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Transcription Factor CUTL1 Is a Negative Regulator of Drug Resistance in Gastric Cancer*

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Background: Transcription factors play crucial roles in drug resistance in cancer.
Results: CUTL1 activity decreased in gastric cancer with a drug resistance phenotype. Modulation of CUTL1 expression led to changes of cancer cell responses to chemotherapy.
Conclusion: CUTL1 modulates multidrug resistance in gastric cancer.
Significance: CUTL1 is a potential target for improving chemotherapeutic efficacy for gastric cancer.

Gastric cancer is one of the leading causes of malignancy-related mortality worldwide, and drug resistance hampered the clinical efficacy of chemotherapy. To better understand the molecular mechanism causing drug resistance, we previously established an isogenic pair of doxorubicin-sensitive and -resistant gastric cancer cell lines, SGC7901 and SGC7901/ADR cells. Here, we investigated how modulation of CUTL1 activity affects the response of gastric cancer to frequently used chemotherapeutic agents. In this study, we demonstrated that CUTL1 transcription activity was significantly reduced in doxorubicin-resistant cells. Furthermore, decreased CUTL1 expression was strongly associated with intrinsic drug resistance in human gastric cancer tissues and could be used as a poor prognosis biomarker. Both gain-of-function (by overexpression of active CUTL1) and loss-of-function (by CUTL1-specific shRNA knockdown) studies showed that increased CUTL1 activity significantly enhanced cell sensitivity to drugs and led to increased apoptosis, whereas decreased CUTL1 expression dramatically reduced cell sensitivity to drugs and thus fewer apoptoses. Importantly, modulation of CUTL1 activity resulted in altered sensitivity to multiple drugs. *In vivo* mouse studies indicated that overexpression of active CUTL1 significantly resulted in increased cancer tissue response to chemotherapy and therefore inhibited growth, whereas knockdown of CUTL1 conferred resistance to chemotherapy. Taken together, our results strongly indicate that CUTL1 activity is inversely associated with drug resistance and thus is an attractive therapeutic target to modulate multidrug resistance in gastric cancer.

Gastric cancer is the second most common cause of death worldwide (1). The nature of advanced cancer entails a tremendous capacity for developing drug resistance to any therapeutic treatment. About 80% of patients who respond to initial chemotherapy frequently evolve to acquire resistance to these agents (2). Additionally, our previous data showed that doxorubicin was ineffective in more than 60% of freshly isolated gastric cancer specimens (3). Therefore, reversing drug resistance in cancer cells presents one of the most promising approaches to cure cancer using the chemotherapeutic drugs available nowadays.

The development of drug resistance is complex and involves numerous factors. Previous reports revealed diverse mechanisms underlying multiple drug resistance (MDR),[5] such as extrusion of drug by cell membrane pumps, increase of drug detoxification or DNA damage repair, redistribution of intracellular drug accumulation, modification of drug target molecules, suppression of drug-induced apoptosis, up-regulation of lipids, and other biochemical changes (4, 5). In our laboratory, we have also identified several MDR-correlated genes such as *PrP*^{c}, *GAS1*, and *ZNRD1* (6–8). However, the precise mechanisms of MDR are far from fully elucidated.

As increasing evidence indicates that transcription factors (TFs) contribute to drug-induced responses and are involved in acquired drug resistance (9), molecular dissection of the functions of TFs would help to elucidate the complexities of drug resistance. Oligonucleotide array-based transcription factor assay (OATFA) is a newly established and quite sensitive technology for the detection of DNA binding activity of TFs

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[5] The abbreviations used are: MDR, multidrug resistance; TF, transcription factor; CUTL1, CCAAT displacement protein, ADR, adriamycin; CDDP, cisplatin; MMC, mitomycin C; OATFA, oligonucleotide array-based transcription factor assay; 5-Fu, 5-fluorouracil; HDRA, histoculture drug response assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

in vitro, which could allow us to simultaneously analyze the activity of multiple TFs and thus facilitate a high throughput profiling (10, 11).

In this study, we applied OATFA to screen TF signatures related to acquired drug resistance to doxorubicin in human gastric cancer. After confirming the decreased activity of CUTL1 in drug-resistant gastric cancer cells and human tissues, we further demonstrated that CUTL1 expression was positively related to the survival time of gastric cancer patients from both retrospective and prospective studies. Through overexpression and knockdown, the role of CUTL1 in regulating drug resistance was then confirmed using *in vitro* cell line-based assays and *in vivo* animal models. Overall, our results clearly demonstrated that CUTL1 is a promising therapeutic target to modulate the chemotherapeutic response against gastric cancer.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Human gastric cancer cell line SGC7901 was obtained from the Academy of Military Medical Science (Beijing, China). SGC7901 cells were gastric adenocarcinoma cells, which originated from the metastatic carcinoma tissues in lymph nodes near the gastric cancer tissues (5, 7, 12). The other gastric cancer cell lines, MKN45 and AGS, were purchased from the ATCC (Manassas, VA). Human doxorubicin-resistant cell variant, SGC7901/ADR, was prepared and characterized in our laboratory previously (13). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and antibiotics. The cells were cultured in a 37 °C incubator at a humidified atmosphere of 5% CO_2. Doxorubicin (0.6 μg/ml) (Haizheng, China) was routinely added to the culture media to maintain drug resistance for SGC7901/ADR cells.

MTT Assays—Doxorubicin (ADR), 5-fluorouracil (5-Fu), cisplatin (CDDP), and mitomycin C (MMC) (Haizheng, China) were all freshly prepared before each experiment. The sensitivity of cells to anticancer agents was evaluated using the MTT assay (7, 14). Briefly, cells were seeded into 96-well flat-bottomed plates (Costar, Cambridge, Mass.) and incubated at 37 °C for 24 h. Anticancer drugs were added at concentrations as follows: 0.04, 0.4, 4, and 40 μg/ml for doxorubicin; 0.2, 2, 20, and 200 μg/ml for 5-Fu; 0.06, 0.6, 6, and 60 μg/ml for CDDP; and 0.05, 0.5, 5, and 50 μg/ml for MMC. Cells were incubated for another 72 h. After incubation for 4 h with 50 μl of 2 mg/ml MTT solution (Sigma), 150 μl of dimethyl sulfoxide (DMSO, Sigma) was added to each well to dissolve crystals. Absorbance at 490 nm was measured on a BP800 microplate reader (Biohit, Helsinki, Finland). Cell survival rates were calculated according to the following formula: survival rate = (mean A_{490} of treated wells/mean A_{490} of untreated wells) × 100%. All of these assays were performed three times.

OATFA—The assay was done as described previously with modifications (10, 15). Briefly, nuclear extracts of SGC7901 and SGC7901/ADR cells were prepared with NE-PER® (nuclear and cytoplasmic extraction reagents from Pierce) following the manufacturer's instructions. Binding was performed in a 20-μl reaction volume using universal binding conditions as follows: 10 mM Tris-HCl, pH 7.5, with 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, 0.5 mM dithiothreitol, 0.05 μg/μl poly(dI-dC) (Amersham Biosciences). 15 μg of nuclear extracts from SGC7901 and SGC7901/ADR cells were separately incubated with the binding buffers for 10 min on ice prior to the addition of the double-stranded transcription factor/probe mixture. After 1 h of incubation at 30 °C, samples were resolved on 2% agarose gels (Sigma) in chilled 0.5× TBE for 25 min at 120 V. The gel area that contained the protein-DNA complex was excised and transferred to a 1.5-ml tube. Gel extraction was performed using QIAEX II® gel extraction kit (Qiagen) according to the manufacturer's instructions. Cy3- and Cy5-labeled T7 promoter sequences were used in single primer amplifications with an initial denaturation step for 5 min at 95 °C; 30–35 cycles of denaturation steps for 30 s at 95 °C, annealing for 30 s at 53 °C, and elongation for 20 s at 72 °C; with a final extension of 72 °C for 10 min. Two single primer amplification products with Cy3 or Cy5 label were mixed and hybridized to microarrays in a total 12-μl mixture. Hybridization was performed at 42 °C for 16 h followed by washing and was spun dry at 1500 rpm for 2 min. Array images were acquired by CapitalBio LuxScan™-10K(A) Dual Color Confocal Scanner (CapitalBio Corp., Beijing, China). The signal intensity of each spot in the scanned images was quantified by using GenePix Pro 4.0 (Axon Instruments, Molecular Devices, CA). OATFAs were performed at three separate times. Data were saved in an Excel spreadsheet, and the ratio of each spot was calculated.

ELISA—100 μl/plate streptavidin (10 μg/ml) was added to a 96-well plate and incubated at 4 °C overnight. Plate was then washed and blocked with 2.5% defatted milk at room temperature for 1 h. The biotinylated TF-binding probes (100 μl) were added at 1 μg/well, and the plate was incubated at room temperature for an additional hour. After removing unbound probes, nuclear extracts of SGC7901 and SGC7901/ADR cells were added to each well at 20 μg/well and incubated at room temperature for 1 h. After thorough washing, anti-CUTL1 antibodies (1:1000 dilution, Santa Cruz Biotechnology) were incubated for 1 h at room temperature followed by adding peroxidase-conjugated goat anti-rabbit IgG. Colorimetric detection was performed at 450 nm on an Ultramark microplate reader (Bio-Rad). The results were normalized by empty control. The data were presented as mean ± S.D. from three replicate experiments.

EMSA—Nuclear protein of cells and tissues was extracted following the instructions of the nuclear extraction kit (Beyotime, China), and protein concentration was quantitated by the BCA protein concentration detection kit (Beyotime, China). An equal amount of protein (1 μg) from each nuclear extract was loaded. The sequences of oligonucleotides used in EMSA are as follows: 5′-CGATATCGAT-3′ and 5′-TCGAGACGATATCGATAAGCTTCTTTTC-3′. EMSA was performed as described previously (16). Binding reactions were performed by combining 10 μl of a protein mixture in KN100 buffer (20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, and 25 mM Hepes/NaOH, pH 7.5) with 10 μl of a DNA mixture (containing 10 fmol of 5′-end-labeled DNA probe or the reconstituted nucleosome probe) for 20 min with the nucleosome-free DNA probe containing 1 μg of poly(dG-dC)·(dG-dC), and reactions with nucleosomal probes were car-

ried out without nonspecific competitors. For supershift experiments, 1 μg of polyclonal antibody against CUTL1 was added to the reaction mixture before the addition of labeled oligonucleotides. Competition assays were performed by adding unlabeled oligonucleotides to the reaction mixture in a 20- and 100-fold excess. The negative control without nuclear protein was used as normalization. The samples were loaded on a 6% polyacrylamide gel (30:1) and separated by electrophoresis at 8 V/cm in 0.5× TBE. The gels were dried and visualized by autoradiography.

Histoculture Drug Response Assay (HDRA)—Human gastric cancer biopsies from endoscopy examinations and corresponding clinical information were collected from Xijing Hospital, China. 35 samples (10 female and 25 male; median age, 63; range, 46–78 years) were collected for the study. Two samples were excluded from the study due to contamination during culture. All the patients had histologically proven gastric cancer and had not received any treatment before collecting specimens. The study protocol followed the Declaration of Helsinki protocols and was approved by the Human Ethics Review Committee of the Fourth Military Medical University. Informed consent was obtained from the patients before tissue samples were collected. Samples were cut into three pieces as follows: one piece was H&E-stained to confirm that the proportion of cancer cells in the specimens was more than 70%, one was subjected to HDRA, and one was used for EMSA analysis. For HDRA analysis, the cancerous portions of the biopsy, excluding necrotic tissue with blood or gastric contents, were scissor-minced into pieces ~0.5 mm in diameter, which were then placed on each of the prepared collagen surfaces in 24-well plates. The plates were subsequently incubated for 7 days at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. The mixture of anticancer agents was added at concentrations as reported: 7.5 μg/ml for MMC, 15 μg/ml for doxorubicin, and 300 μg/ml for 5-Fu (3, 17). After the histoculture, a collagenase and MTT solution, dissolved in phosphate-buffered saline (PBS), was added to each well and incubated for an additional 8 h. The inhibition rate (%) was then determined using the following formula: inhibition rate = $(1 - T/C) \times 100$, where T is the mean absorbance of tumor specimen/g in the drug-treated wells, and C is the mean absorbance of tumor specimen/g in the nondrug-treated control wells. Values were calculated by averaging at least three wells.

Immunohistochemistry—Immunohistochemistry was performed as described previously (22). Dewaxed and rehydrated slides were washed in PBS for 10 min and then incubated in PBS with 10% normal bovine serum for 1 h. After incubating overnight at 4 °C with primary antibodies for CUTL1 (Santa Cruz Biotechnology), the slides were washed in PBS three times for 5 min each time and incubated with secondary antibodies (Zhongshan Goldenbridge Biotechnology Co. Ltd., Wuhan, China) for 40 min at room temperature, before being developed with 3,3′-diaminobenzidine and counterstained with hematoxylin. All slides were examined independently by two experienced pathologists who were blinded to the clinicopathological information. An average value of two independent scores was shown in this study. Expression of CUTL1 was evaluated according to the proportions of positive cells per specimen and staining intensities. The ratio of positive cells was calculated by counting the number of stained tumor cells among the total number of tumor cells as follows: 0 = staining of ≤1%; 1 = staining of 2–25%; 2 = staining of 26–50%; 3 = staining of 51–75%; and 4 = staining of >75% of the cells examined. Staining intensity was graded as follows: 0 = achromatic; 1 = amber; 2 = yellow; and 3 = brown. A total score of 0–12 was finally calculated and graded as negative (−; score, 0–1), weak (+; score, 2–4), moderate (++; score, 5–8), and strong (+++; score, 9–12) (18).

Clinical Response to Chemotherapy in Relation to CUTL1 Expression—Patients with gastric cancer were treated with total (41 cases) or subtotal (58 cases) gastrostomy and underwent systemic chemotherapy after surgery. The survival time of every patient was recorded, and the survival rate was assessed by the Kaplan-Meier method to evaluate the effect of postoperative adjuvant chemotherapy.

Retroviral Infections—Retroviruses were produced by transfecting Phoenix eco cells with the pREV/TRE vector alone or encoding active p110 CUTL1-Tag2. Preparation of the retroviruses and the establishment of stable cell lines were done as described previously (7). SGC7901/ADR cells were infected by the addition of the virus-containing supernatant from eco producer cells, and stable clones were obtained after selection with puromycin.

Short Hairpin RNA—The shRNA pRevsuper plasmid for human CUTL1 targeting (5′-AAGAAGAACACTCCAGAGGATTT-3′), named shRNA1, was a generous gift from Patrick Michl and Julian Downward (19). shRNA2 targeting human CUTL1 (5′-AAGAATCTTCTCGTTTGAAACTT) (20) was cloned as hairpin oligonucleotide into pSuper.retro.puro (Oligoengine) according to the manufacturer's instructions. SGC7901 and MKN45 cells were transfected with the plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions, and stable clones were obtained after selection with puromycin. Control clones were established by transfecting with empty shRNA vector alone.

RT-PCR—The levels of CUTL1 mRNA were determined by RT-PCR. The PCR cycling conditions were 40 cycles of 95 °C for 50 s, 60 °C for 50 s, and 72 °C for 50 s. GAPDH was used as an internal control. Primers for RT-PCR were as follows: CUTL1 forward, 5′-GCTTCACCGAATCCTTTCTCTGTG-3′, and CUTL1 reverse, 5′-TTCCTCATCTGCCCCTTTTACC-3′ (21); GAPDH forward, 5′-CGGATTTGGTCGTATTGGG-3′, and GAPDH reverse, 5′-TCTCGCTCCTGGAAGATGG-3′. PCR was performed three times. The results were analyzed using LightCycler software version 3.5 (Roche Diagnostics).

Western Blot Analysis—CUTL1 protein level was detected by Western blot. Briefly, cells in log phase were harvested, and nuclear extracts were prepared as described previously (22). Equal amounts of nuclear proteins were separated and blotted onto a nitrocellulose membrane (Millipore, Bedford, MA). The blot was blocked in 5% nonfat milk for 1 h followed by overnight incubation with CUTL1 rabbit polyclonal antibodies (Santa Cruz Biotechnology). After extensive rinsing, the blot was incubated in HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for 2 h at room temperature. The blot was detected with the enhanced chemiluminescence reagent

(Amersham Biosciences). β-Actin was used as an equal loading control as described previously (19). The experiment was repeated three times.

Apoptosis Analysis—Doxorubicin was added to different cells at a concentration of 3 μg/ml to induce apoptosis. 18 h later, cells were harvested and washed twice with pre-cooled PBS. After incubation with a mixture containing annexin V and propidium iodide (Roche Diagnostics) in a binding buffer for 15 min, the fluorescence of cells was measured by flow cytometry (FACSCanto^{TM}, BD Biosciences). Apoptosis assay was repeated three times.

In Vivo CUTL1 Effects in Response to Chemotherapy—Forty eight nude mice were randomly divided into four groups; 12 mice in each cohort were subcutaneously injected with the indicated stable cells at a single site. Tumor onset was scored visually and by palpitation at the site of injection by two trained laboratory staff every other day. Tumor sizes were measured with Vernier calipers every 3 days. Four weeks after injection, on day 29, 12 mice in each cohort were randomly divided into two subgroups as follows: one subgroup received chemotherapy, and one subgroup received same amount of saline as control. For the chemotherapy group, a mixture of doxorubicin (30 mg/m^2), MMC (10 mg/m^2), and 5-Fu (600 mg/m^2) was injected through the caudal vein at day 29 and 33. On day 34, all mice were sacrificed by cervical dislocation. The exact length and width of tumor were measured. Tumor size was calculated according to the formula: $S = LW^2/2$, where S is tumor size of nude mice in each group; L is tumor length; W is tumor width. The tumor size change after chemotherapy was calculated according to the formula: $\Delta TS = S_{T2} - S_{T1}$; where S_{T1} is tumor size before giving drugs; S_{T2} is tumor size when the mouse was sacrificed. Tumor weight from each animal was calculated as mean weight ± S.D. in each group. The tumors were fixed in 10% formaldehyde and then verified by hematoxylin and eosin (H&E) staining. The mice were cared for in accordance to the Guide to the Care and Use of Experimental Animals.

H&E Staining—Cell nuclei were stained with hematoxylin. After rinsed in running tap water, slides were differentiated with 0.3% acid alcohol. Then the slides were rinsed again and were stained with eosin for 2 min. Finally, after being dehydrated, cleared, and mounted, they were analyzed under the microscope.

TUNEL Staining—TUNEL staining was performed according to the *in situ* cell death detection kit instructions (Roche Diagnostics). Slides of paraffin-embedded tissues of resected tumors in mice were dewaxed and rehydrated according to the standard protocols. Then tissue sections were incubated for 20 min at room temperature with proteinase K (20 μg/ml). After rinsing the slides twice with PBS, the TUNEL reaction solution was prepared, 50 μl of which was added to the tissue sections in a humidified atmosphere for 60 min at 37 °C. The slides were then rinsed three times with PBS. A 50-μl transforming agent peroxidase was added to the sample for 30 min at 37 °C. After rinsing the slides, diaminobenzidine was added at room temperature, and the slides were washed again. Then slides were counterstained with hematoxylin. Quantitation of apoptotic cells was accomplished by counting the number of apoptotic bodies observed in the microscopic fields.

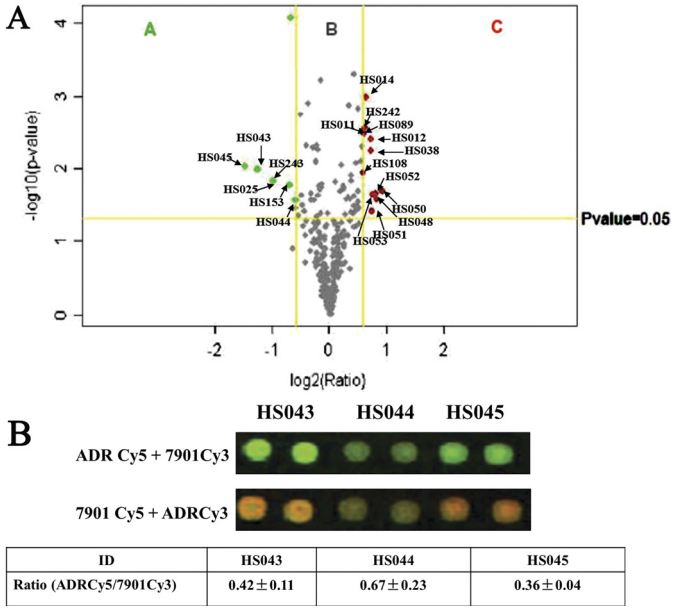
TABLE 1

Drug sensitivity of gastric cells to chemotherapy agents

Gastric cancer SGC7901 cells and SGC7901-derived cells that were cultured with or without drug ADR for 14 days were used in the assay. The sensitivity of cells to different chemotherapeutic agents was evaluated using the MTT assay. The concentration of each drug that caused a 50% reduction in the number of cells (IC_{50}) was calculated. Note: SGC7901/ADR-r represents SGC7901/ADR cells cultured without ADR for 2 weeks; SGC7901/ADR-a represents SGC7901/ADR cells continuously cultured with ADR.

Cells	IC_{50} value			
	Doxorubicin	5-Fu	CDDP	MMC
	μg/ml			
SGC7901	0.16 ± 0.02	4.76 ± 0.20	0.64 ± 0.04	0.62 ± 0.19
SGC7901/ADR-r	6.63 ± 0.27[a]	14.09 ± 0.97[a]	4.40 ± 0.22[a]	2.92 ± 0.65[a]
SGC7901/ADR-a	6.71 ± 032[b]	14.76 ± 1.49[b]	4.79 ± 0.16[b]	3.36 ± 0.54[b]

[a] $p < 0.05$, SGC7901/ADR-r *versus* SGC7901.
[b] $p < 0.05$, SGC7901/ADR-a *versus* SGC7901.



ID	HS043	HS044	HS045
Ratio (ADRCy5/7901Cy3)	0.42±0.11	0.67±0.23	0.36±0.04

FIGURE 1. **Identification of CUTL1 in relation to drug resistance by OATFA.** Nuclear proteins from gastric cancer cell SGC7901 and its doxorubicin-resistant variant SGC7901/ADR were compared in OATFA. *A,* volcano plot from OATFA. 18 probes with different binding activity, corresponding to 13 transcription factor binding domains, were identified. *Green dots* indicated probes with the activity ratio in SGC7901 *versus* SGC7901/ADR cells of more than 1.5, and *red dots* indicated probes with the activity ratio in SGC7901/ADR *versus* SGC7901 cells of greater than 1.5. Additional lists of the 18 differentially binding probes are shown in Table 2. *B,* three probes targeting CUTL1, HS043, HS044, and HS045 showed dramatic changes by comparing signals from SGC7901/ADR *versus* SGC7901.

Statistical Analysis—Data of assays were expressed as means ± S.D. Differences were compared by one-way analysis of variance followed by Dunnett's multiple comparison tests. Student's *t* test and Fisher's exact probability were applied to determine significance between the two groups. Kaplan-Meier method was used to evaluate the survival rate. Linear correlation was applied to analyze the relationship between the two variances. A value of $p < 0.05$ was considered significant.

RESULTS

Characterization of Drug Resistance Phenotype in Doxorubicin-resistant Gastric Cancer Cells—The establishment of drug-resistant cell lines is a well established strategy to understand the molecular mechanisms underlying drug resistance. We have previously established ADR-resistant SGC7901 cells

TABLE 2

List of differentially binding probes in OATFA

Transcription factors with differential activity between SGC7901 and SGC7901/ADR cells were detected by OATFA. C/EBP is CCAAT/enhancer-binding protein.

Probe ID	Corresponding transcription factor(s)	Ratio (ADR/7901)	*p* value
HS045	CUTL1 (31)	0.36	0.009
HS043	CUTL1	0.42	0.010
HS243	PITX2 (32, 33)	0.51	0.015
HS025	AR (34)	0.62	0.017
HS153	HSF (35)	0.66	0.027
HS044	CUTL1	0.67	0.035
HS011	AP-1/c-Fos/c-Jun/Fra-1/Fra-2/JunB/JunD (36, 37)	1.51	0.003
HS108	FOXO/FOXO4 (38)	1.51	0.011
HS089	E4BP4 (39)	1.52	0.003
HS242	AML1,AML2,AML3 (40)	1.54	0.003
HS014	AP-1/c-Fos/c-Jun/Fra-1/Fra-2/JunB/JunD	1.55	0.001
HS012	AP-1/c-Fos/c-Jun/Fra-1/Fra-2/JunB/JunD	1.65	0.004
HS038	AP-1/c-Fos/c-Jun/Fra-1/Fra-2/JunB/JunD	1.66	0.006
HS051	C/EBP (41, 42)	1.67	0.039
HS053	C/EBPδ	1.68	0.023
HS052	C/EBPβ	1.75	0.022
HS048	C/EBP	1.77	0.027
HS050	C/EBPα	1.90	0.021

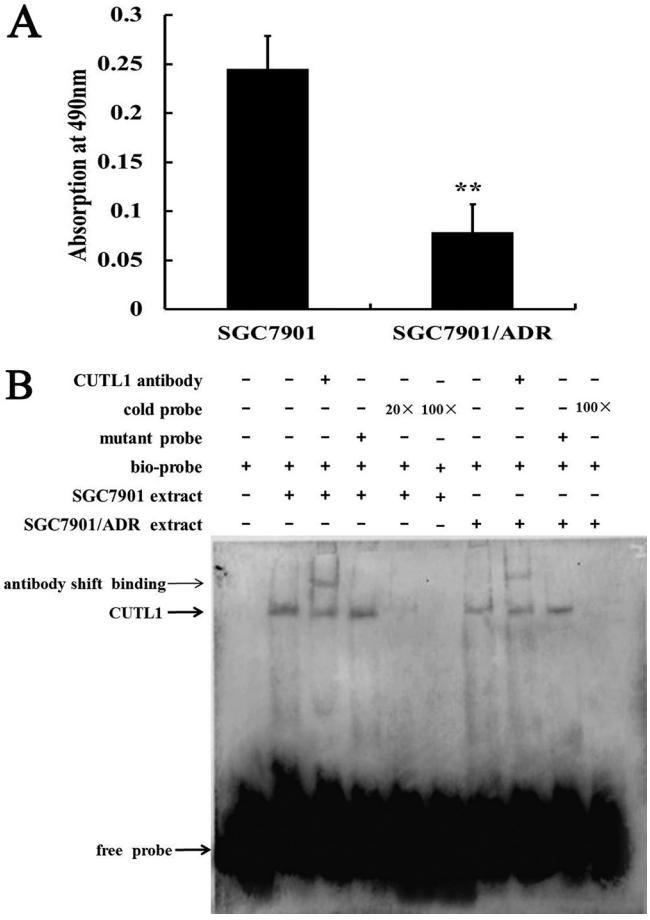


FIGURE 2. **CUTL1 activity decreased in drug-resistant gastric cancer cells.** *A,* gastric cancer SGC7901 cells and its doxorubicin-resistant variant SGC7901/ADR cells were cultured in 96-well plates. 24 h later, the cells were incubated with the biotinylated TF-binding probes (100 μl). Nuclear extracts were prepared after removing the unbound probe and analyzed by ELISA. *Bar graph* analysis of ELISA results were presented as means ± S.D. from three replicate experiments. ** denotes $p < 0.05$ compared with SGC7901 cells. *B,* nuclear protein from SGC7901/ADR and SGC7901 cells was extracted, and 1 μg of each was used for EMSA. *Arrows* indicate the specific binding, CUTL1 binding, and free probe, respectively. Figure was represented from one assay, and similar results were seen in three replicate experiments performed at separate times.

TABLE 3

Clinicopathological data of the patients with gastric cancer

TNM means tumor (T), lymph nodes (N), and metastasis (M).

No.	Sex	Age	TNM stage	Differentiation of tumors	CUTL1 activity	Inhibition rate
		years				
1	Female	58	II	Moderate	75.1	74
2	Male	69	I	Poor	76.7	75
3	Male	66	III	Moderate	128.2	93
4	Male	69	II	Poor	86.3	76
5	Female	72	III	Poor	72.6	68
6	Female	48	III	Well	68.2	61
7	Male	64	I	Poor	69.4	62
8	Male	66	IV	Poor	83.5	68
9	Female	67	III	Moderate	105.7	85
10	Male	55	IV	Poor	99.4	78
11	Male	70	II	Moderate	68.7	60
12	Female	60	III	Poor	109.4	85
13	Male	76	IV	Poor	65.2	58
14	Male	46	IV	Poor	75.9	69
15	Male	62	III	Well	90.3	82
16	Female	74	II	Moderate	88.4	76
17	Female	63	IV	Moderate	54.3	47
18	Male	59	III	Moderate	56.2	48
19	Male	66	IV	Poor	23.1	21
20	Male	78	II	Poor	12.1	17
21	Male	64	III	Moderate	8.4	10
22	Female	60	III	Well	45.9	32
23	Male	57	IV	Poor	34.2	23
24	Male	62	III	Well	32.5	26
25	Male	56	I	Poor	28.9	22
26	Male	78	II	Moderate	44.6	42
27	Female	53	IV	Poor	13.7	18
28	Male	61	I	Moderate	35.6	29
29	Male	68	III	Poor	47.5	45
30	Male	54	III	Moderate	54.2	50
31	Male	65	IV	Well	43.9	36
32	Female	57	III	Moderate	56.9	53
33	Male	56	II	Moderate	60.2	56

(SGC7901/ADR cells) by culturing SGC7901 cells with gradually increasing doxorubicin concentrations, up to 0.6 μg/ml within 3 months. To identify TFs responsible for doxorubicin resistance, it was essential to exclude the possible interactions between TFs and drugs. Thus, we first characterized the drug resistance phenotype of SGC7901/ADR cells cultured under drug-free conditions for 2 weeks. As shown in Table 1, after a 2-week drug-free culture, SGC7901/ADR cells still retained resistance to doxorubicin with IC_{50} at 6.63 ± 0.26 μg/ml, a similar level as observed for those cells that were continually cultured with doxorubicin ($p > 0.05$) but in contrast to its parental SGC7901 cells with IC_{50} at 0.16 ± 0.02 μg/ml ($p < 0.05$). As we had previously found that SGC7901/ADR cells were not only resistant to doxorubicin but also to other drugs, such as 5-Fu, MMC, and CDDP (6, 23), we then tested the cells for drug resistance to these drugs after culturing cells under drug-free conditions, as we did for doxorubicin. Similar results were found for these drugs, as indicated in Table 1, suggesting that SGC7901/ADR cells could still retain resistance to multiple drugs after being cultured under drug-free conditions for 2 weeks.

Identification of TFs Related to Drug Resistance—To gain more understanding regarding how TFs regulate drug resistance in gastric cancer, we detected the activity changes of TFs by OATFA (Fig. 1*A*), an array-based quantitative proteomic approach that could simultaneously analyze the DNA binding activity of TFs (11). Using the TF array that contains 240 different human TFs, we first compared activities of global TFs in SGC7901 cells with that of SGC7901/ADR cells. Using a 1.5-fold change as a selection criterion based on the recommenda-

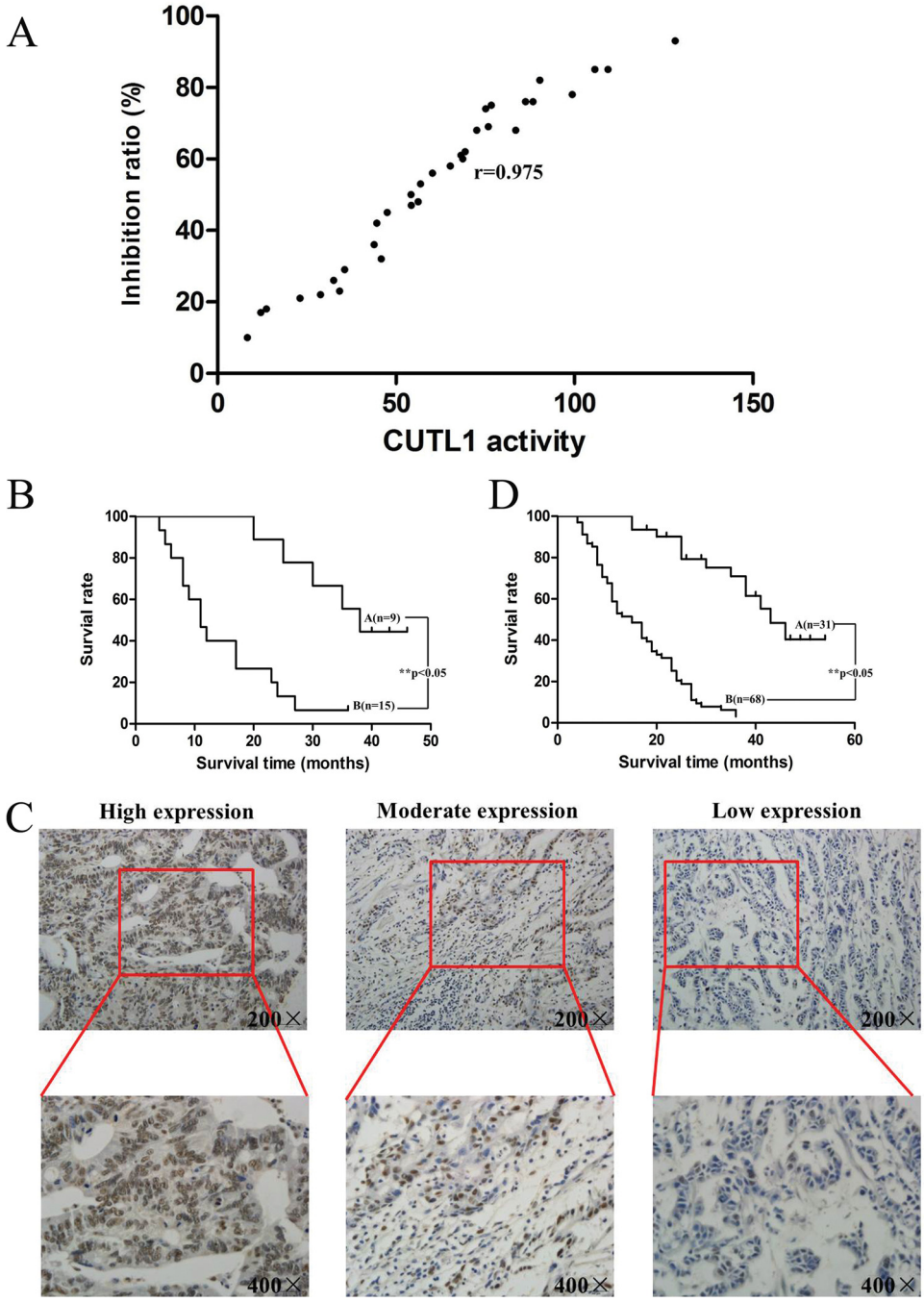


FIGURE 3. **Lower CUTL1 activity was associated with multiple drug resistances in gastric cancer tissues.** *A,* 33 fresh gastric cancer tissues from endoscopic biopsy were collected. Nuclear protein was extracted from part of each sample, and 1 μg of the nuclear extract was used for EMSA. The gray value of each sample was calculated to represent the binding activity. Another part of each sample was placed on the prepared collagen surfaces. 7 days later, the mixture of chemotherapy agents was given, and the inhibition rate of each sample was analyzed by HDRA. The correlation of CUTL1 activity and inhibition rate was analyzed and indicated that reduced CUTL1 activity was related with multiple drug resistance in human gastric cancer tissues ($r = 0.975$). *B,* survival periods of the above 33 patients were tracked. Except for the nine patients that gave up further treatment after diagnosis, the survival rate and time of 24 patients in relation to CUTL1 expression levels were analyzed by the Kaplan-Meier method. ** denotes $p < 0.05$. *C* and *D,* 99 samples from gastric cancer patients that underwent gastrostomy and standard adjuvant chemotherapy were collected, and the expression of CUTL1 was evaluated by immunochemistry (*C*), and the survival rate and time of patients in relation to CUTL1 expression level were assessed by the Kaplan-Meier method (*D*), ** denotes $p < 0.05$. *B* and *D, a* indicates the high CUTL1 activity group, and *b* indicates the low CUTL1 activity group.

tion from the assay (11), six probes from SGC7901 cells and 12 from SGC7901/ADR cells showed significant differences (for the list of probes, see Table 2). Specifically, in SGC7901/ADR cells, the activities of the CCAAT displacement protein (CUTL1/CDP), PITX2, androgen receptor, and heat shock transcription factor 1 (HSF1) were decreased, and those of AP-1(-like) components (including c-Jun, c-Fos, JunB, JunD, Fra-1, Fra2, and CCAAT/enhancer-binding protein $\alpha/\beta/\delta$), acute myeloid leukemia proteins (AML1/2/3), FOXO1α/4, and adenovirus E4-binding protein 4 (E4BP4) were increased (Fig. 1, *A* and *B*). Notably, when comparing signals from SGC7901/ADR *versus* SGC7901, the three probes, HS043, HS044, and

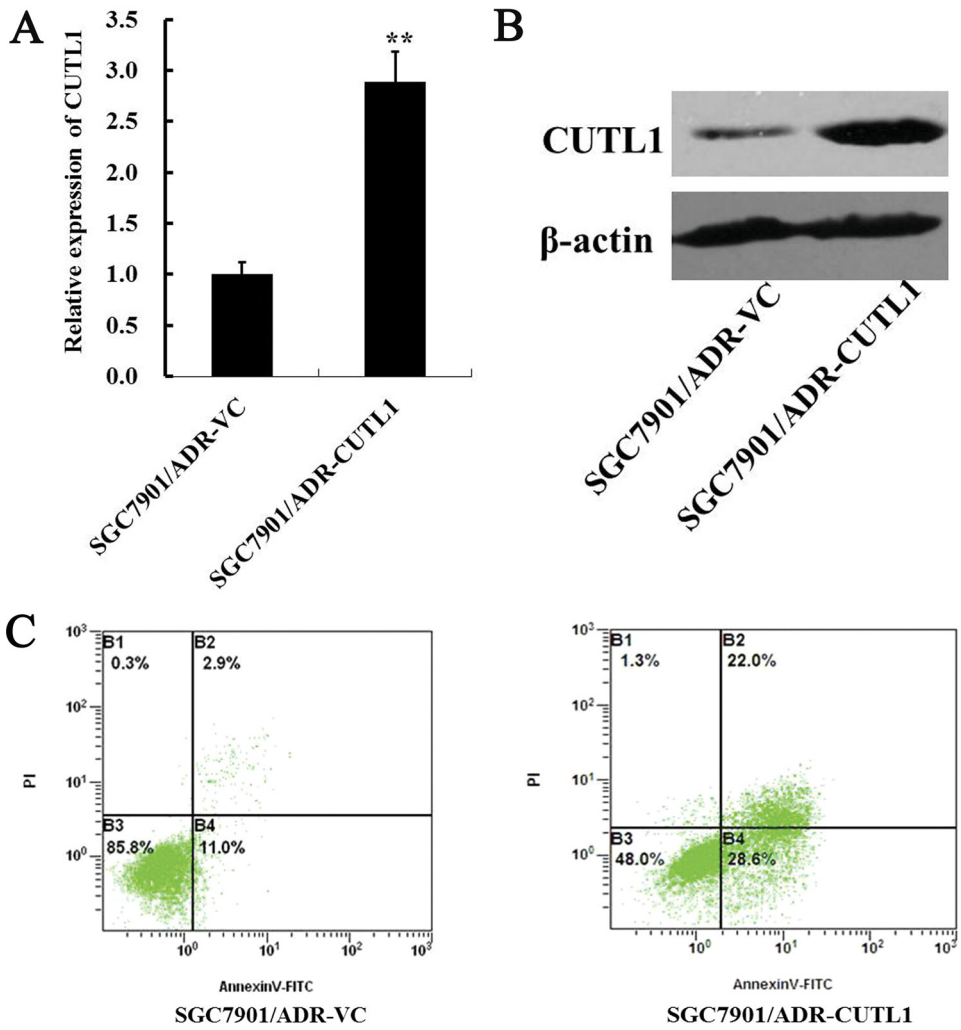


FIGURE 4. **CUTL1 overexpression increased drug sensitivity and apoptosis rate in gastric cancer cells.** *A,* stable SGC7901/ADR cells overexpressing active CUTL1 (*SGC7901/ADR-CUTL1*) or vector control (*SGC7901/ADR VC*) were established by retroviral infections. Total RNA was prepared and analyzed by RT-PCR (*A*) using specific CUTL1 primers. Nuclear proteins were extracted and detected by Western blot using CUTL1 antibody (*B*). Student's *t* test, $n = 3$, ** denotes $p < 0.05$, and *bars* denote S.D. *C,* SGC7901/ADR-CUTL1 and SGC7901/ADR vector control cells were incubated with doxorubicin at a concentration of 3 μg/ml. 18 h later, cell apoptosis was measured by flow cytometry. Representative images from one assay are shown, and similar results were seen in three replicate experiments performed at different times. *PI,* propidium iodide.

TABLE 4

Drug sensitivity of gastric cancer cells with active CUTL1 overexpression

The sensitivity of SGC7901/ADR cells and SGC7901/ADR-derived cells to different chemotherapeutic agents was evaluated using the MTT assay. The concentration of each drug that caused a 50% reduction in the number of cells (IC_{50}) was calculated. Note: SGC7901/ADR-VC represents SGC7901/ADR cells infected with retrovirus vector pREV/TRE alone for overexpression control, and SGC7901/ADR-CUTL1 cells represent SGC7901/ADR cells infected with retrovirus vector pREV/TRE encoding active CUTL1.

Groups	IC_{50} value			
	ADR	5-Fu	CDDP	MMC
			$\mu g/ml$	
SGC7901ADR-VC	7.53 ± 0.46	15.36 ± 1.38	3.68 ± 0.26	4.99 ± 0.57
SGC7901ADR-CUTL1	2.58 ± 0.07[a]	4.36 ± 0.73[a]	1.05 ± 0.52[a]	1.66 ± 0.25[a]

[a] $p < 0.05$, SGC7901/ADR-CUTL1 *versus* SGC7901/ADR-VC.

HS045, targeting CUTL1 showed dramatic changes (SGC7901/ADR *versus* SGC7901 0.42 ± 0.11, 0.67 ± 0.23, 0.36 ± 0.04, respectively) (Fig. 1*B*), suggesting that CUTL1 might be related to constitutive doxorubicin resistance in gastric cancer cells. Therefore, we focused on CUTL1 activity in relation to drug resistance in the following studies.

CUTL1 Activity Decreased in Doxorubicin-resistant Gastric Cancer Cells—To confirm our screening results, ELISA was carried out to study the activity of CUTL1 in relation to drug resistance in gastric cancer cells. Compared with drug-sensitive SGC7901 cells, the activity of CUTL1 in SGC7901/ADR cells showed 72% reduction (0.25 ± 0.02 *versus* 0.07 ± 0.01, $p < 0.05$) (Fig. 2*A*). In agreement with ELISA, results from EMSA also indicated that CUTL1 activity in SGC7901/ADR was less than that in SGC7901 cells (Fig. 2*B*). Altogether, these results, as well as data from OATFA, demonstrated that CUTL1 activity decreased in cells with drug resistance phenotypes.

Decreased CUTL1 Activity Is Related to Multiple Drug Resistances in Gastric Cancer Tissues—Mindful of the artificial nature of screening and limitation of *in vitro* assays, we then interrogated the expression patterns of CUTL1 in gastric cancer lesions for evidence of human relevance, specifically response to drug treatment as criteria for clinicopathological validation. For this purpose, we collected fresh tissues specimens from 33 patients with gastric cancer and performed

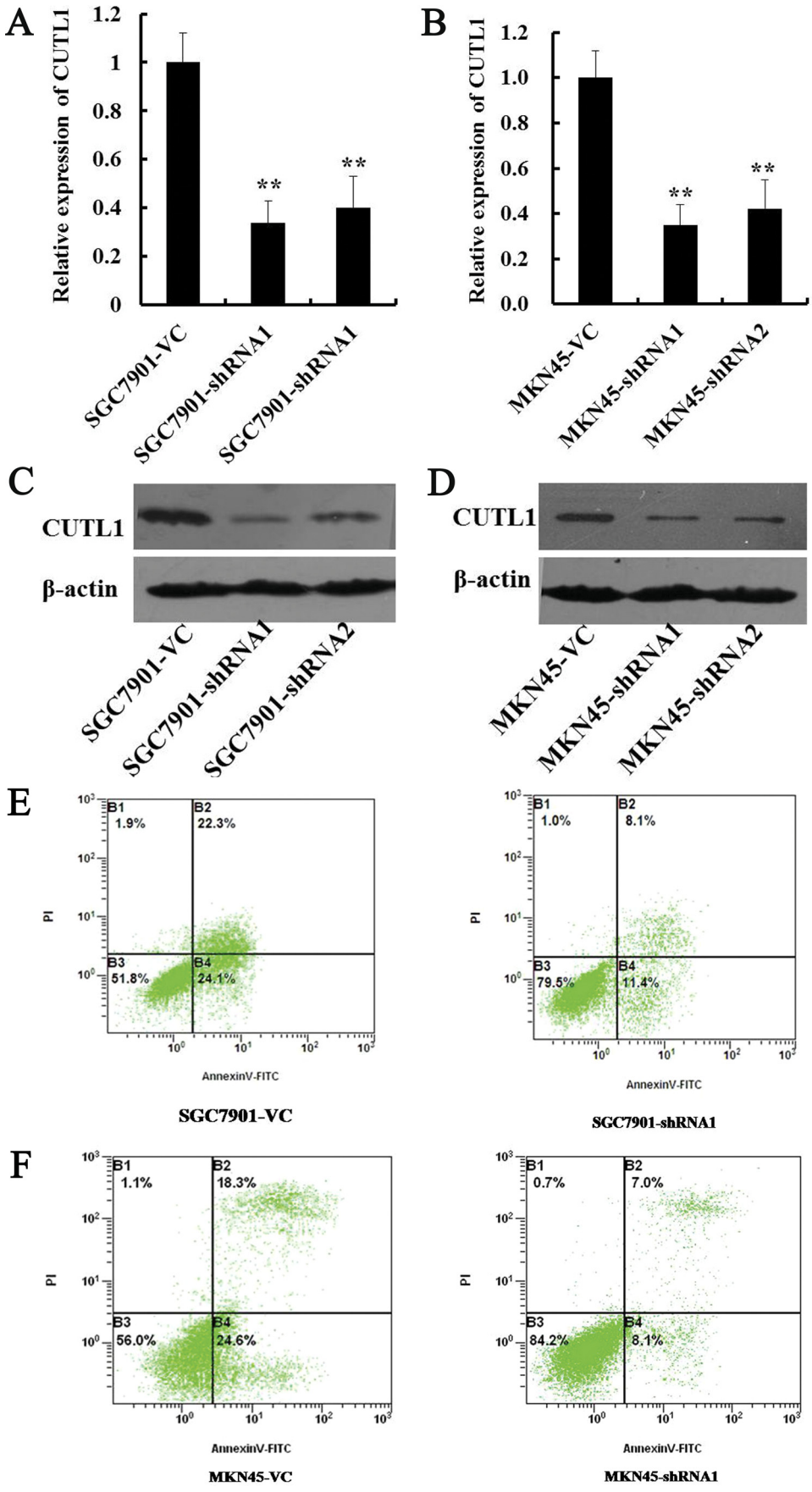

A
Relative expression of CUTL1
1.2
1
0.8
0.6
0.4
0.2
0
**
**
SGC7901-VC
SGC7901-shRNA1
SGC7901-shRNA1
B
Relative expression of CUTL1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
MKN45-VC
MKN45-shRNA1
MKN45-shRNA2
C
CUTL1
β-actin
SGC7901-VC
SGC7901-shRNA1
SGC7901-shRNA2
D
CUTL1
β-actin
MKN45-VC
MKN45-shRNA1
MKN45-shRNA2
E
B1 1.9%
B2 22.3%
B3 51.8%
B4 24.1%
PI
AnnexinV-FITC
SGC7901-VC
B1 1.0%
B2 8.1%
B3 79.5%
B4 11.4%
PI
AnnexinV-FITC
SGC7901-shRNA1
F
B1 1.1%
B2 18.3%
B3 56.0%
B4 24.6%
PI
AnnexinV-FITC
MKN45-VC
B1 0.7%
B2 7.0%
B3 84.2%
B4 8.1%
PI
AnnexinV-FITC
MKN45-shRNA1

TABLE 5

Drug sensitivity of gastric cancer cells with CUTL1 knockdown

The sensitivity of SGC7901 or MKN45 cells and SGC7901 or MKN45-derived cells to different chemotherapeutic agents was evaluated using the MTT assay. The concentration of each drug that caused a 50% reduction in the number of cells (IC_{50}) was calculated. Note: SGC7901-VC or MKN45-VC cells represents SGC7901 or MKN45 cells transfected with pRevsuper vector alone for knockdown control, and SGC7901-shRNA1 or MKN45-shRNA1 cells represents SGC7901 or MKN45 cells transfected with human CUTL1 shRNA in pRevsuper vector.

Groups	IC_{50} value			
	doxorubicin	5-Fu	CDDP	MMC
	μg/ml			
SGC7901-VC	0.31 ± 0.07	3.37 ± 0.45	0.94 ± 0.33	1.25 ± 0.46
SGC7901-shRNA1	1.74 ± 0.25[a]	16.23 ± 0.38[a]	3.22 ± 0.41[a]	4.18 ± 0.74[a]
MKN45-VC	0.22 ± 0.05	4.39 ± 0.38	1.28 ± 0.42	0.38 ± 0.11
MKN45-shRNA1	1.36 ± 0.28[b]	13.85 ± 0.49[b]	4.03 ± 0.37[b]	1.94 ± 0.34[b]

[a] $p < 0.05$, SGC7901-shRNA1 *versus* SGC7901-VC.
[b] $p < 0.05$, MKN45-shRNA1 *versus* MKN45-VC.

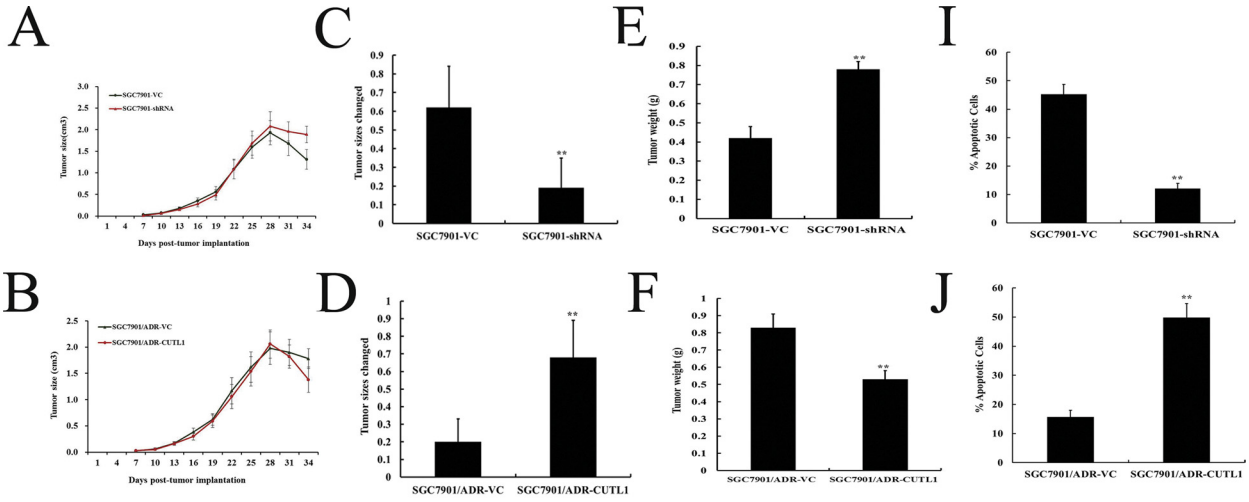
EMSA and HDRA to measure CUTL1 activity in relation to drug response. The gray values of each sample from EMSA were used as the measurement of the CUTL1 activity. Chemosensitivity of these clinical tissues was determined by the inhibition ratio calculated from HDRA measurement. The CUTL1 activity was the average of three times, and the inhibition rate of each sample was the average of three repeated pores. The clinicopathological data and the detection results of the patients are shown in Table 3. The linear correlation was used to analyze the correlation of CUTL1 activity and the inhibition rate of each sample by SPSS 17.0 software. The results demonstrated that reduced CUTL1 activity was related to multiple drug resistance in human gastric cancer tissues ($r = 0.975$) (Fig. 3*A*).

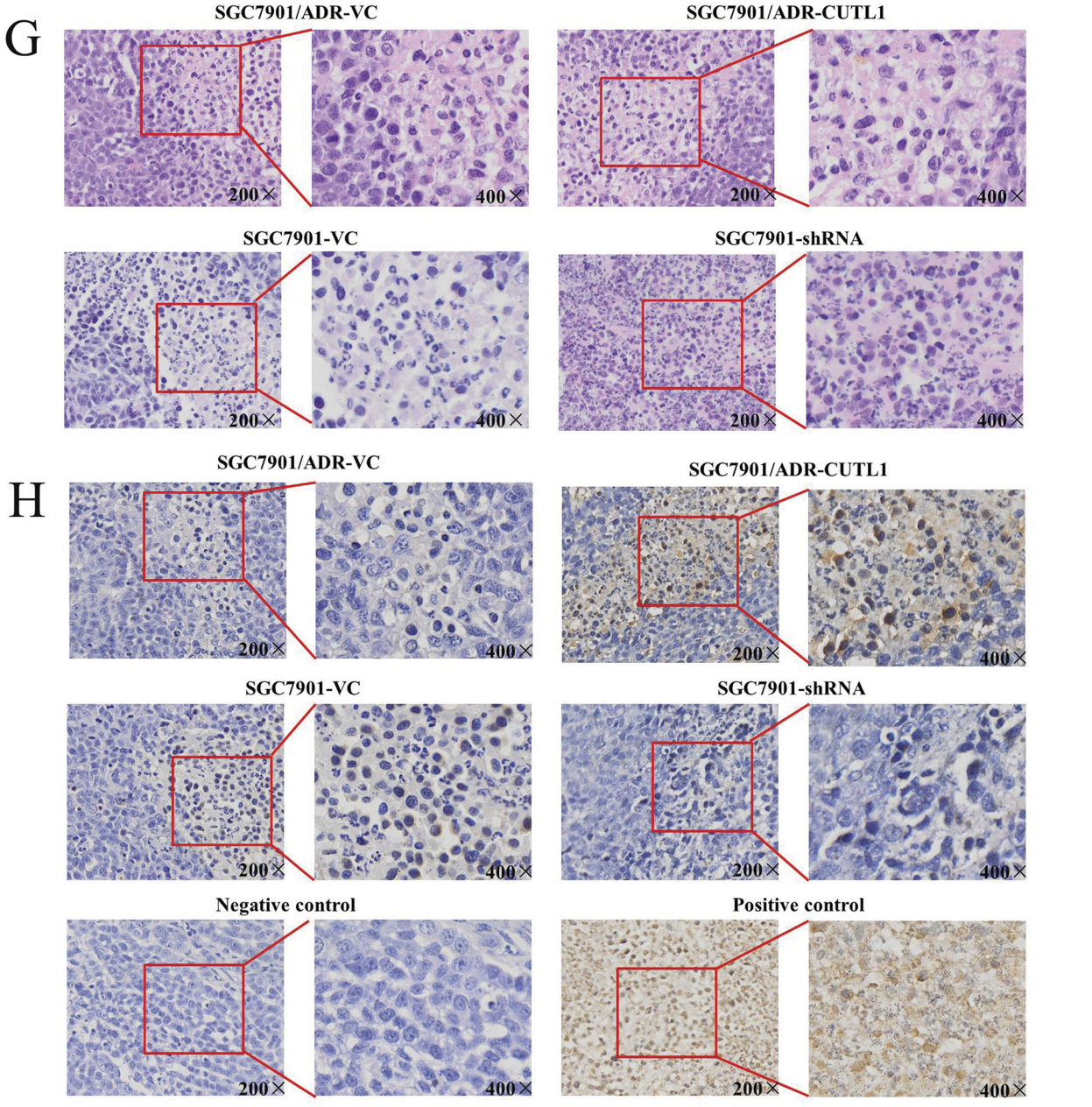
CUTL1 Expression Is Positively Related to Survival of Gastric Cancer Patients—To evaluate the relation of CUTL1 activity with the chemotherapy response in clinical situations, we followed up the survival period of the above patients. Except for nine patients who stopped additional treatment after the diagnosis, we collected data from 24 cases. CUTL1 activity was regarded as high when the gray value was equal to or higher than that of the positive control. Among these 24 patients, 9 cases showed high levels of CUTL1 activity, and 15 cases showed low activity. As shown in Fig. 3*B*, patients with low CUTL1 activity had shorter survival time (average 14.5 months) than those with high CUTL1 activity (average 35.2 months), which indicated that gastric cancer patients with a higher CUTL1 activity responded better to chemotherapy than those with lower activity.

To further confirm the relevance of CUTL1 with drug resistance, we retrospectively collected 99 gastric cancer tissue samples that underwent gastrostomy and standard postoperative adjuvant chemotherapy in the past 5 years at the Xijing hospital. We detected CUTL1 expression in tissues by immunohistochemistry. CUTL1 expression was regarded as high when the staining score was 9 or higher. Among these 99 patients, 31 cases showed high levels of CUTL1 expression, and 68 cases showed low levels of expression (Fig. 3*C*). The survival time of these patients was analyzed by the Kaplan-Meier method, and the results revealed that patients with low CUTL1 expression had shorter survival time (average 16.2 months) than those with high CUTL1 expression (average 34.1 months) (Fig. 3*D*), which suggested that high CUTL1 expression was associated with a better drug response to chemotherapy and thus a longer survival time. Altogether, our results indicated that CUTL1 might be an important biomarker for predicting the chemotherapy outcome and assessing the prognosis of gastric cancer patients. To thoroughly understand how this information could be utilized in a clinical setting, more complete knowledge of the consequences of CUTL1 modulation is needed. We therefore carried out some basic research to modulate CUTL1 expression and study their effects on chemotherapy response.

Role of CUTL1 in Regulating Drug Resistance in Gastric Cancer Cells—The above findings raised the notion that reduced CUTL1 activity would contribute to drug resistance phenotype. If this were the case, then overexpression of active CUTL1 in SGC7901/ADR cells should reverse the cell resistance to doxorubicin. A series of experiments was therefore performed to test this hypothesis. First, we established stable SGC7901/ADR cells overexpressing active CUTL1 (SGC7901/ADR-CUTL1) or vector control (SGC7901/ADR-VC) by retroviral infections. The expression of CUTL1 in SGC7901/ADR-CUTL1 cells was detected by RT-PCR and Western blot, respectively. As shown in Fig. 4, *A* and *B*, a 3-fold increase of CUTL1 expression at the mRNA level was observed in SGC7901/ADR-CUTL1 cells compared with SGC7901/ADR-VC cells, although the 5-fold increase at the protein level was obtained. Next, we investigated the functional activity of CUTL1 in relation to drug resistance. This was done using MTT assay by measuring sensitivity of SGC7901/ADR-CUTL1 cells or SGC7901/ADR-VC cells to multiple anticancer drugs, including doxorubicin, 5-Fu, MMC, and CDDP. As indicated in Table 4, the IC_{50} value of SGC7901/ADR-CUTL1 cells was 34.2% of SGC7901/ADR-VC cells for doxorubicin (2.58 ± 0.07 *versus* 7.53 ± 0.46, $p < 0.05$), 28.4% for 5-Fu (4.36 ± 0.73 *versus* 15.35 ± 1.38, $p < 0.05$), 33.31% for MMC (1.66 ± 0.25 *versus* 4.99 ± 0.57, $p < 0.05$), and 27.2% for CDDP (1.05 ± 0.52 *versus* 3.85 ± 0.26, $p < 0.05$). As a result of increased sensitivity to chemotherapy drugs, the ratio of apoptotic SGC7901/ADR-CUTL1 cells was significantly increased (>3-fold) compared with that observed in SGC7901/ADR-VC cells, as measured by flow cytometry with the paired cell lines (Fig. 4*C*). In addition, similar results were obtained in AGS cells (data not shown). In addition, similar results were obtained in AGS cells (data not shown). Overall, the results indicated that overexpressing active CUTL1 reversed drug resistance and thus increased the apoptosis rate induced by these drugs. More importantly, the reversed drug resistance was not only limited to doxorubicin but also to other structurally unrelated drugs,

FIGURE 5. **CUTL1 expression knockdown decreased drug sensitivity and apoptosis rate in gastric cancer cells.** *A–D,* stable SGC7901 (or MKN45) cells with CUTL1 expression knockdown (*SGC7901/MKN45-shRNA1* and *SGC7901/MKN45-shRNA2*) or vector controls (*SGC7901/MKN45-VC*) were established by transfecting cells with shRNA or vector control. Total RNA was prepared, and CUTL1 expression level was measured at the transcription level by RT-PCR (*A* and *B*). Nuclear proteins were extracted and detected by Western blot (*C* and *D*). These experiments were repeated three times, and data from one assay were present. ** denotes $p < 0.05$ compared with each vector control. *E* and *F,* SGC7901 cells (*E*) and MKN45 cells (*F*) stably transfected with CUTL1 shRNA or vector control were incubated with doxorubicin at a concentration of 3 μg/ml. 18 h later, cell apoptosis upon drug treatment was measured by flow cytometry. Representative images from one assay are shown, and similar results were seen in three replicate experiments performed at different times. *PI,* propidium iodide.


A
SGC7901-VC
SGC7901-shRNA
Tumor size(cm3)
Days post-tumor implantation
C
Tumor sizes changed
SGC7901-VC
SGC7901-shRNA
E
Tumor weight (g)
SGC7901-VC
SGC7901-shRNA
I
% Apoptotic Cells
SGC7901-VC
SGC7901-shRNA
B
SGC7901/ADR-VC
SGC7901/ADR-CUTL1
Tumor size (cm3)
Days post-tumor implantation
D
Tumor sizes changed
SGC7901/ADR-VC
SGC7901/ADR-CUTL1
F
Tumor weight (g)
SGC7901/ADR-VC
SGC7901/ADR-CUTL1
J
% Apoptotic Cells
SGC7901/ADR-VC
SGC7901/ADR-CUTL1


G
SGC7901/ADR-VC
SGC7901/ADR-CUTL1
SGC7901-VC
SGC7901-shRNA
H
SGC7901/ADR-VC
SGC7901/ADR-CUTL1
SGC7901-VC
SGC7901-shRNA
Negative control
Positive control
200×
400×

which suggested that CUTL1 plays an important role in the occurrence of multiple drug resistance.

To further evaluate the functional importance of CUTL1 in the regulation of drug resistance, a separate series of experiments was performed wherein shRNA methodology was used to knock down the expression of CUTL1, and the effects upon drug resistance were measured. Two CUTL1-specific shRNA vectors, namely CUTL1-shRNA1 and CUTL1-shRNA2, were designed and constructed separately. Stable cell lines were established from parental SGC7901 and MKN45 cells and were designated as SGC7901 (or MKN45)-shRNA1, SGC7901 (or MKN45)-shRNA2, or SGC7901 (or MKN45)-VC (vector control), respectively. RT-PCR and Western blot analysis were performed after CUTL1 shRNA targeting (Fig. 5, *A–D*). As CUTL1-shRNA1 down-regulated the expression of CUTL1 more effectively in SGC7901 and MKN45, SGC7901-shRNA1 and MKN45-shRNA1 cells were used in further cellular assays.

MTT assay and flow cytometry assay indicated that knockdown of CUTL1 did not affect cell growth (data not shown). However, as anticipated, SGC7901-shRNA1 cells showed decreased sensitivity to all the above anticancer drugs as indicated in Table 5. Specifically, the IC_{50} value of SGC7901-shRNA1 cells was 5.70-fold of SGC7901-VC cells for doxorubicin (1.74 ± 0.25 *versus* 0.31 ± 0.07, $p < 0.05$), 4.81-fold for 5-Fu (16.23 ± 0.38 *versus* 3.37 ± 0.45, $p < 0.05$), 3.36-fold for MMC (4.18 ± 0.74 *versus* 1.25 ± 0.46, $p < 0.05$), and 3.42-fold for CDDP (3.22 ± 0.41 *versus* 0.94 ± 0.32, $p < 0.05$). Accordingly, the proportion of apoptotic cells in SGC7901-shRNA1 cells was significantly decreased (>2-fold) compared with that in SGC7901-VC cells, as demonstrated by flow cytometry (Fig. 5*E*). Similar results were obtained in MKN45-shRNA1 cells (Fig. 5*F*). In addition, similar results were obtained in AGS cells (data not shown). Therefore, knockdown of CUTL1 resulted in significant multiple drug-resistant phenotypes. These findings strengthened our hypothesis that CUTL1 is regulating multiple drug resistance in gastric cancer cells.

CUTL1 Expression Is Inversely Related to Multiple Drug Resistances in Vivo—Based on the *in vitro* data, we hypothesized that ectopic CUTL1 could regulate drug resistance *in vivo*. For this purpose, a subcutaneous gastric cancer mouse model was adapted as reported previously (18). In this model, the stably transfected cells used above were injected into nude mice subcutaneously. A mixture of doxorubicin (30 mg/m^2), MMC (10 mg/m^2), and 5-Fu (600 mg/m^2) was injected through the caudal vein daily at days 29 and 33. This treatment mimics the clinical therapy scheme routinely used for gastric cancer patients undergoing chemotherapy. Tumor sizes were measured every 3 days for 5 weeks. As shown in Fig. 6, *A* and *C*, after drug treatment, the tumor size in the group injected with SGC7901-VC cells was significantly less than that of the group injected with SGC7901-shRNA1 cells. Conversely, the size of the tumor from SGC7901/ADR-VC group was larger than that from SGC7901/ADR-CUTL1 cells (Fig. 6, *B* and *D*). Furthermore, the end weight of the tumor in each group showed similar changes (Fig. 6, *E* and *F*). All these results indicated that CUTL1 played an important role in modulating cell sensitivity to drugs, which led to a significant difference on tumor outgrowth *in vivo* in nude mice.

Based on the *in vitro* data, we hypothesized that CUTL1 might regulate cell apoptosis by modulating cell sensitivity to chemotherapy drugs. To confirm this hypothesis, all resected tumors were fixed in 10% formaldehyde and embedded with paraffin. Then we detected the apoptosis in cancer tissues from mice by H&E staining and *in situ* cell death detection kit. As shown in Fig. 6, *G* and *J*, apoptotic cells and apoptotic bodies in the tissues injected with SGC7901/ADR-CUTL1 cells were much more than that of the tissues injected with SGC7901/ADR-VC cells. However, the apoptotic cells and apoptotic bodies in SGC7901-shRNA cells were less than that in SGC7901-VC cells. These results showed that overexpression of CUTL1 could increase the cell's response to chemotherapy and thus increase the rate of cell apoptosis, but knockdown of CUTL1 would protect cells from apoptosis by decreasing the cell's response to chemotherapy. In summary, both our *in vitro* and *in vivo* results demonstrated that CUTL1 would modulate the cell's response to chemotherapy in gastric cancer.

DISCUSSION

The intrinsic or acquired multidrug resistance has long been recognized as a major obstacle to successful cancer chemotherapy. Identification of drug resistance modulators is of clinical importance. OATFA is a high throughput proteomic screening technology that enables us to identify TFs with post-translational modification changes (15). Using the previously established isogenic pair of ADR-sensitive and -resistant gastric cancer cell lines, SGC7901 and SGC7901/ADR cells, we identified 13 TFs as differentially expressed by comparing the activities of 248 TFs by OATFA. Although the function of some TFs in drug resistance remains to be explored, some, such as AP-1, have been reported to regulate drug resistance (24). Specifically, acti-

FIGURE 6. **CUTL1 modulated gastric cancer response to chemotherapy and apoptosis *in vivo* in a nude mice model.** Nude mice were randomly divided into four groups, and 12 mice in each cohort were subcutaneously injected with the indicated stable cells (*SGC7901-VC, SGC7901-shRNA1, SGC7901/ADR-VC*, and *SGC7901/ADR-CUTL1*), respectively, at a single site. Among these cells, SGC7901-VC cells were SGC7901 cells transfected with pRevsuper vector alone for knockdown control; SGC7901-shRNA1 cells were SGC7901 cells transfected with human CUTL1 shRNA in pRevsuper vector; SGC7901/ADR-VC cells were SGC7901/ADR cells infected with retroviruses vector pREV/TRE only for overexpression control; and SGC7901/ADR-CUTL1 cells were SGC7901/ADR cells infected with retrovirus vector pREV/TRE encoding active CUTL1. Anticancer agents were administered to mice on days 29 and 33, mimicking clinical therapy scheme. The sizes of the tumor in each group were detected every 3 days. *A,* growth curves of tumors in nude mice that were injected with SGC7901-VC cells (*green line*) or SGC7901-shRNA1 cells (*red line*). *B,* growth curves of tumors in nude mice that injected with SGC7901/ADR-VC cells (*green line*) or SGC7901/ADR-CUTL1 cells (*red line*). *C* and *D,* changes of the tumor sizes from each group before and after chemotherapy. *C,* knockdown group; *D,* overexpression group. Student's *t* test, $n = 12$, ** denotes $p < 0.05$, *bars* indicate S.D. *E* and *F,* final tumor weight from each group was measured, and the average tumor weight of each group is shown. Student's *t* test, $n = 12$, ** denotes $p < 0.05$, *bars* indicate S.D. *G* and *H,* paraffin-embedded tissues slides of resected tumors in mice from each group were detected by H&E staining (*G*) and TUNEL assay (*H*), and the representative pictures from each cohort are shown. *I* and *J,* quantification of TUNEL-positive cells in tissue samples. Our results indicated that apoptotic cells and apoptotic bodies in the tissues injected with SGC7901/ADR-CUTL1 cells increased more than that of the tissues injected with SGC7901/ADR-VC cells, whereas the apoptotic cells and apoptotic bodies in SGC7901-shRNA cells were less than that in SGC7901-VC cells.

vated c-Jun was reported to up-regulate MDR expression (25), and increased CCAAT/enhancer-binding protein transactivated the human *MDR1* gene (26). All these results indicated the success of our screening using OATFA. We focused on CUTL1 as its change was the most dramatic one from our screening, and its role in drug resistance remains largely unknown.

A key finding of the present studies is that we identified CUTL1 from a high throughput screen as an important regulator for multidrug resistance. Studies in human gastric cancer tissues indicated that higher CUTL1 activity endowed gastric cancer tissues with a higher response to clinical chemotherapy, and thus a longer survival time. A functional relationship between CUTL1 expression level and drug resistance phenotype was corroborated by CUTL1 overexpression or knockdown studies that directly showed enhanced or reduced gastric cancer cell sensitivity to drugs, respectively. Importantly, the change in drug sensitivity was not just limited to doxorubicin but also extended to other commonly used but structurally different drugs, including 5-Fu, MMC, and CDDP, indicating a potential role of CUTL1 in modulating multidrug resistance of gastric cancer. The clinical relevance of our work is highlighted by the fact that we further demonstrated enhanced chemotherapy effects in mice injected with cancer cells overexpressing active CUTL1 and vice versa. These results support our hypothesis that CUTL1 modulates multidrug resistance in gastric cancer.

Although little is known about how CUTL1 activity is controlled, complex post-translational modifications that modulate CUTL1 DNA binding activity were reported (27). It is interesting to note that CUTL1 is a transcriptional target of the TGβ signaling system that enhances cell motility and invasiveness on NIH3T3 fibroblasts, as well as multiple other cancer cells (19). Nevertheless, authors from the same group also reported later that when CUTL1 was phosphorylated at Ser^{1215} in NIH3T3 cells by PKA, cell cycle progression and cell motility were diminished (28). These data emphasized the complex network for regulating CUTL1 activity and the importance of studying the effects of CUTL1 in the light of cellular context and its upstream modulators.

While our work was ongoing, Michl and co-workers (29) reported their new findings for CUTL1 as a target of AKT signaling and mediator of resistance to apoptosis in pancreatic cancer. The underlying mechanism for our conflicting results is unclear. The fact that CUTL1 exists in multiple isoforms with distinct DNA binding and transcriptional properties presents one possible explanation that the function of CUTL1 may vary along with the histology of the cancer. We hypothesize that CUTL1 plays different roles in different cancers because of the histology, which was supported by the studies in transgenic mice (30). In homozygous mutant mice where both CUTL1 alleles were deleted, thymic cellularity was dramatically reduced due to the enhanced apoptosis. However, in contrast to the lymphoid demise, these mice demonstrated myeloid hyperplasia. Resolving the above apparent discrepancies will require a better understanding of the particular role of CUTL1 and its potential to interact with specific cellular environments and function in different types of tissues. We are in the process of investigating how CUTL1 activity is regulated in gastric cancer.

The experimental significance of our findings is highlighted by the fact that although CUTL1 was selected based on doxorubicin resistance, alteration of CUTL1 activity not only resulted in altered sensitivity to doxorubicin but also to multiple other structurally unrelated drugs. This phenomenon suggested that, to some extent, cells became drug-resistant through the same or overlapping mechanisms. Our previous studies have shown that MDR expression was up-regulated in ADR-resistant cells and ZNRD1-mediated resistance of gastric cancer cells to methotrexate by regulation of IMPDH2 and Bcl-2 (31). Our studies are ongoing to investigate the mechanism underlying CUTL1-mediated reversal of MDR and other related molecules.

Collectively, we reported for the first time that lower CUTL1 activity was associated with drug resistance in gastric cancer. The absence of CUTL1 activity in untreated gastric cancer tissues suggests that CUTL1 might be responsible for intrinsic drug resistance. We also found in human gastric cancer that higher CUTL1 expression is associated with longer survival times. Our further work indicated that, both *in vitro* and *in vivo*, expression of active CUTL1 in gastric cancer could effectively reverse the multidrug resistance phenotype, and knockdown of CUTL1 resulted in increased drug resistance. Thus, we envision that targeting CUTL1 is a potentially practical strategy for enhancing the response to chemotherapy in gastric cancer. Moreover, it would be prudent to consider the availability of therapeutic targets and components of the required cellular processes prior to targeting CUTL1 in combination with current and future chemotherapeutic agents.

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