Reversal of multidrug resistance of gastric cancer cells by down-regulation of ZNRD1 with ZNRD1 siRNA

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ABSTRACT

The over-expression of a new zinc ribbon (*ZNRD*1) gene has been shown previously to promote a multidrug-resistant phenotype in gastric cancer cells through the up-regulation of permeability-glycoprotein (P-gp). In the present study, siRNA eukaryotic expression vectors of ZNRD1 are constructed and transfected into SGC7901/VCR cells to examine whether or not down-regulation of ZNRD1 increases cell sensitivity towards chemotherapeutic drugs. After transfection, ZNRD1 expression decreased dramatically in ZNRD1 siRNA transfectants compared with that in parental cells and empty vector control cells. Down-regulation of ZNRD1 significantly enhanced the sensitivity of SGC7901/VCR cells to vincristine, adriamycin and etoposide, but not to 5-fluorouracil and cisplatin. Cell capacity to efflux adriamycin decreased markedly in ZNRD1 siRNA transfectants, and correlation between ZNRD1 down-regulation and increased multidrug resistance 1 (*MDR*1) gene transcriptional activity was observed. These results suggest that the ZNRD1 siRNA constructs down-regulate the expression of ZNRD1 effectively and reverse the resistant phenotype of gastric cancer cells. Furthermore, ZNRD1 might influence transcription of the *MDR*1 gene and thus play an important role in multidrug resistance in gastric carcinoma.

KEY WORDS: Drug resistance, neoplasm. Genes, MDR. Genes, ZNRD. Stomach neoplasms.

Introduction

The zinc ribbon domain-containing 1 (*ZNRD*1) gene is a transcription-associated gene that encodes a protein consisting of two zinc ribbon domains.[1] The C-terminal domain (Cx2Cx24Cx2C) of ZNRD1 is well conserved in many organisms as a transcription-associated protein, which is folded as three β-sheets stabilised by a zinc ion instead of finger-like helices,[2] forming a zinc ribbon structure that fits into the TFIIS zinc ribbon fold (C-terminal residues 231–281) very well.[3] Analogous zinc ribbon motifs occur in many other transcription-associated proteins, indicating that ZNRD1 might be involved in gene regulation.

Previously, it has been shown that ZNRD1 expression is related to the multidrug resistance of stomach cancer. *ZNRD*1 has been shown to be an up-regulated gene in the multidrug-resistant gastric cancer cell line SGC7901/VCR by subtractive hybridisation, reverse-transcriptase polymerase chain reaction (RT–PCR) and Western blot.[4] *In vitro* drug sensitivity assay showed that gastric cancer cells transfected with antisense nucleic acid for ZNRD1 were more sensitive to vincristine and adriamycin than were non-transfectants,[5] and Western and Northern blot techniques showed that over-expression of ZNRD1 could promote a multidrug-resistant phenotype of gastric cancer cells through up-regulation of permeability-glycoprotein (P-gp).[6]

The results of these studies indicate that the *ZNRD*1 gene might effect the occurrence and development of a multidrug-resistant phenotype in gastric carcinoma. Further analysis of biological functions of ZNRD1 in the multidrug resistance of gastric cancer might be helpful for further understanding the mechanisms of multidrug resistance and the development of possible strategies to reverse it in gastric carcinoma.

The present study aims to investigate the role and the underlying mechanisms of ZNRD1 in multidrug resistance in gastric cancer cells. Eukaryotic expression vectors of ZNRD1 small interference RNA (siRNA) are constructed to examine the role of ZNRD1 in the modulation of drug resistance in gastric cancer.

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Materials and methods

Cell culture

The human VCR-resistant gastric adenocarcinoma cell line SGC7901/VCR was developed by exposing the parental SGC7901 cells to step-wise increasing concentrations of anticancer drugs.[7] All cells were routinely cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum in a 37°C humidified incubator in a mixture of 95% air and 5% CO_2. For SGC7901/ VCR cells, the medium also contained 1 μg/mL VCR to maintain its drug-resistance phenotype. Before use, the SGC7901/VCR cells were cultured in drug-free medium for two weeks.

Plasmid construction and transfection

Two pairs of hairpin siRNA oligos for ZNRD1 containing Bbs I and Xba I sites were designed according to work reported

Table 1. IC_{50} values of five cancer drugs for gastric cancer cells.

	Vincristine	Adriamycin	Etoposide	5-fluorouracil	Cisplatin
SGC7901/VCR	49.74 ± 0.28	5.31 ± 0.37	9.26 ± 0.21	12.18 ± 0.17	9.73 ± 0.12
SGC7901/VCR-mU6pro	41.62 ± 0.41	4.39 ± 0.33	7.94 ± 0.26	9.67 ± 0.11	8.81 ± 0.19
SGC7901/VCR-ZND1siRNA	6.09 ± 0.25	0.92 ± 0.13	2.21 ± 0.33	9.11 ± 0.24	8.31 ± 0.13

Data represented as mean ± SD of four independent experiments.

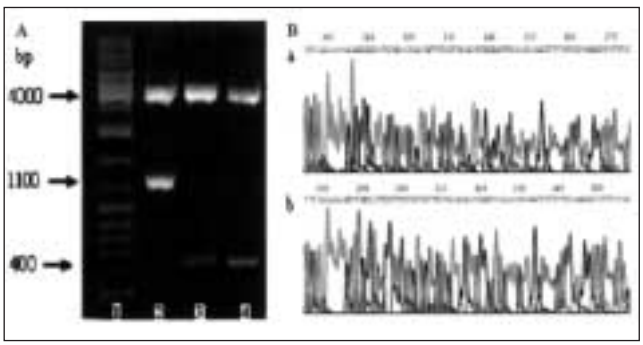

Fig. 1. Identification of ZNRD1 siRNA expression vectors. A: electrophoresis of ZNRD1 siRNA plasmids digested with Hind and Xba endonucleases – lane 1: DNA marker; lane 2: mU6pro vector; lane 3: ZNRD1 oligo1; lane 4: ZNRD1 oligo2. B: DNA sequencing maps – graph a and graph b represent ZNRD1 siRNA1 and ZNRD1 siRNA2, respectively.

previously.[1] For oligo-1, sense: 5′-TTTGAGCCGCAATCTGAA CAGA ACATCTGTTCAGATTGCGGCTCTTTTT-3′, antisense: 5′-CTAGAAAAAGAGCCGCAATCT GAACAGATGTTCTGTT CAGATTGCGGCT-3′; for oligo-2, sense: 5′-TTTGTATGCC ATTCCTTCATGAACATCATGAAGGAATGGCATACTTTTT-3′, antisense: 5′-CTAGAAAAAGTATGCCATTCCTTCATGATGT TCATGAAGGAATGGCATA-3′.

Target sequences were aligned to the human genome database in a BLAST search to ensure that the sequences chosen were not highly homologous with other genes. For annealing to form DNA duplexes, 100 mol/L each sense and antisense oligos were used. Oligos were incubated at 95°C for 5 min and then cooled to room temperature (RT). Duplexes were diluted 1 in 4000 in 0.5x annealing buffer and 1 μL of the dilution was taken for ligation with 50 ng mU6pro vector (previously digested by the Bbs I/Xba I restriction enzyme and gel purified) in a 10 μL volume at RT for 30 min. The products were transformed into DH5α competent cells.

Ampicillin-resistant colonies were chosen, identified by restriction digestion with Hind and XbaI and further confirmed by DNA sequencing. SGC7901/VCR cells were planted in six-well plates and grown in drug-free medium. At 70–80% confluence, cells were washed (x2) with phosphate-buffered saline (PBS) and grown in 2 mL RPMI1640 without antibiotics.

MU6pro-ZNRD1 siRNA plasmids (2 μg) were transfected into SGC7901/VCR cells using Lipofectamine 2000 reagent (Invitrogen), following the manufacturer's instructions. SGC7901/VCR cells transfected with mU6pro vector alone served as a negative control. After 48 h, cells were placed in growth medium containing G418 (Gibco) for clone selection. The ZNRD1 expression levels in G418-resistant clones were evaluated by Western blot analysis.

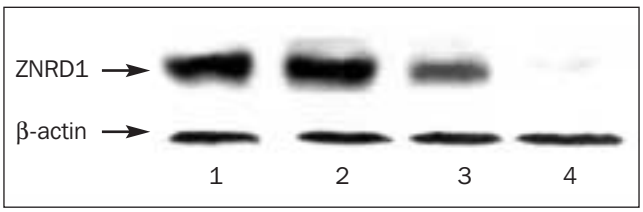

Fig. 2. Identification of siRNA vector by Western blot. Lane 1: SGC7901/VCR; lane 2: SGC7901/VCR-mU6pro vector; lane 3: SGC7901/VCR-ZNRD1 oligo1; lane 4: SGC7901/VCR-ZNRD1 oligo2.

Western blot analysis

Cells were trypsinised and total cellular proteins were prepared with lysis buffer (pH 8.0) containing 1% NP-40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1 mmol/L phenylmethylsulfonyl fluoride and 1 μg/mL aprotinin. Total protein was quantified by the Bradford method. Electrophoresis was performed in 12% SDS-PAGE and blotted on a nitrocellulose membrane. Membranes were blocked with 5% fat-free milk powder at RT for 2 h and incubated overnight with monoclonal antibody specific for ZNRD1 (clone H6) at 4°C.

After washing (x3) for 15 min in Tris-PBS plus Tween 20 (PBS-T), the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Wuhan, Hubei, China) for 1 h at RT. The membrane was washed again in PBS-T, enhanced chemiluminescence (ECL, Amersham) reagent was added and the membrane was monitored for the development of colour. The H6 monoclonal antibody was previously prepared and characterised in-house,[8] and mouse anti β-actin was obtained from Boster Biotechnology (Wuhan, Hubei, China).

Drug sensitivity assay

Vincristine, adriamycin, etoposide, 5-fluorouracil and cisplatin were prepared freshly before each experiment. Drug sensitivity was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, cells were trypsinised and diluted with culture medium to the seeding density ($1x10^4$ cells/well), suspended in 96-well flat-bottomed plates (200 μL/well; Costar) and incubated at 37°C for 24 h. After anchorage, cells were then incubated for 72 h with and without various concentrations of the anticancer agents in 200 μL medium. Each was assayed in triplicate.

After cells were cultured for 72 h, 50 μL 2 mg/mL MTT (Sigma, St. Louis, MI, USA) was added to each well and culture continued for a further 4 h. The supernatant was discarded and 150 μL DMSO (Sigma) was added to each well to dissolve the crystals. Absorbance (A_{490}) was measured with a microplate reader (BP800, Biohit).

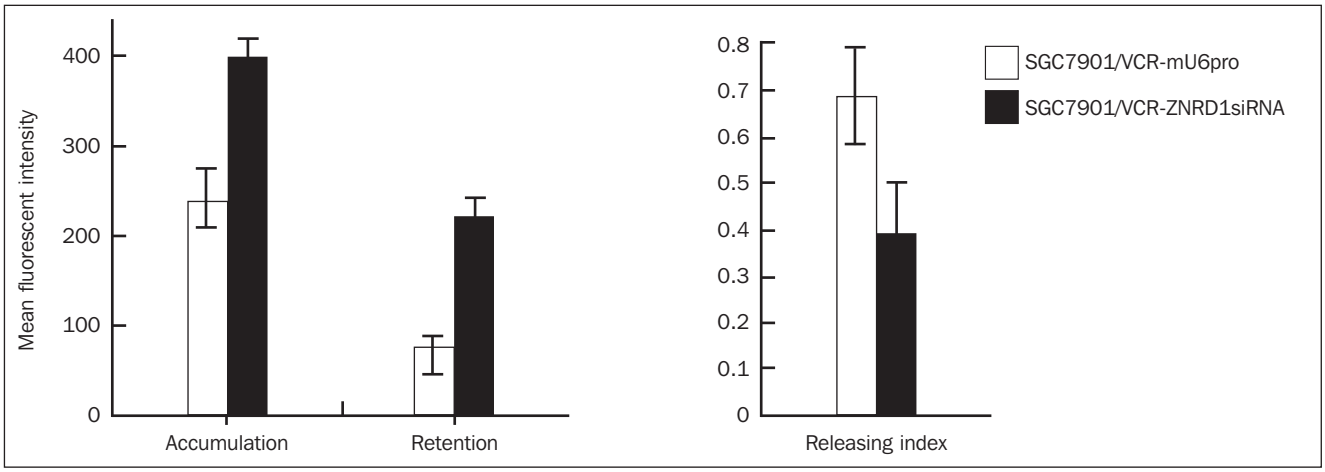

Fig. 3. Effects of ZNRD1 siRNA on the capacity of cells to extrude the fluorescence dye ADR. A: fluorescence intensity analysis of intracellular ADR. B: ADR releasing index of gastric cancer cells. Releasing index = accumulation value – retention value/accumulation value. Data presented as means ±SD (n=3).

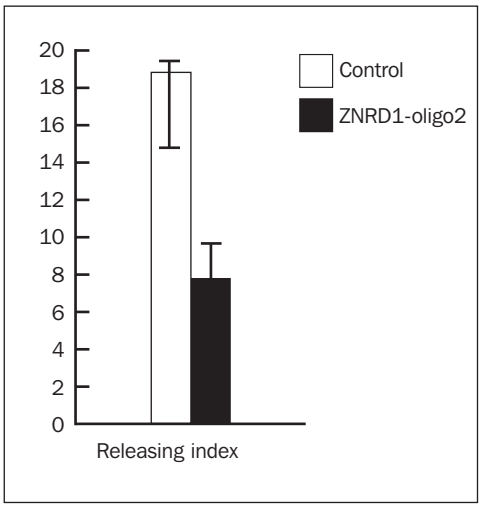

Fig. 4. Down-regulation of ZNRD1 protein expression decreases MDR1 transcriptional activity in SGC7901/VCR cells. Data presented as means ±SD (n=3).

Cell survival rates were calculated according to formula: survival rate = (mean A_{490} treated wells/mean A_{490} untreated wells) x100%. Finally, dose-effect curves of anticancer drugs were drawn on semi-logarithm coordinate paper and IC_{50} values were determined.

Intracellular adriamycin concentration analysis

Fluorescence intensity of intracellular adriamycin was determined by flow cytometry. Briefly, gastric cancer cells in log phase were seeded into six-well plates (1x10^6 cells/well) and cultured overnight at 37°C. After addition of adriamycin to the final concentration of 5 μg/mL, culture continued for 1 h. Cells were then trypsinised and harvested (for detection of adriamycin accumulation) or cultured in drug-free RPMI1640 for a further 1 h, followed by trypsinisation and harvesting (for detection of adriamycin retention).

Cells were washed with PBS and the mean fluorescence intensity of intracellular adriamycin was detected using flow cytometry at an exciting wavelength of 488 nm and emission wavelength of 575 nm. Experiments were performed three times. Finally, the adriamycin-releasing index of the gastric cancer cells was calculated using the formula: releasing index = (accumulation value–retention value)/accumulation value.

Reporter gene assay

SGC7901/VCR cells were plated in six-well dishes and grown in maintenance medium. At 70–80% confluence, cells were co-transfected with the siRNA complexes and pGL3-MDR1. Cells co-transfected with the siRNA complexes and empty pGL3 vector served as a negative control. After 48 h, cells were lysed and luciferase activity was determined using the Dual-Luciferase system. The pGL3-MDR1 vector (MDR1 promoter) and the control vector were provided by Dr Changcun Guo.

Statistical analysis

Data were expressed as mean (± SD). Differences were compared by one-way ANOVA analysis, followed by Dunnett's multiple comparison tests. $P<0.05$ was considered significant.

Results

Identification of ZNRD1 siRNA vector

To verify the mU6pro-ZNRD1 siRNA vectors, the recombinants were identified by endonuclease digestion with Hind and Xba I. The resulting fragments (400 bp) obtained were as expected (Fig. 1A) and were confirmed by DNA sequencing (Fig. 1B).

Down-regulation effect of ZNRD1 siRNA on endogenous ZNRD1

ZNRD1 siRNA oligo-2 significantly down-regulated ZNRD1 expression by almost 87%, compared with that obtained with ZNRD1 siRNA oligo-1 (Fig. 2).

In vitro drug sensitivity assay

Table 1 shows that SGC7901-ZNRD1 siRNA cells exhibited significantly decreased IC_{50} values ($P<0.01$) for vincristine, adriamycin and etoposide (P-gp-related drugs). However, the IC_{50} values for 5-fluorouracil and cisplatin (not P-gp-related drugs) showed no significant difference among these cell lines ($P>0.05$). The data indicated that lower ZNRD1 expression correlated with a decreased cellular ability to resist the chemotherapeutic drugs.

Adriamycin content in ZNRD1 siRNA transfectants

Accumulation of adriamycin in SGC7901/VCR cells

transfected with ZNRD1 siRNA showed a marked increased compared with that in empty vector control cells (Fig. 3). Down-regulation of ZNRD1 was accompanied by a reduction in the amount of adriamycin being pumped out of the cell. The adriamycin-releasing index of ZNRD1 siRNA transfectants was significantly lower than that of empty vector control cells (Fig. 4).

Down-regulation of ZNRD1 decreases MDR1 transcriptional activity

Considerably less luciferase activity was observed in pGL3-MDR1 transfected cells than in the control transfectants. Thus, ZNRD1 might be involved in regulation of MDR1 transcription.

Discussion

The *ZNRD*1 gene was cloned from the human MHC class I region.[1] The zinc ribbon domain at the C-terminal of ZNRD1 protein, similar to the reported zinc ribbon motif in human and yeast TFIIS,[3] is well conserved throughout evolution, including archaea, yeast, drosophila, nematodes, amphibians and mammals.

The zinc ribbon domain is a ubiquitous motif in archaeal and eucaryal transcription and has proved to be a functional domain needed for biological activities in TFIIS and TFIIB.[9–13] Analogous Cys4 structural motifs have been found to occur in many other transcription-associated proteins and play important roles in promoting cleavage of the nascent transcript and read-through past the block to elongation.[14–19]

Recently, ZNRD1 expression was found to be related to the multidrug resistance of gastric cancer cells[4–6] and the carcinogenesis of gastric cancer.[8] Taken together, the *ZNRD*1 gene was assumed to be associated with transcription regulation and might play potential roles in mediating some physiological and pathological functions.

Multidrug resistance is the main obstacle to effective chemotherapy for malignant tumours, especially gastric cancer. In previous work, four resistant sublines from the human gastric cancer cell line SGC7901 were established.[7] The studies also revealed that the VCR-resistant cell line SGC7901/VCR over-expressed P-gp, with cross-resistance to several anticancer drugs, suggesting MDR1/P-gp-mediated classical multidrug resistance. ZNRD1 was found to be over-expressed in SGC7901/VCR cells and might influence the multidrug-resistant phenotype of gastric cancer cells through regulation of P-gp.[6] However, the role of the *ZNRD*1 gene in the multidrug resistance of gastric cancer remains unclear and the present study sought to examine whether or not its down-regulation increased the sensitivity of cells to chemotherapeutic drugs.

At present, blockade of *ZNRD*1 occurs mainly at the DNA and mRNA levels. The former includes DNA trap and gene knockout technology; the latter mostly includes antisense technology and RNAi, which is a new form of gene silencing that has emerged over the past five years. Approaches using antisense techniques have proved useful but are not generally effective.

Previous study proved that RNAi has a much greater effect than antisense oligonucleotides in silencing the target gene and its inhibition is more specific, which is comparable to gene knockout.[20–22] However, the difficulty and expense of knockout techniques limited wide usage. RNAi may be a more promising technique in the study of gene function because it is simple, rapid and economic.

To further explore the role of ZNRD1 in the multidrug resistance of gastric cancer, siRNA technology was used and ZNRD1 siRNA eukaryotic expression vectors were designed to establish the effective transient transfectants. The results obtained confirm previous data which show that ZNRD1 could be involved in tumour resistance to chemotherapeutic drugs through regulation of P-gp expression.

Each case of P-gp-related multidrug resistance has been related to an increased human *MDR*1 mRNA level, which can be linked to gene amplification and/or increased gene transcription.[23] It is believed that alterations in the MDR1 promoter are important for P-gp function.[24] Results of the present study suggest that ZNRD1 might regulate transcription of the chromosomal *MDR*1 gene through direct binding to the MDR1 promoter, which suggests an alternative approach to the control of multidrug resistance. □

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