Identification of GAS1 as an Epirubicin Resistance-related Gene in Human Gastric Cancer Cells with a Partially Randomized Small Interfering RNA Library*5

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Epirubicin has been widely used for chemotherapeutic treatment of gastric cancer; however, intrinsic and acquired chemoresistance remains an obstacle to successful management. The mechanisms underlying epirubicin resistance are still not well defined. Here we report the construction and application of a partially randomized retrovirus library of 4 × 106 small interfering RNAs to identify novel genes whose suppression confers epirubicin resistance in gastric cancer cells SGC7901. From 12 resistant cell colonies, two small interfering RNAs targeting GAS1 (growth arrest-specific 1) and PTEN (phosphatase and tensin homolog), respectively, were identified and validated. We identified a previously unrecognized chemoresistance role for GAS1. GAS1 suppression resulted in significant epirubicin resistance and cross-resistance to 5-fluorouracil and cisplatin in various gastric cancer cell lines. GAS1 suppression promoted multidrug resistance through apoptosis inhibition, partially by up-regulating the Bcl-2/Bax ratio that was abolished by Bcl-2 inhibition. GAS1 suppression induced chemoresistance partially by increasing drug efflux in an ATP-binding cassette transporter and drug-dependent manner. P-glycoprotein (P-gp) and BCRP (breast cancer resistance protein) but not MRP-1 were up-regulated, and targeted knockdown of P-gp in GAS1-suppressed gastric cancer cells resulted in significant epirubicin resistance and cross-resistance to 5-fluorouracil and cisplatin in various gastric cancer cell lines. GAS1 suppression induced multidrug resistance through apoptosis inhibition, partially by up-regulating the Bcl-2/Bax ratio that was abolished by Bcl-2 inhibition. GAS1 suppression induced chemoresistance partially by increasing drug efflux in an ATP-binding cassette transporter and drug-dependent manner. P-glycoprotein (P-gp) and BCRP (breast cancer resistance protein) but not MRP-1 were up-regulated, and targeted knockdown of P-gp and BCRP could partially reverse GAS1 suppression-induced epirubicin resistance. Verapamil, a P-gp inhibitor, could reverse P-gp substrate (epirubicin) but not non-P-gp substrate (5-fluorouracil and cisplatin) resistance in GAS1-suppressed gastric cancer cells. BCRP down-regulation could partially reverse 5-fluorouracil but not cisplatin resistance induced by GAS1 suppression, suggesting 5-fluorouracil but not cisplatin was a BCRP substrate. These results suggest that GAS1 might be a target to overcome multidrug resistance and provide a novel approach to identifying candidate genes that suppress chemoresistance of gastric cancers.

Despite a recent decrease, gastric cancer remains the second most frequent cancer in the world and accounts for substantial morbidity and mortality worldwide. The outcome among patients with advanced gastric cancer is poor. Surgery combined with chemotherapy is the current treatment of choice. The combination of epirubicin, cisplatin, and 5-fluorouracil has been considered as one of the current standard chemotherapy regimes for gastric cancer (1–3). Accordingly, epirubicin remains to be one of the most widely used first-line chemotherapy agents in gastric cancer, but like many other anticancer drugs, intrinsic and acquired resistance to epirubicin treatment has limited its clinical use. Our previous data showed doxorubicin, a structurally related chemotherapy agent to epirubicin, was ineffective in more than 60% of freshly isolated gastric cancer specimens (4). Unlike cisplatin and fluorouracil, whose resistance mechanisms in malignant tumors have been relatively well characterized, the mechanisms of epirubicin resistance remain largely unknown. To date, few molecules have been correlated with the epirubicin resistance of gastric cancer. In previous work, we have identified multiple molecules related to multidrug resistance (MDR) through suppression-subtractive hybridization and differential display PCR (5, 6). However, the mechanism of drug resistance is too complicated to be elucidated only by genotype-based screens because of the fact that numerous unrelated or nonessential molecules may mix with the real key regulators; therefore, phenotype-based genome-wide functional screens might be ideal for functional annotation of both known and novel genes implicated in the drug resistance process (7).

Since the discovery that 21–23-bp double-stranded RNAs (dsRNA) could mediate sequence-specific, post-transcriptional gene silencing of a homologous gene, RNA interference (RNAi) has been utilized as a powerful tool for inhibiting the expression of genes of interest (8). Genome-scale RNAi screens can reveal novel or unexpected pathways and gene families that can induce specific phenotypes when inhibited and simultaneously provide a large background set of genes for the determination of biological effects (9–14). Recently, RNAi screens in model organisms and human cells have successfully identified genes that modulate cell growth,

*5 The abbreviations used are: MDR, multidrug resistance; RNAi, RNA interference; dsRNA, double-stranded RNA; siRNA, small interfering RNA; shRNA, short hairpin RNA; CDDP, cisplatin; 5-FU, 5-fluorouracil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EPI, epirubicin; VCR, vincristine; ADR, adriamycin; ABC, ATP-binding cassette; GFP, green fluorescent protein; BCRP, breast cancer resistance protein; P-gp, P-glycoprotein.

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apoptosis, chemoresistance, and chemosensitivity (9, 15–19). This approach has been particularly successful for the isolation of several genes that encode proteins involved in cancer-relevant pathways such as the p53 and NF-κB pathways, phosphatidylinositol 3-kinase signaling, and RAS-dependent transformation (20–24). We hypothesized that siRNA (small interfering RNA) library screens could identify genes whose loss of function would confer decreased sensitivity to chemotherapy drugs. Identification of such genes could possibly provide targets for gene therapy that, either alone or in combination with conventional chemotherapeutic agents, might help to reverse drug resistance (9).

Here we constructed a partially randomized siRNA retrovirus library and performed RNAi screens in the presence of epirubicin to identify genes whose loss of function desensitized gastric cancer cells to chemotherapeutic agent-induced cell death. For the first time, to the best of our knowledge, we have successfully screened hits targeting functional genes related to a special phenotype (drug resistance in our work) using a randomized siRNA library-based strategy. We further provide evidence that GAS1 (growth-arrest-specific 1), a putative tumor suppressor gene, plays a previously unappreciated role in regulating cellular drug chemosensitivity. This study strongly supports the feasibility of this novel approach to the identification of drug resistance-related genes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmid Transfection, and Retroviral Infection**

Phoenix™-Eco packaging cells were purchased from Orbigen (catalog number RVK-10001). The human gastric adenocarcinoma cell line SGC7901 was obtained from the Academy of Military Medical Science (Beijing, China). Other gastric cancer cell lines, MKN28, MKN45 and AGS, were purchased from the ATCC (Manassas, VA). Multidrug-resistant cell variants, SGC7901/VCR and SGC7901/ADR, were established and maintained in our laboratory. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. All transfections were performed by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. SGC7901-rec, a SGC7901 variant to facilitate retrovirus infection, was generated by transfection of an ecotropic receptor as described previously (21). Ecotropic retroviral supernatants were collected 48 h after transient transfection of Phoenix™-Eco cells, and cells were incubated at 32 °C to obtain higher yields of virus after transfection. Viral supernatants were filtered through a 0.45-μm filter, and infections were performed in the presence of 4 μg/ml Polybrene (Sigma). Fluorescent microscopy was used to detect the transfection and infection efficiency 48 h post-transfection and 72 h post-infection.

**Oligonucleotide Design and Partially Randomized Plasmid Library Construction**

Before constructing the random siRNA library, the siRNA retrovirus expression vector psiRNA-lib was constructed, and psiRNA-lib harboring the Cdc2 siRNA sequence (psiRNA-lib-Cdc2) was established to validate the effectiveness of the vector (see supplemental Fig. S1). Fig. 1 outlines our procedure for constructing the siRNA retrovirus plasmid library, which can roughly be divided into four steps.

**Step A, Partially Randomized Oligonucleotide Design**

siRNA sequences were designed with an algorithm developed to increase the silencing efficiency of the siRNAs as follows: T at position 1, T at position 2, T at position 7, A at position 10, T at position 11, C at position 19, and G at position 21 are characteristic of active siRNAs (25). Based on these rules, a partially randomized library primer with a SalI site and AAAAA at the 5’ end and TTTT as well as a ClaI site at the 3’ end was synthesized. Double-stranded DNA was formed by annealing with a short extension primer (AAAAATAGCTAATGATG) that was complementary to the 3’-terminal sequence.

**Step B, Primer Extension**

Fifty microliters of reaction mixture was prepared (2 μl of polymerase Taq DNA polymerase (Promega), 9 μl of 10 μmol/liter library primer, 18 μl of 50 μmol/liter extension primer, 6 μl of dNTP). Extension was carried out with the following parameters: 95 °C for 2 min, gradual increase from 35 to 72 °C (5 °C every 8 s), and extension at 55 °C for 2 h.

**Step C, Purification of SalI/ClaI DNA Duplexes**

The resulting DNA duplexes were cleaved with SalI and Clai, followed by purification with a QIAquick nucleotide removal kit (Qiagen, Germany). A 15% polyacrylamide gel was used to determine the size of resulting DNA products.

**Step D, Ligation of DNA Duplexes to the Vectors**

Purified DNA duplexes as inserts were ligated into psiRNA-lib vectors by DNA ligase (New England Biolabs). Ligation reactions were transformed into competent DH5α bacteria. Bacterial cultures were grown overnight on 10-cm Petri dish, and all clones were collected for plasmid DNA isolation, thus generating the siRNA plasmid library. Meanwhile, 20 independent clones were picked for EcoRI digestion and sequence analysis for library identification.

**Screening of siRNAs That Conferred Resistance to Epirubicin**

Phoenix™-Eco cells were seeded in four 10-cm plates at a density of 5.5 × 10⁶ cells per plate at 18 h prior to transfection. For each plate, the cells were transfected with 12 μg of siRNA library plasmid (equivalent to 10⁶ plasmids). Ecotropic retroviral supernatants were collected 48 h post-transfection and used to transduce four 10-cm dishes of SGC7901-rec cells in the presence of 4 μg/ml Polybrene. A plasmid encoding a negative control siRNA duplex denoting psiRNA-lib-cont was established (sense, 5′-TTCAGCTAGATTCTAAGTCTG-3′; antisense, 5′-CAGACCTGAATCAGCTGAA-3′); it does not target any known mRNA in mammalian cells evaluated by blast search. This negative control plasmid was introduced into SGC7901-rec cells under the same conditions as those of the siRNA library. Three days post-infection, pools of transfected SGC7901-rec cells were trypsinized and resuspended in four 10-cm dishes in medium containing 1.9 μg/ml epirubicin (EPI), a concentration that was lethal to parental SGC7901 and SGC7901-rec cells (data not shown). After 8 weeks of plating, all the surviving colonies from four dishes were trypsinized, collected, pooled, and then transferred into a 10-cm dish. In all, the resistant colonies were cultured and expanded for about 8 weeks before sequence identification and analysis. The screen was repeated eight times totally.
Recovery of siRNA Inserts

Genomic DNA was isolated from expanded colonies using DNAzol (Invitrogen). PCR amplification of the siRNA inserts was performed using primers adjacent to the siRNA cloning site with forward primer 5'-GGATCTCGAGAAGATCCTTTGAT-3' and reverse primer 5'-GGATCTCGAGAAGATCCTTTGAT-3'. PCR products were digested with XhoI and cloned back into pRetro-GFP digested by SalI. Products were sequenced using a psiRNA-lib-seq primer, 5'-GCATGGACGAGCTGTACAAG-3. Ligation reactions were transformed into competent DH5a bacteria. Bacterial cultures were grown overnight, and plasmid DNA was isolated and subjected to sequencing.

Western Blot Analysis

Western blotting was performed as described (26). Anti-Cdc2 antibody was from Amersham Biosciences. Anti-human GAS1 polyclonal antibody was purchased from R & D Systems (catalog number AF2636). Anti-human PTEN, Bcl-2, Bax, MR1, P-pg, and BCRP antibodies were purchased from Santa Cruz Biotechnology. Western blotting for β-tubulin (Sigma T4026) was used as an internal control. Each experiment was repeated three times, and the ratios of various proteins against β-tubulin were calculated. SGC7901 cells transfected with psiRNA-lib-cont (SGC7901/cont) were used as a control in all experimental procedures. Each experiment was repeated three times.

Quantitative RT-PCR

The mRNA expression and silencing effects were quantified by quantitative real time RT-PCR on a Bio-Rad Chromo 4 instrument with a SYBR Green real time RT-PCR master mix kit (Takara, China) for detection of GAS1. GAPDH was used as an endogenous control. Forward (F) and reverse (R) primer sequences were as follows: GAS1 (F) 5'-CTGGGGTTGTTTACGTTACTAC-3', where N represents bases with different ATGC compositions. The single-stranded library primers were annealed to extension primer 5'-AAAAATAGCTAATGATG-3', and double-stranded fragments were generated by primer extension. The double-stranded fragments were digested with Sall and Clal and cloned into the same sites in plasmid psiRNA-lib-Cdc2.

![Sequence of 20 randomly picked clones from the library](image)

The direction of the sequence is from 5' to 3' on the antisense strand. The nonrandom nucleotides (according to previous design) are boxed in gray. N=A or G or C or T. † represents sequences inserted with the five T termination signal.

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Intracellular Epirubicin Concentration Analysis

The fluorescence intensity of intracellular EPI was determined using flow cytometry as described previously (28). In brief, SGC7901/cont and SGC7901/siGAS1 cells in log phase were plated onto 6-well plates (1 × 10^6 cells/well). After the addition of EPI to a final concentration of 1.9 μg/ml, cells were cultured for 1 h. Then cells were harvested to detect EPI accumulation or were continuously cultured in drug-free medium for another 2 h, followed by harvesting to detect EPI retention. Finally, cells were washed twice with cold phosphate-buffered saline, and the mean fluorescence intensity of intracellular EPI was detected using flow cytometry with an excitation wavelength of 488 nm and emission wavelength of 575 nm.

Apoptosis Analysis by Flow Cytometry

Twenty-four hours after transfection, EPI was added into SGC7901-derived cells to a final concentration of 0.8 μg/ml. 18 h later, cells were harvested and washed twice with pre-cooled phosphate-buffered saline. After incubation with a mixture containing annexin V and propidium iodide (Roche Diagnostics) in binding buffer for 15 min, the fluorescences of cells were measured using flow cytometer (BD FACS<sup>™</sup> Canto). Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane.

Statistical Analysis

Data are expressed as means ± S.D. Differences between means were analyzed with Student's <i>t</i> test. All statistical analyses were performed using SPSS11.0 software (Chicago). Differences were considered significant at <i>p</i> < 0.05.

RESULTS

Construction and Validation of Partially Randomized siRNA Retrovirus Library—After siRNA retrovirus expression vector psiRNA-lib was constructed, and the effectiveness was confirmed by introducing Cdc2 siRNA sequence (see supplemental Fig. S1). We cloned the primer extension product of the synthesized 21-nucleotide partially randomized sequence into the cloning site of psiRNA-lib. Library construction steps are summarized in Fig. 1. To increase the likelihood of obtaining a significant inhibition of gene expression, the 21-bp partially randomized siRNA library primer was synthesized based on guidelines suggested by Huesken et al. (25). Thereafter, the 21-bp library primer was annealed with the extension primer, and primer extension was performed to form complementary DNA duplexes that were further ligated with psiRNA-lib. For each 10-cm Petri dish, as many as 8 × 10^5 clones were obtained when using 50-ng vector at a ratio of 1:10 relative to the siRNA insert (data not shown). In total, ligation products were plated in 500 Petri dishes, and 4 × 10^6 clones were obtained finally.

To validate the library, we isolated 20 independent clones for EcoRI digestion (data not shown), and sequence information of the inserts was obtained (Fig. 2). Of these, 18 constructs contained inserts of the appropriate size and all were unique, although one clone had no insert and one clone had a TTTTTT termination sequence. Average G + C content was 41.1%. These results suggest that the library we constructed contained sufficient complexity (4 × 10^6) for screening purposes. After library validation, we collected the pools of clones and extracted the plasmids.

Screening of siRNAs That Conferred Resistance to Epirubicin—To validate the random siRNA screening approach, we developed a cell system to screen for EPI-resistant molecules in gastric cancer cells. Phoenix<sup>™</sup>-Eco cells were transfected with 12 μg of siRNA library plasmid (equivalent to 10^6 plasmids) per 10-cm plate to generate the siRNA retrovirus library. In total, four plates of the virus library should then be used to infect four plates of SGC7901-rec cells and selected with 1.9 μg/ml EPI to ensure relatively low plasmid/cell ratio (roughly 1:1) and enough coverage of library capacity. Fluorescence microscopy showed that the infection frequency was ~20% by analysis of GFP fluorescence (see supplemental Fig. S1). The first resistant pools of colonies were observed 2 weeks after retroviral transduction and, considering that some cells might survive whereas

TABLE 1

Sequences of 12 siRNAs obtained by screening a partially randomized siRNA retrovirus library

| Clone No. | Sequence (5′–3′) |
|-----------|-----------------|
| siRNA 001 | TCCATTTCGTAGAACCACCG |
| siRNA 002 | TCTGATCGCGG |
| siRNA 003 | TTTCGATTCGATCGTCATCCTG |
| siRNA 004 | TTGCGCTAAATTCGATCGCGG |
| siRNA 005 | TTTCGGATGGATAACGCCACTG |
| siRNA 006 | TTTAGTTCAATATGTTGTCCG |
| siRNA 007 | TTCCGATCCATGCAATTCCAG |
| siRNA 008 | TTCCGATCCATGCAATTCCAG |
| siRNA 009 | TTAGTTCAATATGTTGTCCG |
| siRNA 010 | TTTCGATTCGATCGTCATCCTG |
| siRNA 011 | TTTCGATTCGATCGTCATCCTG |
| siRNA 012 | TTTCGATTCGATCGTCATCCTG |

FIGURE 3. Chemosensitivity of transfected SGC7901 cells to EPI determined by MTT analysis. A, SGC7901 cells were transfected with 12 individual siRNAs obtained from the screening of a random siRNA library. SGC7901 and SGC7901/cont cells were used as controls. **, <i>p</i> < 0.05. B, Transfection efficiency of each siRNA was obtained by observation of GFP-positive cells using fluorescent microscopy. Totally four fields at random positions were collected, and average percentage of GFP-positive cells was counted.
others might die gradually, we allowed all colonies to proliferate for 8 weeks under EPI selection pressure before further characterization. A plate of SGC7901-rec cells infected with the psiRNA-lib-cont virus was included as a parallel negative control to eliminate the possibility of acquired resistance because of prolonged culture with EPI. The screen was repeated eight times to collect as many surviving clones as possible. Twelve clones were collected from a total of 32 plates in the presence of 1.9 \( \mu \)g/ml EPI, and no spontaneously resistant clones grew under these selective culture conditions. Subsequently, the genomic DNA of surviving cells was isolated individually, and PCR was performed to amplify the siRNA expression cassettes; 12 siRNA sequences were obtained (Table 1). To detect genes involved in drug resistance, we compared the sensitivities of SGC7901 cells transfected with psiRNA-lib plasmids harboring every candidate siRNA and psiRNA-lib-cont (SGC7901/cont) with different concentrations of EPI by MTT assay. Normalized by transfection efficiency, IC\(_{50}\) values of SGC7901 cells transfected with all siRNAs were higher than those of SGC7901/cont cells, with siRNA 001 (4.3-fold) and siRNA 003 (3.9-fold) the most significant (Fig. 3), indicating these siRNAs might suppress genes involved in EPI resistance. BLAST was used to compare nucleotide sequences with human transcript data bases and to calculate the statistical significance of matches. Of the 12 siRNA sequences obtained, siRNA 001 and siRNA 003 produced the most significant alignments, whereas the other 10 siRNAs failed to target any transcripts in the human genome. The sense strand of siRNA 001 (TTCATTTCCATGAGCCACCG) has 95% homology to site 204–224 (TTCATTTCCAGAGCCACCG) of the human GAS1 (NM_002048.1) transcript, with only one nucleotide mismatch (T at position 11 instead of G) (Fig. 4A). The sense strand of siRNA 003 (TTTAACTGTATTATTTGGCAG) has 95% homology to site 5221–5241 (TTTAACTGTAGTATTTGGCAG) of the PTEN (NM_000314.4) transcript, with one nucleotide mismatch (T at position 11 instead of G) (Fig. 4B). These two genes are likely to be the target genes for our isolated clones, because previous findings have shown that several mismatches can be tolerated between siRNAs and targets (29, 30).

Silencing of GAS1 Yielded Significant Resistance to Various Anticancer Agents in Gastric Cancer Cells—Because PTEN has been intensively studied for its role in drug resistance to gastric cancer (31, 32), we focused on GAS1, a putative tumor suppressor gene that had not been previously reported to be involved in drug resistance. GAS1 blocks entry to S phase and prevents cycling of normal and transformed cells. To confirm that the EPI-resistant phenotype was because of specific inhibition of the GAS1 gene, we first introduced psiRNA-lib-GAS1(+) plasmids harboring the GAS1 siRNA sequence with one nucleotide mismatch. Figure 4. Sequence analysis and effectiveness confirmation of GAS1 or PTEN siRNA, and validation of their roles in EPI chemoresistance. A and B, sequence analysis of siRNAs obtained from the screening of a random siRNA library targeting GAS1 (A) and PTEN (B), respectively. Inhibition of GAS1 and PTEN expression in SGC7901 cells by transfection with psiRNA-lib-GAS1, psiRNA-lib-GAS1(+), psiRNA-lib-PTEN, and psiRNA-lib-PTEN(+), respectively, is shown. SGC7901/cont was established by transfection with psiRNA-lib-cont in SGC7901 cells. Relative expression levels of GAS1 (C) and PTEN (D) were determined by real time RT-PCR. All data were normalized by GAPDH. Means ± S.D. are shown. E, Western blot analysis of GAS1 expression in SGC7901/siGAS1 and SGC7901/siGAS1(+) cells. F, Western blot analysis of PTEN expression in SGC7901/siPTEN and SGC 7901/siPTEN(+) cells. SGC7901 and SGC7901/cont cells were used as controls; β-tubulin was used as a loading control. Chemosensitivities of GAS1 (G) and PTEN (H) siRNA plasmid-transfected SGC7901 cells to EPI were determined by MTT analysis. **, \( p < 0.05 \). (+) GAS1 or PTEN siRNA with one nucleotide mismatch.
mismatch as well as a fully matched counterpart psiRNA-lib-GAS1 into separate SGC7901 cell lines, thus establishing SGC7901/siGAS1 and SGC7901/siGAS1 cell lines. The forward and reverse strands of the “perfect match” target sequence of GAS1 were ordered separately and annealed to form a ready-to-ligate target sequence. Real time RT-PCR (Fig. 4C) and Western blot (Fig. 4E) analysis showed that GAS1 expression was significantly down-regulated by more than 80% in both

FIGURE 5. psiRNA-lib-GAS1 and pcDNA3-GAS1 plasmid transfections regulate resistance to multiple structurally unrelated chemotherapeutic agents. A, Western blot analysis of GAS1 expression in protein lysates from MKN28, SGC7901, MKN45, and AGS cell lines. B, up-regulation and down-regulation of GAS1 expression after pcDNA3-GAS1 transfection in MKN28 cell lines were confirmed by Western analysis. MKN28, MKN28/pcDNA3, and MKN28/psiRNA-lib-cont were used as controls. MTT assay showed cell viability upon combination of chemotherapy and pcDNA3-GAS1 transfection in MKN28 and AGS cells treated with low dose EPI (C), CDDP (D), and 5-FU (E), respectively. MTT assay showed cell viability in psiRNA-lib-GAS1 transfected four gastric cancer cells treated with EPI (F), CDDP (G), and 5-FU (H), respectively. **, p < 0.05.
SGC7901/siGAS1 and SGC7901/siGAS1 (+) cells compared with SGC7901/cont control cells. These results indicate that SGC7901/siGAS1 (+) was capable of inhibiting GAS1 expression to the same degree as SGC7901/siGAS1, suggesting that the mutated sequences still retain the silencing ability. MTT assay also validated decreased epirubicin sensitivity by GAS1 suppression in SGC7901 cell lines (Fig. 4G). A similar approach to the PTEN gene generated the similar results observed for GAS1 (Fig. 4, D, F, and H), indicating that our library has the ability to screen functional siRNAs.

To determine whether GAS1 was involved in chemoresistance of gastric cancer, we first examined the effect of GAS1 on the sensitivities of different gastric cell lines with varying levels of GAS1 expression (Fig. 5A), to various structurally unrelated anticancer agents. AGS and MKN28, which showed the lowest background expression of GAS1 among the four cell lines, were chosen for studies by up-regulating GAS1 expression. Cells were transfected with pcDNA3-GAS1 plasmid 48 h before treatment with 0.08 μg/ml EPI, 2.5 μg/ml 5-FU, or 0.4 μg/ml CDDP, respectively. Cells were then cultured for an additional 48 h, and cell viability was determined by MTT assay. Up-regulation of GAS1 protein expression by pcDNA3-GAS1 transfection in four cell lines was confirmed by Western analysis. Fig. 5B showed the MKN28 results. We observed enhanced cell death after chemotherapeutic agents in cells transfected with pcDNA3-GAS1, as compared with those treated with low dose chemotherapeutic agents alone, which displayed little effect on cell death (Fig. 5, C–E). This phenomenon happened independently of the cytotoxic mechanisms of the anticancer agents, suggesting that the enhanced cell death might be due to the GAS1-related pathway.

To further determine the role of GAS1 down-regulation in MDR, we tested whether GAS1 down-regulation was able to protect against death induced by EPI, 5-FU, and CDDP. We transfected SGC7901, MKN45, MKN28, and AGS cells with psRNA-lib-GAS1 separately to inhibit GAS1 expression. Cells were cultured for 48 h before treating with low dose EPI, 5-FU, and CDDP, respectively. Down-regulation of GAS1 expression after psRNA-lib-GAS1 transfection in four gastric cancer cell lines was confirmed by Western analysis, and Fig. 5B showed the MKN28 result. We observed enhanced cell survival after chemotherapy in cells transfected with GAS1 siRNA plasmid, and this increase was correlated with GAS1 expression (Fig. 5, F–H). It was also noted that GAS1 down-regulation had little effect on the protection against drug-induced death in AGS cells, which showed little endogenous expression of GAS1, indicating this effect was specific to GAS1 inhibition. We further examined GAS1 expression in two drug-resistant cell lines we established previously, SGC7901/ADR and SGC7901/VCR (33), which also display cross-resistance to various drugs, including EPI. It was found that compared with SGC7901/cont cells, GAS1 was significantly down-regulated in both SGC7901/ADR (>10-fold, \( p < 0.05 \)) and SGC7901/VCR (>10-fold, \( p < 0.05 \)) (Fig. 6, A and B), and up-regulating GAS1 expression by pcDNA3-GAS1 transfection could partially reverse EPI resistance mediated by GAS1 inhibition when compared with controls (Fig. 6C).

**FIGURE 6.** GAS1 expression in SGC7901 and its resistant variants, SGC7901/ADR and SGC7901/VCR. A, real time RT-PCR was performed to determine relative GAS1 expression in the SGC7901 cell line and in its resistant cell lines. All data were normalized by GAPDH. Means ± S.D. are shown. B, Western blot analysis of GAS1 expression in SGC7901 and resistant cell lines. β-Tubulin was used as a loading control. C, chemosensitivities of pcDNA3-GAS1-transfected SGC7901/ADR and SGC7901/VCR cells to EPI were determined by MTT analysis. ***, \( p < 0.05 \).

GAS1 Down-regulation Promotes Drug Resistance by Inhibition of Apoptosis—The development of drug resistance in various cancer cells has been linked from reduced susceptibility to drug-induced apoptosis (34). Considering that GAS1 expression has been reported to induce tumor apoptosis and inhibit tumor growth (35, 36), we focused the apoptosis-promoting role of GAS1 in regulating chemosensitivity of gastric cancer cells. Fluorescence-activated cell sorter detection of annexin V showed that the proportion of apoptotic SGC7901/siGAS1 cells was significantly decreased (>2-fold) when compared with the proportion observed in SGC7901/cont cells (Fig. 7, A and B). This finding suggests that GAS1 could inhibit EPI-induced apoptosis in gastric cancer cells. Subsequently, two important mol-
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A

![Bar chart showing apoptosis rate comparison between 7901/cont and 7901/siGAS1.

B

![Scatter plots comparing apoptosis rates between 7901/cont and 7901/siGAS1.

C

![Western blots showing expression of Bax, Bcl-2, and Tubulin in 7901, 7901/cont, and 7901/siGAS1.

D

![Bar chart showing IC50 values for different treatments in 7901, 7901/cont, 7901/siGAS1, and their respective controls.

* * *
ecules in apoptosis, Bcl-2 and Bax, were investigated for possible involvement. Western blotting indicated that inhibition of GAS1 could up-regulate Bcl-2 without affecting the expression level of Bax (Fig. 7C). Furthermore, when SGC7901 cells were exposed to either EPI or 5-FU and CDDP, inhibition of Bcl-2 expression by siRNA transfection could significantly decrease the effect of GAS1 inhibition on the survival of cells (Fig. 7D). Knockdown of Bcl-2 protein expression after siRNA transfection was confirmed by Western analysis (see supplemental Fig. S2). Thus, GAS1 might partially inhibit the multiple cancer drugs-induced apoptosis of gastric cancer cells by increasing the Bcl-2/Bax ratio. However, decreased expression of GAS1 per se promotes cell proliferation (see supplemental Fig. S3), and we cannot exclude that the enhanced proliferation might also contribute to the resistant phenotype.

**GAS1 Down-regulation Promotes Drug Resistance by Regulation of Drug Transport**—Drug transport is another common mechanism that might contribute to multiple drug resistance of tumor cells. Therefore, we attempted to determine whether GAS1 affects intracellular drug accumulation. To evaluate this hypothesis, the influence of GAS1 on intracellular drug accumulation and retention was evaluated, using EPI as a probe. The fluorescence intensity of intracellular EPI was determined by flow cytometry. As shown in Table 2, SGC7901/siGAS1 cells showed significantly decreased fluorescence intensity compared with SGC7901/cont cells, indicating that GAS1 inhibition regulated the active transportation of drugs.

We further explored whether GAS1 inhibition could affect the expression of P-gp and the other two members of the ABC transporter family, MRP-1 and BCRP, which have been reported to participate in EPI resistance (37). Western blotting revealed that P-gp and BCRP but not MRP-1 was up-regulated by GAS1 inhibition in gastric cancer cell lines (Fig. 8, A–C). AGS cells were not included in this study because of relatively low expression of GAS1. To determine whether inhibition of transporter expression affects GAS1 down-regulation-induced drug resistance, we performed MTT assay in which expression of P-gp, BCRP, or both were inhibited by siRNA transfection. Knockdown of P-gp and BCRP protein expression after siRNA transfection was confirmed by Western analysis (see supplemental Fig. S4). It was indicated that inhibition of transporter expression could partially reverse the EPI-resistant phenotype mediated by GAS1 inhibition when compared with controls, with the greatest effect observed in cells in expression of both P-gp and BCRP was inhibited. However, cells with GAS1 inhibition still had a weak survival advantage over control cells, even in the presence of P-gp and BCRP siRNAs (Fig. 8, D–F). Furthermore, we examined whether resistance of SGC7901/siGAS1 cells to P-gp substrates EPI, ADR, and VCR (vincristine) could be modulated by verapamil, a P-gp inhibitor and chemosensitizer. MTT assay showed that verapamil could partially reverse the EPI, ADR, and VCR resistance by GAS1 down-regulation (Table 3), although it exerted little effect on GAS1 down-regulation-mediated 5-FU and CDDP resistance, which were non-P-gp substrates (data not shown). P-gp knockdown by P-pg siRNA transfection also exerted no reverse effect on 5-FU and CDDP resistance mediated by GAS1 inhibition (see Fig. 8G). Interestingly, although there are few reports concerning BCRP-mediated 5-FU and CDDP resistance, our findings suggested that knockdown of BCRP expression by siRNA transfection could partially reverse 5-FU resistance mediated by GAS1 inhibition when compared with controls in SGC7901 cells; however, no influence on the CDDP-resistant phenotype was observed by BCRP knockdown analysis (see Fig. 8G). These findings indicate that GAS1 down-regulation promotes MDR partially by increasing drug efflux by ABC transporters.

**DISCUSSION**

Although the genome-wide screening approach has been well established in mammalian cells, relatively few screens utilize a random siRNA library (38–41). In this work, we have successfully constructed a partially randomized siRNA retrovirus library and applied it to identify GAS1, whose loss of function led to marked epirubicin resistance in SGC7901 cell lines. These results demonstrate the potential power of random siRNA libraries in the identification of genes involved in drug resistance.

Many studies have been focused on generating siRNA libraries from either cDNAs (42–45) or chemically synthesized siRNAs (21, 46, 47), and many functional genes have been successfully identified. However, given the complicated technical manipulation and relatively low efficiency for enzymatic preparation of siRNA libraries from cDNAs, as well as the high cost of chemical synthesis, the random siRNA library strategy might provide a favorable alternative. The random siRNA library strategy represents an easy approach that can be used for screening at lower cost. Several groups have successfully carried out genetic screens using random siRNA libraries, further suggesting the feasibility of this approach. Chen et al. (38) reported the construction of a 19-mer fully randomized siRNA plasmid library that was used to screen genes related to cell proliferation; multiple siRNAs that could significantly enhance cell growth were identified. Recently, Wang et al. (40) constructed a full 29-mer random shRNA (short hairpin RNA) library to screen three functional siRNAs that could promote cell survival. This group also made some improvements using a retroviral vector, as we did, which allows long term expression.

**TABLE 2**

| Fluorescence intensity | SGC7901/cont | SGC7901/siGAS1 |
|------------------------|-------------|---------------|
| Accumulation           | 100.1 ± 15.3| 42.8 ± 9.2    |
| Retention              | 46.7 ± 10.2 | 26.6 ± 9.4    |

*p < 0.05 is compared with the respective control cell.

**FIGURE 7. Effects of GAS1 inhibition on EPI-induced apoptosis and expression levels of Bcl-2 and Bax.** A, annexin V staining was used to evaluate cell apoptosis by flow cytometry. B, percentage of apoptotic cells was shown (mean ± S.D.). C, Western blot analysis showed that Bcl-2 was significantly up-regulated in SGC7901/siGAS1 cells, whereas Bax was not. SGC7901 and SGC7901/cont were used as control cells. D, altered chemosensitivity of SGC7901/siGAS1 cells to EPI, 5-FU, and CDDP by Bcl-2 siRNA transfection was determined by MTT analysis. **, p < 0.05 compared with control cells.
of the integrated siRNAs. Furthermore, the low infection efficiency (20% in our work and 30% in theirs) could ensure the delivery of approximately 1 siRNA construct per cell, thereby maximizing the chances of identifying effective siRNAs with weaker effects (40). The differences between our strategy and that of Wang et al. (40) lie in the selection of siRNA versus shRNA and the length of the siRNA sequence. Although it was reported that shRNA (expressed from one promoter in transfected cells) was more effective than inhibition by tandem siRNA (expressed from separate promoters) (48), shRNA has some limitations. shRNA is difficult to synthesize in bacteria and sequence. Moreover, the oligonucleotides needed to generate shRNA can be costly and error-prone (49, 50). In addition, the hairpin length and sequence might affect the ability of the siRNA to inhibit gene expression (51). To overcome these limitations, we have constructed an siRNA expression plasmid derived from pHippy (50), which can express siRNAs from convergent opposing promoters and is thus well suited to generate a random siRNA library (38). It has been demonstrated that the pHippy system could be used for random siRNA screening (50). We have also demonstrated that our newly established siRNA expression plasmid psiRNA-lib based on pHippy was as effective as a shRNA plasmid (data not shown). Some reports showed that shRNAs or siRNAs of 27–29 bp were more potent inducers of RNAi than constructs of 19–21 bp (52). However, one concern is that sequences longer than 23 bp are more likely to induce nonspecific interferon responses (53). Two other groups also reported the construction of a randomized shRNA library using either the pre-hairpin or the PCR method, but no subsequent screening procedures were carried out (39, 41).

One of the limitations for the reported siRNA libraries is that they failed to identify target genes by screening. One possible reason is that a fully randomized sequence might decrease the possibility of achieving successful RNA interference. In our study, we established a 21-mer partially randomized siRNA library instead of a hairpin siRNA library
TABLE 3

Effect of the P-gp inhibitor verapamil on GAS1 suppression-mediated drug resistance

The sensitivity of SGC7901-derived cells to different chemotherapeutic agents accompanied by 25 μg/ml verapamil (VP) was evaluated using MTT assay. The concentration of each drug that caused a 50% reduction in number of cells (IC50) was calculated.

| Cell lines          | IC50 value   |
|---------------------|--------------|
|                     | EPI | ADR | VCR       |
| SGC7901/cont        | 0.81 ± 0.35 | 0.53 ± 0.13 | 3.11 ± 1.13 |
| SGC7901/SiGAS1      | 3.48 ± 0.73 | 2.76 ± 0.71 | 9.86 ± 2.09 |
| SGC7901/SiGAS1 + VP | 1.43 ± 0.46a | 1.17 ± 0.41 | 4.19 ± 1.23a |

*a p < 0.05.

FIGURE 8. Effects of GAS1 inhibition on the expression of ABC transporter proteins. Western blot detection of GAS1, P-gp, BCRP, and MRP-1 in pSiRNA-lib-GAS1 transfected MNK28 (A), SGC7901 (B), and MKN45 (C) cells, respectively. β-Tubulin was used as a loading control. MTT assay examined the altered chemosensitivity of pSiRNA-lib-GAS1-transfected MNK28 (D), SGC7901 (E), and MKN45 (F) cells to EPI by transfection with P-gp siRNA, BCRP siRNA, or a combination of both. G, MTT assay examined the altered chemosensitivity of pSiRNA-lib-GAS1-transfected SGC7901 cells to 5-FU and CDDP by transfection with BCRP siRNA. **, p < 0.05 compared with untreated control cells.

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harboring a longer siRNA sequence. The design strategy of the library was based on effective siRNA design algorithms as reported previously (25), which likely improved the potency of the library and increased the possibility of screening functional siRNAs. To the best of our knowledge, we have constructed the first random siRNA library used to successfully identify functional target genes.

The library capacity is a concern for random siRNA library screening. Ideally, a random siRNA library should be extensive enough to cover most genes, but limited enough to facilitate screening (38). Chen et al. (38) achieved a satisfactory capacity of 5 × 106 fully randomized siRNAs; this is also the theoretical capacity for our library. However, this capacity will likely make manipulation of the library difficult during screening. To reduce the complexity, we constructed a partially randomized library of 4 × 106 siRNAs, which should facilitate screening while still ensuring effectiveness. Theoretically, there could be multiple functional siRNAs against a single gene and a relatively small library could cover a substantial part of the genome. In practice, we achieved a capacity greater than that attained by Wang et al. (40) for a 29-mer fully randomized library (3 × 106) and also comparable with that of enzymatically generated siRNA libraries (105–106 as previously reported) (43–45). Notably, we have identified some functional siRNAs as well as two novel genes involved in drug resistance, indicating that the capacity of our library is large enough to screen functional genes.

For random siRNA library screens, the “pooled screen” approach is favored. Compared with the “arrayed screen” approach, the pooled screen is straightforward and facilitates whole genome screens without requiring automated aids or software analysis. In contrast, we applied drugs at a concentration lethal for parental cells, and the surviving resistant cells were collected for further analysis; this process was less labor intensive. It has been suggested that the pooled screen approach can be performed by assaying enrichment from pools (positive selection), but this limits the range of phenotypes that can be addressed (18). Although the arrayed screen approach, often combined with the barcode screening approach, can be used for both negative and positive selection, we used a phenotypic filter (i.e., drug resistance) that reflects cell survival and only selected resistant colonies (not sensitive ones). The limitation of the pooled screen approach is that it appears not to be as sensitive as the arrayed screen approach, and a large number of false negatives or false positives can be obtained during screening (54). However, false positives can be easily excluded in further validation studies. Therefore, we consider that extensive tests with a set of secondary assays should be performed to increase the number of positive hits in the pooled screen (55).

According to previous reports, siRNA has a high level of mismatch tolerance. In this study, we found that siRNA with one or two nucleotide mismatches was well tolerated, and the target site was silenced almost as efficiently as its fully matched counterpart, which agreed with a previous report (30). In this work, we isolated 12 clones, all of which were shown to be somewhat effective for mediating epirubicin resistance; however, only the two most prominent siRNAs were found to target genes. It has been suggested that BLAST software might not be suitable for specificity analysis of siRNAs because it can miss some potential targets (56). Therefore, the use of advanced alignment algorithms and an understanding of the mechanism of silencing are required for random siRNA library-based screening to produce more reliable results.

Our screening identified siRNAs that target PTEN and GAS1. PTEN is a well known tumor suppressor gene that has been extensively implicated in tumor drug resistance (31, 32, 57, 58). We therefore concentrated on GAS1, which plays a previously unappreciated role in regulating cellular drug resistance. GAS1 is inserted into the cell membrane through a glycosylphosphatidylinositol anchor at its carboxyl terminus (59) and has been directly related to cell cycle arrest and/or apoptosis in different cell systems (60, 61). The role of GAS1 has been implicated in embryonic development, stem cell renewal, and cancer growth (61). Our study further broadens the understanding of its biological functions by identifying its role in mediating MDR phenotype.

Regulation on apoptosis pathway and drug efflux are two common mechanisms leading to MDR. We showed that GAS1 down-regulation might promote MDR through inhibition of apoptosis, partially by up-regulating the Bcl-2/Bax ratio. Inhibition of Bcl-2 expression by siRNA transfection could significantly abolish the inhibition of GAS1 on cell survival, which is also consistent with the known role of Bcl-2 as a primary regulator of apoptosis. The phenomenon that apoptosis inhibition through up-regulating Bcl-2 correlates positively with CDDP and 5-FU resistance in various malignant tumors has been reported previously (62–66), which is consistent with our results. Although it has been indicated that GAS1 could inhibit apoptosis in some systems (67), many studies were supportive that GAS1 was correlated with cell cycle arrest and could act as a tumor suppressor or an inducer of cell death and apoptosis (61). All these data suggest that GAS1 is a pleiotropic gene and...
could regulate apoptosis in a context-dependent manner. In this study, we showed that GAS1 might act as a putative tumor suppressor gene in gastric cancer cells. Consistent with our results, a previous study did genome-wide shRNA screening to identify GAS1 as a novel melanoma metastasis suppressor gene that displayed proapoptotic activity (68). A more recent study also demonstrated that GAS1 could inhibit cell proliferation and induce apoptosis in human primary gliomas (69).

We also found that GAS1 down-regulation could mediate MDR partially by increasing drug efflux in an ABC transporter and drug-dependent manner. GAS1 down-regulation increases the expression of P-gp and BCRP, but not MRP-1, and targeted knockdown of P-gp and BCRP expression by shRNA could partially reverse GAS1 down-regulation-induced EPI chemoresistance. Verapamil, a P-gp inhibitor, could reverse P-gp substrate (i.e. EPI)-related resistance. In contrast, cross-resistance to non-P-gp substrates, such as 5-FU or CDDP in our study, was not influenced by verapamil, indicating that GAS1 down-regulation might confer MDR phenotypes in gastric cancer cells by either P-gp-related or P-gp-unrelated pathways. P-gp knockdown by siRNA transfection also exerted no reverse effect on 5-FU and CDDP resistance mediated by GAS1 inhibition. Interestingly, although there is little information concerning BCRP-mediated 5-Fu and CDDP resistance, our findings indicated knockdown of BCRP expression by shRNA could partially reverse 5-FU but not CDDP resistance mediated by GAS1 inhibition, suggesting 5-FU but not CDDP might be a BCRP substrate. A recent study also identified 5-FU as a specific substrate for BCRP-mediated drug resistance of breast cancer, which is consistent with our findings (70).

Our data suggest that GAS1 may be a target to overcome MDR and increase chemotherapeutic response when applied in combination with anticancer drugs. Regulation of drug efflux and apoptosis are both important mechanisms by which GAS1 inhibition promotes chemoresistance, but they are not the sole causal factors. It has been shown that GAS1 regulates cell cycle progression, Hedgehog pathway, and glial cell line-derived neurotrophic factor pathway, which also likely contribute to GAS1 inhibition-mediated chemoresistance (71–73).

In conclusion, we constructed a partially randomized siRNA retrovirus library that was used to successfully identify two genes involved in drug resistance. This practical platform could provide a useful tool for the investigation of novel or unexpected pathways and gene families related to a specific phenotype in a large number of cell types and a broad variety of organisms.

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