6. HDAC1 reduces doxorubicin–DNA interaction

All results presented above indicated that changes in HDAC1 protein expression are relevant to the anti-cancer effect of doxorubicin. In the next, we wonder how HDAC1 impacted on the response of cancer cells to doxorubicin. Doxorubicin can directly interact with double strand DNA to induce DNA damage and disrupt genome replication and gene transcription [14]. We first determined the intracellular level of γH2AX, an early marker of DNA damages, in doxorubicin-treated cells with or without exogenous HDAC1 expression. As shown in Fig. 6A, the intracellular level of γH2AX in both MKN45 and MKN28 cells were reduced in the presence of exogenous HDAC1, indicating that HDAC1 attenuates doxorubicin-induced DNA damage.

Since HDAC1 participates in chromosome condensation [15,16], we speculated that HDAC1 may interfere with the interaction of doxorubicin with the chromosome. Doxorubicin itself relieves a particular fluorescence at a particular fluorescence (excitation 488 nm/emission 575 nm). When doxorubicin interacted into DNA, its fluorescence intensity of doxorubicin will decrease accordingly [17–19]. Thus, we detected doxorubicin–DNA interaction by measuring fluorescence (excitation 488 nm/emission 575 nm) intensity with the flow cytometry [20]. As shown in Fig. 6B, fluorescence intensity in MKN45 and MKN28 cells transfected with HDAC1 expressing plasmid were higher than fluorescence intensity in cells without exogenous HDAC1 expression, indicating that exogenous HDAC1 indeed impaired doxorubicin–DNA interaction. When miR-520h inhibitor was applied to rescue down-regulation of HDAC1 after doxorubicin treatment, less γH2AX was accumulated (Fig. 6C).

3. Discussion

Although doxorubicin has been widely used as a chemotherapy drug in cancer treatments for many years, molecular mechanisms underlying its anti-cancer effect, side effects and drug-resistance are not well defined. Our studies demonstrated that doxorubicin at therapeutic concentration will epigenetically reduce HDAC1 expression to accelerate DNA damage, and forced expression of HDAC1 will help gastric cancer cells to escape from doxorubicin-induced DNA damage and subsequent cell death.

By deacetylating histones and other cancer-related proteins [21], HDAC1 has emerged as a crucial participant in the development of human cancers. Upregulation of HDAC1 is prevalent in various kinds of malignant tumors including gastric cancer [22–24]. Acetylation of histone would neutralize positive charge, resulting in an open state of the chromatin [25]. Furthermore, HDAC1/2 directly interact with DNA topoisomerase II [15], an enzyme known critical in chromatin organization [26]. Interestingly, DNA topoisomerase II can also be inhibited by doxorubicin. Clinical trials of combined HDACi and doxorubicin in advanced solid tumors indicate synergized effect of HDAC inhibitor and doxorubicin. In the current study, we found that doxorubicin induces HDAC1 down-regulation specifically, thus loosing chromatins to expose more potential doxorubicin binding sites (Fig. 6). When doxorubicin induced down-regulation of HDAC1 is rescued by exogenous HDAC1 expression, DNA double strand damage is decreased accordingly. Therefore, the upregulation of HDAC1 may lead to the resistance to doxorubicin. Indeed, it has been demonstrated

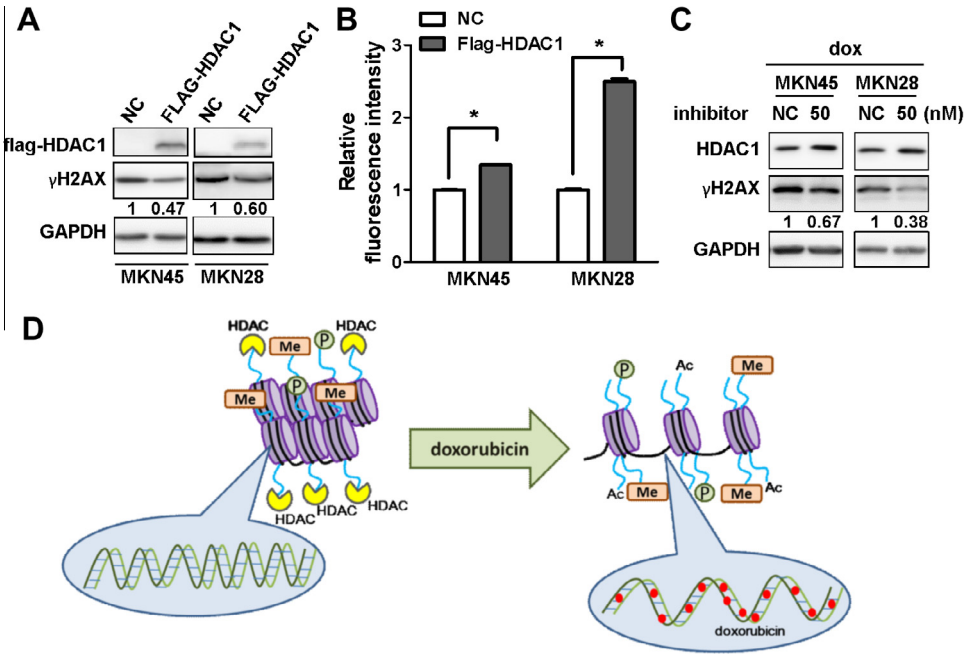

Fig. 6. Up-regulation of HDAC1 reduced doxorubicin–DNA interaction. (A) Western blot analysis of γH2AX level in MKN45 and MKN28 transfected with flag-HDAC1 expressing plasmid 48 h before doxorubicin treatment. (B) Flow cytometry analysis of fluorescence intensity in doxorubicin-treated MKN45 and MKN28 cells transfected with or without HDAC1 expressing plasmid. The asterisks indicate statistical significance ($P < 0.05$). All experiments were repeated for three times. Error bars represent the SD of data points generated from experiments. (C) Western blot analysis of γH2AX level in MKN45 and MKN28 preincubated with miR-520h inhibitor 48 h before doxorubicin treatment. All experiments were repeated three times. (D) Proposed Model: doxorubicin downregulates HDAC1 expression to aggravate DNA-doxorubicin interaction by inducing the expression of HDAC1-targeting miR-520h.

that HDAC1 protects cells from DNA-damage [8]. Specific and potent inhibitors of HDAC1 can synergize with doxorubicin to inhibit the expression and activity of HDAC1 and exacerbate DNA damage to induce cell death. Moreover, HDAC1 inhibitors may also be used as the second line treatment for the patients with drug-resistance to doxorubicin and HDAC1 upregulation might be the indicative biomarker for such target therapies.

MicroRNAs (miRNAs) mediate gene silencing by binding the 3′ or 5′ untranslated regions (UTRs) of its target mRNA. Abnormal expression of miRNAs contribute to the development of human cancers [27–29]. MiRNAs are also implicated in the regulation of DNA damage response by targeting checkpoint proteins [30,31]. Cellular survival and checkpoint response after DNA damage were severely affected once miRNA-mediated gene-silencing was inhibited. For example, miR-185 enhances radiation-induced apoptosis and inhibition of proliferation by repressing ATR pathway.

Moreover, several miRNAs have been proved to regulate chemosensitivity in cancer cells [32]. For example, miR-129 promotes apoptosis and enhances sensitivity of colorectal cancer cells to 5-fluorouracil [33]. In this study, we find a new miRNA to regulate DNA damage response and drug sensitivity. MiR-520h is upregulated by doxorubicin to target HDAC1 and sensitizes gastric cancer cells to doxorubicin (Fig. 6).

The sensitivity of cancer cells to doxorubicin can be regulated by some known miRNAs such as miR-451 [34], indicating that induction or activation of such miRNAs might be the potential approach to improve the clinical efficacy of doxorubicin based treatment.

Certainly, the result of this in vitro study should be confirmed in vivo. In addition, although MTT or MTS based cell viability assays have been widely used to evaluate the growth inhibitory effect of doxorubicin, other examinations on cell cycle or cell death should be performed to investigate the inhibition effect of doxorubicin on cell growth.

In summary, doxorubicin down-regulates HDAC1 expression to aggravate DNA–doxorubicin interaction by inducing the expression of HDAC1-targeting miR-520h. The upregulation of HDAC1 may contribute to drug resistance of human cancer cells and targeting HDAC1 is a promising strategy to increase the clinical efficacy of DNA damage-inducing chemotherapeutic drugs.

4. Materials and methods

4.1. Cell culture and doxorubicin treatment

MKN45 and MKN28 human gastric cancer cells were obtained from RIKEN BioResource center (Ibaraki, Japan). MKN45 represented the poorly differentiated gastric cancer cells and MKN28 represented the well differentiated cells. MKN45 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum and MKN28 cells were cultured in DMEM medium (Invitrogen) with 10% fetal bovine serum. Both of them were incubated at 5% CO_2, 37 °C, and 95% humidity. In the cell viability analysis, cells were cultured with 0.25 μM, 0.5 μM, 1 μM, 2 μM and 4 μM doxorubicin respectively for 24 h, 48 h, 72 h. In the Western blot analyses, cells were cultured with 4 μM doxorubicin for 8 h.

4.2. Cell viability assay (MTS)

Cell viability was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA). The cell viability was measured following instructions provided [35] by using a colorimetric method with 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS).

4.3. Western blotting

Cell lysates harvested by 1 × SDS–PAGE sample buffer (120 mM Tris–HCl, pH 6.8, 4% SDS, 50 mM DTT, 2% glycerol and 0.01% bromophenol blue). The same amount of protein from each lysate was boiled for 10 min. The boiled lysates were resolved by SDS–PAGE, transferred to PVDF membranes. Then the membranes were incubated with the primary antibodies and corresponding second antibodies. Digital images were acquired with enhanced chemiluminescence from Millipore (Billerica, MA, USA). Anti-GAPDH antibodies were obtained from Cell Signaling Technology (Boston, MA, USA). Anti-HDAC1, anti-HDAC2 and Anti-γH2AX antibodies were from Millipore. Anti-HDAC3 antibodies were from Epitomics (Burlingame, CA, USA). Anti-HDAC 8 antibodies were from Proteintech (Chicago, IL, USA). Anti-Flag antibodies were from Sigma (St. Louis, MO, USA). Laser density quantification was performed by Quantity One.

4.4. Plasmid transfection

Flag-HDAC1 plasmid was obtained from Gene Copoecia (Rockville, MD, USA). Cells were seeded overnight in six-well plates (2×10^5/well) and 2 μg of plasmids were transfected with FuGENE HD (Roche Applied Science, Mannheim, Germany). Cells were transfected for 48 h in six-well plates, and then transferred into a 96-well plate to pre-incubated with doxorubicin for 24 h before analyzed by MTS assay. For other analyses, cells were harvested for protein extraction after indicated timings.

4.5. RNA isolation and quantitative RT–PCR

Total RNA were isolated from cells grown in six-well plates and pre-incubated with doxorubicin for 8 h to characterize HDAC1 mRNA expression and miR-520h expression in MKN45 and MKN28 cells. The TRIzol RNA extraction kit (Invitrogen) was used for mRNA extraction and miRNeasy Mini Kit (Qiagen, Hilden, Germany) for microRNA extraction Total RNA was isolated using the TRIzol RNA extraction kit (Invitrogen) and microRNA was extracted RNA concentrations were quantified by NanoDrop 1000 (Nanodrop, Wilmington, Del, USA). The RNA integrity was evaluated by agarose gel electrophoresis in addition to Nanodrop analysis. Moreover, we designed primers for RT-PCR in different exons so that DNA contamination should not be a concern. Reverse transcription reaction was performed using 1 μg of total RNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) or miScript miRNA detection system (Qiagen). Real-time quantitative RT-PCR was performed using SYBR Green Real-Time PCR System (Applied Biosystems). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 was used as an internal control for mRNA or miRNA quantification. Real-time quantitative RT-PCR for miRNAs was detected by SYBR Green miScript System (Qiagen). Primers used for RT-PCR were listed in Table 1.

4.6. Luciferase activity assay

HDAC1 3′ UTR fragments with wild or mutant miR-520h binding sites were cloned by PCR. Primers used were included in Table 1. PCR products were inserted into pMD18T vector (Takara, Dalian, LN, China) for sequence validation. The correct insert was sub-cloned into pMIR-REPORT Luciferase vectors (Applied Biosystems). Cells (1×10^5) were transfected with different pMIR-REPORT Luciferase vectors into MKN45 cells in the absence or presence of miR-520h mimics. 48 h after transfection, the activities of firefly luciferase and renilla luciferase were measured using the Dual-Glo™ luciferase assay system (Promega, USA) in accordance

Table 1
Primers used in this study.

Primers	
hHDAC1-F	GGTTCTGTGGCAAGTGCTGT
hHDAC1-R	CTTGGCGTGTCCTTTGATAG
hMIR-511	GTGTCTTTTGCTCTGCAGTC
hMIR-449a	GGCAGTGTATTGTTAGCTGGT
hMIR-583	CAAAGAGGAAGGTCCCATTAC
hMIR-570-3p	CGAAAACAGCAATTACCTTTGC
hMIR-526a	TCTAGAGGGAAGCACTTTCTG
hMIR-578	CTTCTTGTGCTCTAGGATTGT
hMIR-548b-3p	CAAGAACCTCAGTTGCTTTTGT
hMIR-520g	ACAAAGTGCTTCCCTTTAGAGTGT
hMIR-519c-5p	CTCTAGAGGGAAGCGCTTTC
hMIR-512-5p	CACTCAGCCTTGAGGGCAC
hMIR-410	AATATAACACAGATGGCCTGT
hMIR-520h	ACAAAGTGCTTCCCTTTAGAGT
HDAC1-3′ UTR-F	GGACTAGTCCAGGGACAGAAACCAAG
HDAC1-3′ UTR-R	GCGAAGCTTGGAGAAGACAGACAGAGGG
HDAC1-3′ UTR-mutant-F	CTTGCCACCCATTCTTCCCGTTCTTAAAGGTGAAC CATAAAGGGTGC
HDAC1–3′ UTR-mutant-R	GCACCCTTTATGGTTCACCTTTAAGAACGGGAAGA ATGGGTGGCAAG
Precusor miR	TCCCATGCTGTGACCCTCTAG
Primary miR	TTCTGGATTCCAGAAAATATGC
Primary miR	TCCCAAACAGTAACTCTAAAGG

with the manufacturer's protocols. Relative luciferase activity was normalized with renilla luciferase activity and comparison between wild and mutant pMIR-REPORT Luciferase vector was made [29].

4.7. Flow cytometry analysis

Cells were washed twice with cold 1 × PBS and then suspended in 1 × PBS at a concentration of 1×10^6 cells/ml. The samples were then sent out for analysis by flow cytometry (excitation 488 nm/emission 575 nm) immediately. The normalized mean fluorescence intensity was calculated as $(c - a)/(d - b)$ [20]. (a) Fluorescence intensity of positive-fluorescence cells without doxorubicin treatment, without transfected with HDAC1 expressing plasmid (represented for background fluorescence intensity of HDAC1 low-expressed cells). (b) Fluorescence intensity of positive-fluorescence cells without doxorubicin treatment, with transfected with HDAC1 expressing plasmid for 48 h (represented for background fluorescence intensity of HDAC1 high-expressed cells). (c) Fluorescence intensity of positive-fluorescence cells with doxorubicin treatment, without transfected with HDAC1 expressing plasmid. (d) Fluorescence intensity of positive-fluorescence cells with doxorubicin treatment, with transfected with HDAC1 expressing plasmid for 48 h.

4.8. Statistical analysis

Data were presented as the mean ± S.D. from at least three independent experiments. Unless specifically indicated, the non-parametric Mann–Whiney test was used for a comparison between two groups. $P < 0.05$ indicates statistical significance.

Disclosure

All authors declare no conflict of interest.

Author Contributions

QS, QY, LF, JS, HL, YM, LL, FW, JL and YY performed experiments; QS, HJ and XW conceived the project, analyzed data and drafted the manuscript.

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