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ORIGINAL ARTICLE

MicroRNA-141 confers resistance to cisplatin-induced apoptosis by targeting *YAP1* in human esophageal squamous cell carcinoma

Yukako Imanaka[1], Soken Tsuchiya[2], Fumiaki Sato[2], Yutaka Shimada[3], Kazuharu Shimizu[2] and Gozoh Tsujimoto[1]

MicroRNAs (miRNAs) are endogenous non-coding RNAs that function as negative regulators of gene expression. Alterations in miRNA expression have been shown to affect tumor growth and response to chemotherapy. In this study, we explored the possible role of miRNAs in cisplatin resistance in esophageal squamous cell carcinoma (ESCC). First we assessed the sensitivity of nine human ESCC cell lines (KYSE series) to cisplatin using an *in vitro* cell viability assay, and then we compared the miRNA profiles of the cisplatin-sensitive and -resistant cell lines by miRNA microarray analysis. The two groups showed markedly different miRNA expression profiles, and 10 miRNAs were found to be regulated differentially between the two groups. When miR-141, which was the most highly expressed miRNA in the cisplatin-resistant cell lines, was expressed ectopically in the cisplatin-sensitive cell lines, cell viability after cisplatin treatment was increased significantly. Furthermore, we found that miR-141 directly targeted the 3′-untranslated region of *YAP1*, which is known to have a crucial role in apoptosis induced by DNA-damaging agents, and thus downregulated *YAP1* expression. Our study highlights an important regulatory role for miR-141 in the development of cisplatin resistance in ESCC.
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Keywords: apoptosis; cisplatin resistance; esophageal carcinoma; microRNA; *YAP1*

INTRODUCTION

MicroRNAs (miRNAs) are a class of small (~22 bp) endogenous non-coding RNAs that are well conserved, and function as negative regulators of gene expression. miRNAs bind to complementary sequences in the 3′-untranslated region (UTR) of target messenger RNAs and regulate their expression by cleavage and/or translational inhibition.[1] miRNAs are predicted to regulate the expression of up to one-third of human protein-coding genes,[2–5] and they have been shown to have crucial roles in diverse biological processes, including development, differentiation, apoptosis and proliferation.[6–8] A growing number of studies have provided strong evidence that aberrant miRNA expression is involved in the genesis and progression of cancer,[9] and that miRNAs might function as a novel class of oncogenes or tumor-suppressor genes.[10–13] Altered expression of miRNAs in primary human cancers has been used for tumor diagnosis, classification, staging and prognosis.[14] Furthermore, the involvement of miRNAs in the response of tumor cells to chemotherapeutic agents has also been confirmed,[15–17] which suggests that miRNAs could have a broad effect on the response of cancer cells to chemotherapy.

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer deaths worldwide.[18] In spite of comprehensive available treatment, including chemotherapy, surgery and radiotherapy, the overall 5-year survival rate for patients with esophageal squamous cell carcinoma (ESCC), the most common form of esophageal cancer, remains low, at 10–40%, because of advanced disease, metastasis and resistance of the tumor to chemotherapy and radiotherapy.[19–21] Cisplatin is the most frequently used chemotherapeutic agent for ESCC. However, given that resistance to cisplatin limits the success of treatment, elucidation of the mechanisms that regulate cisplatin resistance in ESCC is urgently needed. In the present study, we studied the biological function of miRNAs in the development of cisplatin resistance in ESCC using the KYSE series of ESCC cell lines as a model, and focused, in particular, on the regulation of apoptosis.

MATERIALS AND METHODS

Cell lines and cultures

Human KYSE cell lines that had been established from primary tumors at our institution as described previously were cultured in RPMI 1640

[1]Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan; [2]Department of Nanobio Drug Discovery, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan and [3]Department of Surgery and Science, Graduate School of Medicine and Pharmaceutical Sciences for Research, Toyama University, Toyama, Japan
Correspondence: Dr G Tsujimoto, Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan.
E-mail: gtsuji@pharm.kyoto-u.ac.jp
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(Life Technologies, Gaithersburg, MD, USA) and Ham's F12 (Nissui Pharmaceutical, Tokyo, Japan) with 5% fetal bovine serum.[22] HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum. Cells were cultured at 37°C with 5% CO_2.

In vitro cell viability assay

KYSE cell lines were seeded in 96-well plates and incubated for 24 h. The medium was then removed and replaced with fresh medium that contained cisplatin (Calbiochem, San Diego, CA, USA) or Dimethyl sulfoxide (DMSO) (vehicle control) and the cells were incubated for a further 48 h. Cell viability was examined using the 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt (WST-1) assay.

miRNA microarray analysis

Total RNA was isolated from the KYSE cell lines with IsoGen lysis buffer (Nippon Gene, Toyama, Japan) followed by precipitation with isopropanol, and the size of the miRNA fractions was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The miRNAs were then labeled with Hy5 using a miRCURY LNA microRNA Power Labeling Kit (Exiqon, Woburn, MA, USA) and hybridized with a Human miRNA Oligo chip (Toray, Tokyo, Japan). Arrays were scanned using a ProScanArray laser scanning system (Perkin-Elmer, Waltham, MA, USA), and processed and analyzed with Genepix Pro 4.0 software (Axon Instruments, Sunnyvale, CA, USA). The GEO database accession code of the miRNA microarray data is GSE25464.

TaqMan RT-PCR for miRNA quantification

Expression levels of mature miRNAs were analyzed by real-time PCR using the TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA, USA). Reactions were performed using an Applied Biosystems 7300 instrument with an initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

In vitro drug sensitivity assay

KYSE960 cells (1.7×10^4 per well) were seeded in 96-well plates and transfected with either Pre-miR miR-141 precursor or Pre-miR miRNA Precursor-Negative Control #1 (AM17110) (Ambion, Austin, TX, USA) using the HiPerFect Transfection Reagent (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. At 24 h after transfection, cells were treated with cisplatin (7.5, 15, 30, 60 or 120 μM) or DMSO for a further 48 h and then collected for analysis. Cell viability was assessed by the WST-1 assay.

Trypan blue dye exclusion assay

At 24 h after transfection, cells were treated with cisplatin (30 μM) or DMSO and collected for analysis after 0, 24, 48 and 72 h. An equal volume of 0.4% Trypan Blue Stain (Invitrogen, Carlsbad, CA, USA) was added to the cell suspensions, which were then allowed to stand for 5 min at room temperature. Stained cells (10 μl) were placed in a hemocytometer and the number of viable (unstained) cells was counted for each individual time point.

Western blotting

At 72 h after transfection, total protein was extracted from the cells using RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, protease inhibitor cocktail). Equivalent amounts of total protein extract were separated on 8% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. The blots were probed for 1 h at room temperature with antibodies against YAP1 (sc-15407, Santa Cruz, Santa Cruz, CA, USA; 1:200) or β-actin (Sigma-Aldrich; 1:1000), which was used as an internal control for protein loading. The protein bands were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer).

Quantitative RT-PCR

In parallel, total RNA was isolated from transfected cells as described above. Total RNA (3 μg) was exposed briefly to RNase-free DNase I, and reverse transcribed to cDNA using random primers and SuperScriptII Reverse Transcriptase (Invitrogen). Subsequently, real-time PCR was performed in triplicate using the SYBR Premix Ex Taq II reagent (TAKARA BIO, Shiga, Japan) and a DNA Engine Opticon 2 System (Bio-Rad, Hercules, CA, USA). The PCR primers used for *YAP1* were 5′-GTAGCCAGTTACCAACACTG-3′ and 5′-CTGTTCAGGAAGTCATCTGG-3′. The housekeeping gene *GAPDH* was used as an endogenous control for RNA normalization. The following PCR conditions were used: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 20 s and extension at 72°C for 20 s. PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. All messenger RNA quantification data were normalized to GAPDH.

Target *in vitro* reporter assay

Constructs were generated in which the wild-type (pGL3-YAP1-3′UTRWT) and mutated (pGL3-YAP1-3′UTRmut) 3′-UTR of *YAP1* was inserted downstream of a luciferase reporter. The 3′-UTR of human *YAP1*, which contains a putative target site of miR-141, was amplified by PCR from human genomic DNA using the primers 5′-ATGGTTGATGGAGCACATTG-3′ and 5′-CCTAACATATGAGCATGCTC-3′, and inserted into the *Eco*RI site, immediately downstream of the luciferase gene in the pGL3-promoter vector (Promega, Madison, WI, USA). Three point mutations in the miR-141 seed region of the *YAP1* 3′-UTR were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The following primers were used for the mutagenesis of the miRNA-binding site: 5′-CAGAATTCATACCAATCTGAGATGAAACTCAAACATTGC-3′ and 5′-GCAATGTTTGAGTTTCATCTCAGATTGGTATGAATTCTG-3′. Wild-type and mutant inserts were verified by DNA sequencing. HEK293 cells (2×10^5 per well) were co-transfected in 24-well plates with 1 μg of the firefly luciferase reporter vector and 100 ng of pRL-TK (a control vector that contains *Renilla* luciferase; Promega), as well as with 5 pmol of miR-141 or a control precursor (Ambion) or with 25 pmol of miR-141 or a control inhibitor (Dharmacon, Lafayette, CO, USA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase activity was measured at 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega). For each well, firefly luciferase activity was normalized to *Renilla* luciferase activity.

Apoptosis assay

At 24 h after transfection, cells were treated with cisplatin (30 μM) or DMSO for a further 48 h and then collected for analysis. Apoptosis was assayed using a FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Apoptotic cells were indicated by high levels of Annexin V-conjugated FITC fluorescence and low levels of propidium iodide fluorescence.

Statistical analysis

Statistical significance was assessed using an unpaired Student's *t*-test. $P<0.05$ was considered to be statistically significant. Data were expressed as mean ± s.e.m.

RESULTS

Cisplatin-sensitive and -resistant human ESCC cell lines

To classify the ESCC cell lines (KYSE series) into cisplatin-sensitive and -resistant cell lines, the cell viability of nine KYSE cell lines in the presence of cisplatin was analyzed using the WST-1 assay (Figure 1). Cisplatin treatment (66.7 μM, 48 h) resulted in a decrease in cell viability of 0–80%, as compared with cells treated with the vehicle DMSO. Among the cell lines examined, KYSE890 and KYSE960 showed the highest sensitivity to cisplatin treatment (~20% cell viability), whereas KYSE450 and KYSE520 showed the lowest sensitivity to cisplatin treatment (~80–100% cell viability). We consequently designated the cell lines KYSE890 and KYSE960 as cisplatin sensitive and KYSE450 and KYSE520 cell lines as cisplatin resistant.

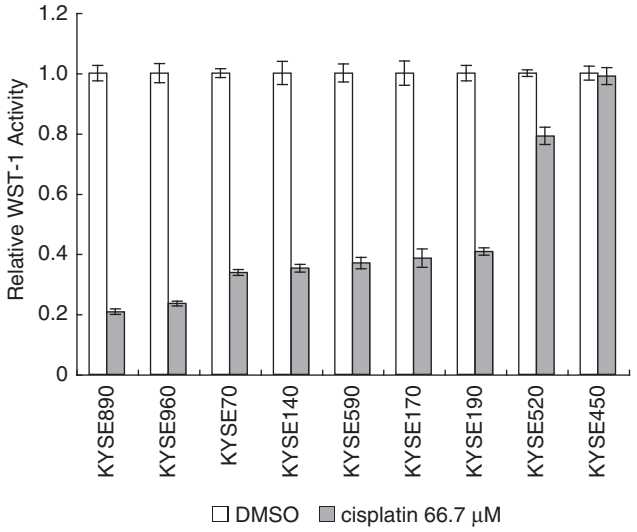

Figure 1 Designation of cisplatin-sensitive and -resistant human ESCC cell lines. The WST-1 activity of cells treated with DMSO was designated as 1 and the relative WST-1 activity is shown.

miR-141 is highly expressed in cisplatin-resistant ESCC cell lines

We then compared the expression of miRNAs in cisplatin-sensitive and -resistant KYSE cell lines using miRNA microarray analysis. Total RNA was isolated from the KYSE cell lines and hybridized to a custom miRNA microarray platform that contained 849 miRNAs. The global miRNA expression analyses (hierarchical clustering and principal component analysis) showed that the expression profiles of the miRNAs differed between the cisplatin-sensitive and -resistant cell lines (Figure 2), and expression levels of 45 miRNAs were changed by more than fourfold in cisplatin-sensitive cell lines as compared with cisplatin-resistant cell lines (Supplementary Table 1). Subsequently, the expression levels of the 10 miRNAs that were selected according to the miRNA microarray data and literature search[23–39] were validated by quantitative reverse transcription-PCR. This confirmed that miR-141, miR-21, miR-19b, miR-200a, miR-19a, miR-27a, miR-20a and miR-20b were expressed at significantly higher levels in the cisplatin-resistant lines, and miR-205 and miR-224 at significantly lower levels than in the cisplatin-sensitive cell lines ($P<0.05$) (Table 1). Notably, miR-141 was upregulated the most in the cisplatin-resistant lines as in contrast with the cisplatin-sensitive lines (87-fold, $P=0.01$) (Figure 3).

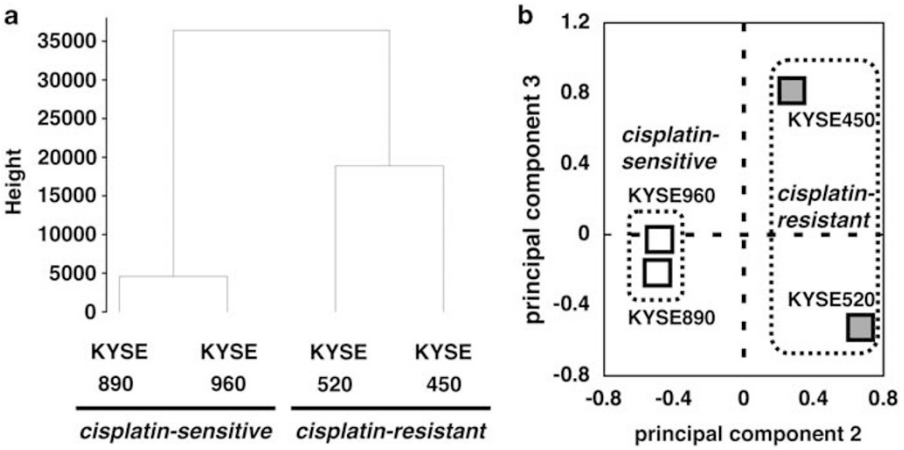

Figure 2 Global miRNA expression analysis of ESCC cell lines. Hierarchical clustering (**a**) and principal component analysis (**b**) of global miRNA expression in ESCC cell lines. These analyses reveal different miRNA expression profiles between cisplatin-sensitive and -resistant cell lines.

Table 1 List of miRNAs that were expressed differentially in cisplatin-sensitive and cisplatin-resistant cell lines

	Expression level of miRNA (mean ± s.e.m.)		*Fold change*	P-*value*
miRNAs	*Cisplatin-sensitive cell lines*	*Cisplatin-resistant cell lines*	*Resistant/sensitive*	*Resistant vs sensitive*
miR-141	189.67 ± 24.95	16587.80 ± 4145.71	87.45	0.0108
miR-21	64.57 ± 10.62	3157.66 ± 511.71	49.90	0.0018
miR-19b	1717.65 ± 526.16	59809.56 ± 5297.46	34.82	0.0001
miR-200a	161.26 ± 45.91	2024.54 ± 105.95	12.55	0.0001
miR-19a	383.92 ± 95.89	27437.61 ± 1074.00	71.47	0.0001
miR-27a	84.33 ± 19.15	2313.07 ± 278.58	27.43	0.0005
miR-20a	1620.13 ± 435.78	79664.13 ± 15638.85	49.17	0.0041
miR-20b	57.90 ± 18.89	1063.99 ± 225.53	18.38	0.0065
miR-205	22770.74 ± 3752.22	2360.90 ± 623.31	0.10	0.0026
miR-224	1680.99 ± 131.29	444.79 ± 283.08	0.26	0.0054

Abbreviation: miRNA, microRNA.

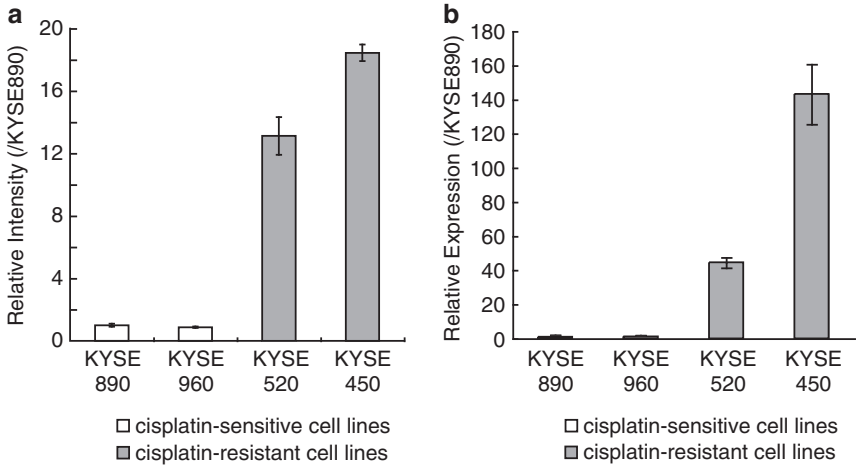

Figure 3 miR-141 is highly expressed in cisplatin-resistant KYSE cell lines. (**a**) miRNA microarray analysis. The data shown present the signal intensity of miR-141 relative to the signal intensity of miR-141 in KYSE890, which was set as 1. (**b**) Quantitative reverse transcription-PCR analysis of miR-141 was carried out to validate the microarray results. The data shown present levels of miR-141 relative to that in KYSE890, which was set as 1.

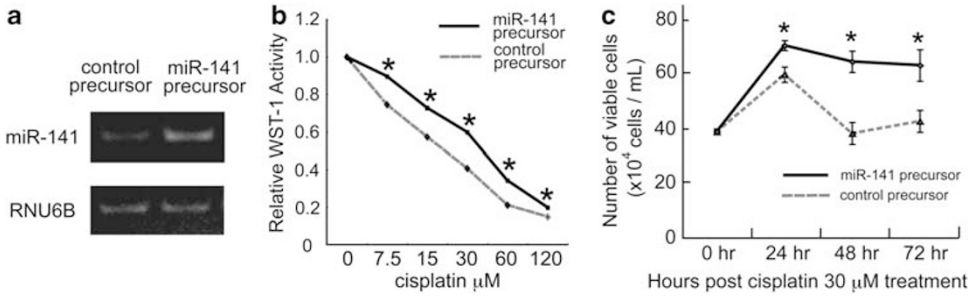

Figure 4 Ectopic expression of miR-141 induces cisplatin resistance in KYSE960 cell lines. (**a**) Ectopic expression of miR-141. KYSE960 cell lines, which express relatively low levels of endogenous miR-141, were transfected with the miR-141 or control precursor. Quantitative reverse transcription-PCR was used to analyze the expression of miR-141 (top) and *RNU6B* (bottom) in these cells. (**b**) Cell viability was assessed by the WST-1 assay. The WST-1 activity of cells treated with DMSO was taken as 1. (**c**) Trypan blue dye exclusion assay. The time at which the cells were treated with cisplatin was considered to be time 0. The number of viable cells at the respective time points is shown. $*P<0.05$, between miR-141 precursor-transfected cells and control precursor-transfected cells.

Ectopic expression of miR-141 confers cisplatin resistance in cisplatin-sensitive cell lines

To investigate whether miR-141 is involved directly in the development of cisplatin resistance, we examined the effects of miR-141 on cisplatin sensitivity. We expressed the miR-141 precursor ectopically in the cisplatin-sensitive cell lines, because they express relatively low levels of endogenous miR-141, and examines whether miR-141 expression rendered the cells resistant to cisplatin-induced cell death. Following transfection of the miR-141 precursor, the cells were collected and ectopic expression of miR-141 was confirmed by quantitative reverse transcription-PCR (Figure 4a). Cells transfected with the control precursor were used as controls. The results of the WST-1 assay showed that the KYSE960 cells that had been transfected with the miR-141 precursor exhibited a markedly reduced sensitivity to varying concentrations of cisplatin (7.5, 15, 30, 60 or 120 μM) (Figure 4b). Similar findings were obtained with KYSE890 cells (data not shown). Moreover, the trypan blue dye exclusion assay revealed that the proportion of KYSE960 cells, which remained viable after treatment with cisplatin (30 μM), was significantly ($P<0.05$) elevated at each time point in cells that overexpressed miR-141: 18, 68 and 48% at 24, 48 and 72 h after cisplatin treatment, respectively (Figure 4c). These results indicated that ectopic expression of miR-141 could confer cisplatin resistance in KYSE cell lines by enhancing their growth and viability.

miR-141 represses *YAP1* expression post transcriptionally

In an effort to elucidate the mechanism of induction of cisplatin resistance by miR-141, we searched for potential target(s) of miR-141 using the TargetScan database (http://www.targetscan.org/). Among the predicted 429 candidate genes, we studied the functional role of human Yes-Associated Protein (*YAP1*) (NM_006106) further, because it has been reported to be a cisplatin-induced apoptosis-related gene.[40] First, we investigated the effects of transfection of the miR-141 precursor on *YAP1* expression in cisplatin-sensitive KYSE cell lines. The quantitative reverse transcription-PCR and western blotting analyses revealed that expression levels of YAP1 messenger RNA and protein were decreased in miR-141 precursor-transfected cells as compared with control precursor-transfected cells (Figure 5a), which indicated that the expression of *YAP1* was inhibited by miR-141. Furthermore, a significant ($P<0.01$) decrease in relative luciferase activity was noted when the miR-141 precursor was co-transfected with the wild-type, but not with the mutant, YAP1-3′-UTR reporter (Figures 5b and c).

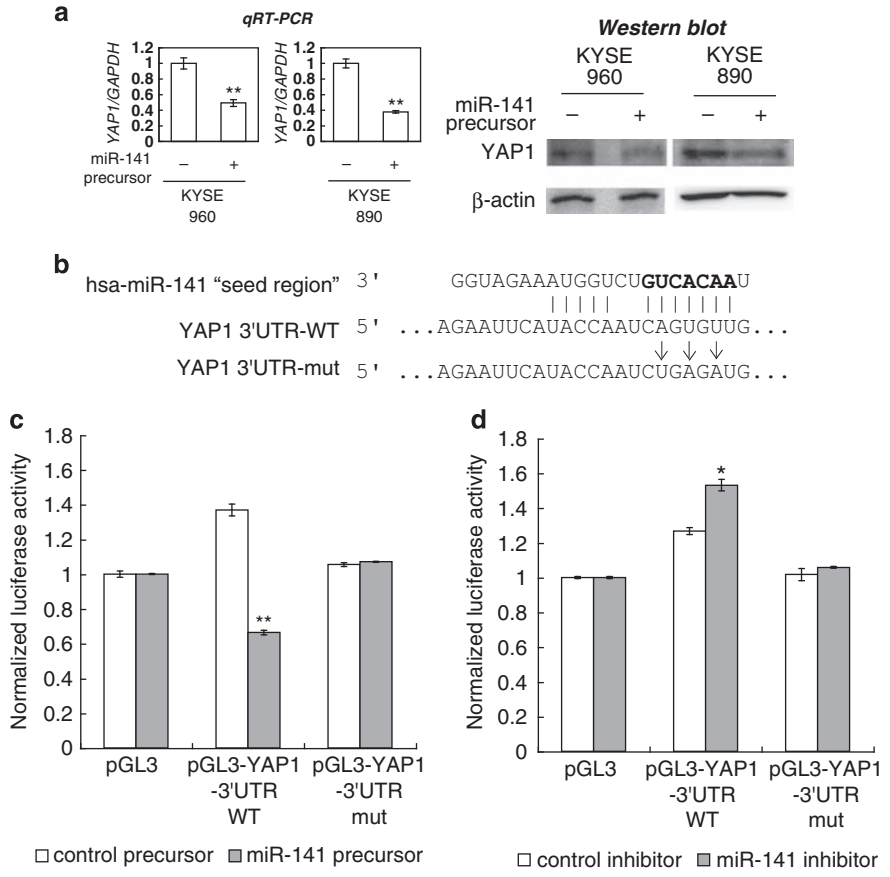

Figure 5 miR-141 negatively regulates YAP1 by binding to a complemetary site in the *YAP1* 3′-UTR. (**a**) miR-141 reduces the expression levels of YAP1 messenger RNA and protein. Expression levels of YAP1 messenger RNA (left panels) and protein (right panels) were assessed 72 h after transfection of the miR-141 precursor or control precursor in cisplatin-sensitive KYSE cell lines. GAPDH for messenger RNA levels and β-actin for protein levels were used as controls. (**b**) Sequence alignment of human miR-141 with the 3′-UTR of *YAP1*. The seed sequence of miR-141 (top) was complementary to a sequence in the 3′-UTR of *YAP1* (middle). Bottom, three point mutations were introduced into the 3′-UTR of *YAP1* to create the mutant luciferase reporter construct. (**c**) miR-141 inhibits the wild-type YAP1-3′UTR reporter but not the mutated version. (**d**) The activity of the wild-type reporter but not the mutated version is upregulated by knockdown of miR-141. For each sample, firefly luciferase activity was normalized to *Renilla* luciferase activity. $^{**}P<0.01$, $^{*}P<0.05$, between miR-141 precursor-transfected cells and control precursor-transfected cells.

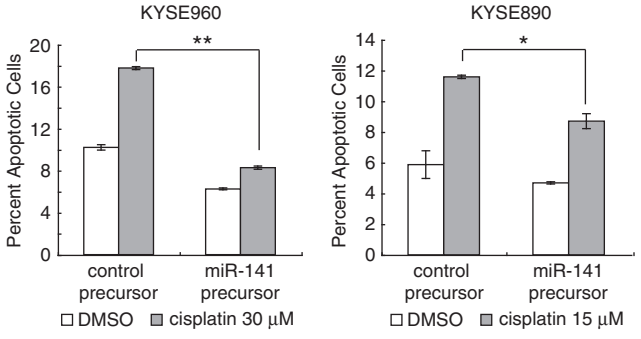

Figure 6 Ectopic expression of miR-141 renders cisplatin-sensitive cell lines resistant to cisplatin-induced apoptosis. KYSE960 and KYSE890 cell lines were transfected with the miR-141 or control precursor. After 24 h, cisplatin was added in fresh medium and the cells were incubated for a further 48 h. The cells were then labeled with FITC-Annexin V and propidium iodide, and apoptosis was analyzed by flow cytometry. The percentage of apoptotic cells is shown. $^{**}P<0.01$, $^{*}P<0.05$, between miR-141 precursor-transfected cells and control precursor-transfected cells.

In contrast, when the wild-type reporter was co-transfected with the miR-141 inhibitor, the relative luciferase activity of the reporter was significantly ($P<0.05$) enhanced (Figure 5d). These results show that miR-141 interacts directly with the predicted target sequence in *YAP1*.

miR-141 exerts an anti-apoptotic effect that confers cisplatin resistance in ESCC cell lines

Given that one of the target genes of miR-141 is *YAP1*, which is a transcriptional factor that promotes the expression of proapoptotic genes during apoptosis induced by DNA-damaging agents, we explored the regulatory mechanism by which miR-141 inhibits cisplatin-mediated apoptosis further. The Annexin V/propidium iodide assay showed that apoptosis of the cisplatin-sensitive ESCC cell lines (KYSE960 and KYSE890) in response to cisplatin was enhanced markedly compared with that of the cisplatin-resistant cells (data not shown). In both KYSE960 and KYSE890 cells, transfection of the miR-141 precursor, but not the control precursor, significantly decreased the percentage of cisplatin-induced apoptotic cells (Figure 6). Taken collectively, these results show that the anti-apoptotic effect of miR-141, perhaps through inhibition of *YAP1*,

might explain how miR-141 confers cisplatin resistance in ESCC cell lines.

DISCUSSION

In the present study, we explored the possible role of miRNAs in cisplatin resistance in ESCC. By comparing the expression of miRNAs in cisplatin-sensitive and -resistant KYSE series, we found 10 miRNAs that were expressed differentially between these lines. Among them were some miRNAs, such as miR-21, miR-20b, miR-205, miR-224, miR-27a, miR-200a and miR-141, which are known to be associated with cancer. miR-21 has been reported to be ubiquitously overexpressed in diverse tumors, including both esophageal adenocarcinoma and ESCC,[23] and it regulates proliferation and invasion in ESCC.[24] In addition, inhibition of miR-21 has been shown to increase the sensitivity of NCI60 and cholangiocarcinoma cell lines to chemotherapeutic agents.[17,25] miR-20b is highly overexpressed in ESCC and gastric cancer,[26,27] and its high-expression level is associated with a lower probability of survival.[28] The expression of miR-205 is highly specific for squamous epithelium,[29] and it has been shown to be downregulated in both esophageal adenocarcinoma and ESCC.[30] miR-205 has also been found to function as an oncosuppressor in breast cancer and to improve responsiveness to tyrosine kinase inhibitor therapies.[31] Furthermore, miR-224, miR-27a and miR-200a have also been associated with hepatocellular carcinoma, ESCC and ovarian cancer, respectively.[32–34] miR-141 is associated with various types of cancer.[35–39] Given that miR-141 was found to be either upregulated (ovarian and colorectal cancers)[35,36] or downregulated (prostate, hepatocellular and renal cell carcinoma)[37–39] in various cancers, it appears that miR-141 might have different roles, as either an oncogene or as a tumor-suppressor gene, in different cancer types of cancer. Therefore, most of the differentially expressed miRNAs identified in this study by comparing miRNA expression in cisplatin-sensitive and -resistant human ESCC cell lines appear to show some involvement in cancer; however, none of these miRNAs has previously been found to be associated with the development of cisplatin resistance.

Our study further showed that miR-141, which was the most upregulated miRNA in cisplatin-resistant ESCC cell lines, conferred cisplatin resistance in ESCC. Upon ectopic expression of miR-141, the viability of the cisplatin-sensitive cell lines after cisplatin treatment was elevated significantly. This effect was due to the inhibition of cisplatin-induced apoptosis by miR-141, which indicated that miR-141 is an anti-apoptotic factor. Furthermore, we found that miR-141 negatively regulates the expression of *YAP1*. *YAP1* is a well-documented proapoptotic transcriptional factor, and inhibition of its expression greatly reduces cisplatin-induced apoptosis.[40,41] Given that the results of our present study showed that miR-141 targets *YAP1* and negatively regulates the expression of *YAP1*, it is likely that miR-141 exerts its anti-apoptotic effect, at least in part, through repressing *YAP1* expression.

In summary, our study provides the first evidence that miR-141 has a key role in cisplatin resistance in ESCC, because of its anti-apoptotic properties. Our study highlights the potentially important role of miRNAs in the development of drug resistance, and suggests that miRNAs might serve as biomarkers for response to chemotherapy.

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