Tumor suppressor gene Runx3 sensitizes gastric cancer cells to chemotherapeutic drugs by downregulating Bcl-2, MDR-1 and MRP-1

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The Runx3 gene is a member of the runt domain family transcription factors, key regulators of development and differentiation in metazoan. Recently, Runx3 was identified as a tumor suppressor gene. Loss of Runx3 was found to be associated with genesis and progression of gastric cancer. In this study, we transfected the gastric cancer cell line SGC7901 with eukaryotic expression vector of Runx3. In vitro drug sensitivity assay suggested that SGC7901/Runx3 cells were more sensitive to various chemotherapeutic drugs. Blocking Runx3 expression in immortalized stomach mucosal cells (GES-1) or gastric cancer cells (SGC7901) by Runx3-specific small interfering RNA conferred the cells resistance to chemotherapeutic drugs. Flow cytometry examination suggested that expression of Runx3 in gastric cancer cells increased the intracellular accumulation and retention of adriamycin. Semi-quantitative RT-PCR and Western blot suggested that Runx3 downregulated expression of Bcl-2, MDR-1 and MRP-1. Binding of Runx3 to promoter sequences of Bcl-2, MDR-1 and MRP-1 gene was detected by eletrophoretic mobility shift assay (EMSA) and supershift EMSA. We cloned the MDR-1 and MRP-1 gene promoters containing Runx binding sites and constructed the luciferase reporter vectors of these 2 promoters. Luciferase reporter assay suggested that Runx3 inhibited the promoter activity of the MDR-1 and MRP-1 promoter in SGC7901 cells. Taken together, our findings suggested that downregulation of Runx3 could sensitize gastric cancer cells to chemotherapeutic drugs by downregulating the Bcl-2, MDR-1 and MRP-1.

Primers and antibodies
Primers used to amplify the Runx3 full-length cDNA and perform semi-quantitative RT-PCR were shown in Table I. Rabbit polyclonal antibody against human Runx3 used for Western blot was purchased from Oncogene (San Diego, CA). Rabbit polyclonal antibody against human Runx3 used for eletrophoretic mobility shift assay (EMSA) was purchased from Active Motif (Carlsbad, CA). Mouse antihuman Bcl-2, Bax, MRP-1, MDR-1 and β-actin antibody, HRP-labeled goat antirabbit secondary antibody, HRP-labeled goat antimouse secondary antibody were purchased from Santa Cruz (Santa Cruz, CA).

Semiquantitative RT-PCR and Western blot
cDNA from the cell lines was prepared and semi-quantitative RT-PCR was used to examine Runx3 expression. The PCR conditions were 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Twenty-five cycles were performed. β-actin was used as internal control. Cells were lysed and whole cell proteins were obtained. The cell lysates underwent Western blot for Runx3 expression examination.

Cell transfection
GES-1 and SGC7901 cells were transfected with Runx3 eukaryotic expression vector (pcDNA3.1/Runx3) or Runx3 siRNA using the Lipofectamine 2000 (Invitrogen, Lidinggo, Sweden) according to the protocol provided by the manufacturer. Empty pcDNA3.1/V5-his B was transfected into the respective cells as control. Cells were screened with G418 for 2 months and stable transfectants were obtained. Semiquantitative RT-PCR and Western blot were used to examine the expression of Bcl-2, Bax, MDR-1 and MRP-1.

Material and methods
Cell lines
SGC7901 is a moderately differentiated stomach adenocarcinoma cell line. GES-1 is an immortalized fetal gastric mucosal cell line transformed with SV40. It has a phenotype much similar to normal stomach mucosal cells and does not grow into colonies in soft agar or tumor masses in nude mice. Other gastric cancer cell lines, MKN28, MGC803, MKN45 and AGS, were purchased from ATCC (Rockville, MD).

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3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay
Log phase-transfected cells and their control cells were trypsinized into single cell suspension and passaged into 96-well plates
at a density of $8 \times 10^3$ cells/well. The total volume of culture medium in each well was 200 μL. After incubation overnight, 8 chemotherapeutic drugs were added separately into the culture medium of the cells at different concentrations. Cells untreated with drugs were set as controls. All cells underwent another 72-hr incubation before 20 μl with drugs were added. The absorbance at 490 nm (A490) of each well was read on a spectrophotometer. Cell survival rates were calculated according to the following formula: survival rate = (mean A490 of treated wells/mean A490 of untreated wells) \times 100%. Finally, dose-effect curves of the anticancer drugs were drawn on semilogarithm coordinate paper and IC50 values were determined. Each experiment was performed in quadruplicates.

Flow cytometry

The fluorescence intensity of intracellular adriamycin was determined using flow cytometry as described previously. In brief, cells in log phase were plated into a 6-well plate (1 \times 10^6 cells/well) and cultured overnight at 37°C. Adriamycin was added to the cells to a final concentration of 5 mg/L. Cells were cultured for another 1 hr. Then the cells were trypsinized and harvested or, alternatively, cultured in drug-free RPMI-1640 for another 30 min. Finally, the cells were washed twice with precooled PBS, and the mean fluorescence intensity of intracellular adriamycin was determined by flow cytometry (Coulter, Miami, FL) with an

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**TABLE I – PCR PRIMERS**

| Gene                  | Primer 1            | Primer 2            |
|-----------------------|---------------------|---------------------|
| Runx3                 | 5'-PO4-r-GCGGTACACCCTCGACATCACATCGACATCTCC-3'OH | 5'-PO4-r-CTCGAGGAAGCTCTTCCGAGCTCAACCAGTACATGAG-3'OH |
| Bcl-2                 | 5'-PO4-r-AGGATTGGCGCCCTTCTGTA-3'OH | 5'-PO4-r-GAGAAGCAGCAGGAAAAATCAAAG-3'OH |
| Bax                   | 5'-PO4-r-CGAGATGCAAGGCGGCCGAACTG-3'OH | 5'-PO4-r-GCATATGGAAGGAGCTTT-3'OH |
| MRP-1                 | 5'-PO4-r-CTCGAGGAATCCGAGCATCAG-3'OH | 5'-PO4-r-ATACCTGCTGTTCGGATTT-3'OH |
| MDR-1                 | 5'-PO4-r-CTCGAGGAATCCGAGCATCAG-3'OH | 5'-PO4-r-GCTTGAGGAGTCTCACCCAACCAAC-3'OH |
| MDR-1 promoter        | 5'-PO4-r-GCTTGAGGAGTCTCACCCAACCAAC-3'OH | 5'-PO4-r-CTCGAGGAATCCGAGCATCAG-3'OH |
| MRP-1 promoter        | 5'-PO4-r-GCTTGAGGAGTCTCACCCAACCAAC-3'OH | 5'-PO4-r-CTCGAGGAATCCGAGCATCAG-3'OH |

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**TABLE II – siRNA FRAGMENTS**

| Fragments          |
|--------------------|
| Runx3 fragment sense 1 | 5'-PO4-r-TTGGCGGAGTAGTTTCTCGATATCCATGAGGACACTACTCCCGCTTTTTT-3'OH |
| Runx3 fragment antisense 1 | 5'-PO4-r-CTAGAAAACGGGAGTTTCTCTGCTATCTGAACTGACAGGAGGAACTCGG-3'OH |
| Runx3 fragment sense 1 | 5'-PO4-r-CTTGGCGGAGTAGTTTCTCGATATCCATGAGGACACTACTCCCGCTTTTTT-3'OH |
| Runx3 fragment antisense 2 | 5'-PO4-r-CTAGAAAACGGGAGTTTCTCTGCTATCTGAACTGACAGGAGGAACTCGG-3'OH |

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**FIGURE 1 – Runx3 expression in gastric cancer cell lines and immortalized gastric epithelial cell.** Semiquantitative RT-PCR was performed to determine Runx3 expression in the cell lines. In total, 25 cycles were run. The products were confirmed to be Runx3 by sequencing.

**FIGURE 2 – Runx3 expression in the stably transfected cells.** The transfected cells were identified by semiquantitative RT-PCR and Western blot. β-actin was used as internal control. (a) Runx3 expression in the transfected cells determined by semiquantitative RT-PCR. (b) Runx3 expression in the transfected cells determined by Western blot. Rabbit antihuman Runx3 antibody was from Oncogene. The blot was visualized by ECL system. 7901/c: SGC7901 cells transfected with empty pcDNA3.1/V5-his B vector; 7901/Runx3: SGC7901 cells transfected with pcDNA3.1/Runx3; 7901/siRunx3: SGC7901 cells transfected with Runx3 siRNA vector; GES/c: GES-1 cells transfected with empty pcDNA3.1/V5-his B; GES/siRunx3: GES-1 cells transfected with Runx3 siRNA vector.
excitation wavelength of 488 nm and receiving wavelength of 575 nm.

**EMSA and supershift EMSA**

EMSA and supershift EMSA assays were performed as described using LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL). Briefly, nuclear extract from SGC7901 cells was prepared and stored at −80°C until used. DNA oligos containing the RUNX sites and their complementary fragments were synthesized and labeled with biotin at the 5′ end. They were annealed to get the double-stranded probes. The oligos used were as follows: Bcl-2, CCGGAGCAGTCATCTGTGGTGAGGCTGATTGGCT-3′, 5′-PO4-r-TAGAAGCCCGCGCTGTGT-PO4-r-CGCGTCACCCCCACCCCAGATCCTCCA-3′; MDR-1, 5′-PO4-r-CCCCCCCCTTCGCCGCACCACACACAGCG-CGGGCTTCTA-3′; Aracytidine, 5′-PO4-r-CGCGTCACCCCCACCCCAGATCCTCCA-3′; Adriamycin, 5′-PO4-r-TCCCCACCCCTCGCCGCACCACACACAGCG-PO4-r-CGCGTCACCCCCACCCCAGATCCTCCA-3′; Vincristine, 5′-PO4-r-TCCCCACCCCTCGCCGCACCACACACAGCG-PO4-r-CGCGTCACCCCCACCCCAGATCCTCCA-3′; Cyclophosphamide, 5′-PO4-r-TCCCCACCCCTCGCCGCACCACACACAGCG-PO4-r-CGCGTCACCCCCACCCCAGATCCTCCA-3′.

A total of 5 μg of SGC7901 nuclear extract was incubated with 30 fmol of labeled probe at room temperature for 20 min for EMSA assay. For supershift EMSA, 2 μl rabbit antihuman RUNX3 antibody (~2 μg IgG; Active Motif) or normal rabbit IgG; Active Motif) or normal rabbit antibody was added to the reaction mixture. The reactions were electrophoresed on 6% polyacrylamide gel at 4°C in 0.5 × TBE buffer for 0.5 hr, then transferred to nylon membrane at 15 V for 30 min. The nylon blots were crosslinked on a 312 nm cross-linker for 15 min, then underwent chemiluminescent visualization to detect the biotin-labeled DNA probes.

**Luciferase reporter assay**

Promoter sequences of MDR-1 and MRP-1 were amplified from the genomic DNA of periphery blood mononuclear cell (PBMC) by PCR. The primers used are shown in Table I. The promoter sequences were then cloned into pGL3 enhancer vector (Promega, Madison, WI) to construct the reporter vectors (named as pGL-MDR and pGL-MRP, respectively). SGC7901 cells were passaged into 24-well plates at a density of 5 × 10^4 cells/well and incubated to 90% confluence. pcDNA3.1/Runx3 or empty pcDNA3.1/V5-his B plasmid was cotransfected into SGC7901 cells with pGL-MDR or pGL-MRP using the Fu gene transfection reagent (Roche, Indianapolis, IN). pRLE-TK was used as transfection efficiency control. Luciferase reporter assays were performed using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer’s instructions. Each experiment was performed in triplicates and repeated twice.

**Statistical analysis**

All the values of the in vitro drug sensitivity assay, flow cytometry assay and luciferase reporter assay were expressed as mean ± standard deviation. ANOVA analysis was performed with statistical software SPSS (version 10.0; SPSS, Chicago, IL) to evaluate the differences. Differences were considered significant when p < 0.05.

**Results**

Among the cell lines examined, GES-1 has the highest expression of Runx3; AGS and MKN28 show no expression of Runx3; MGC803, SGC7901 and MKN45 have a weak expression of Runx3 (Fig. 1). GES-1 and SGC7901 were used as the target cells for transfection. SGC7901 transected with pcDNA3.1/Runx3 (SGC7901/Runx3) had an increased Runx3 expression compared with empty pcDNA3.1/V5-his B vector control (7901/c); transfecting SGC7901 cells or GES-1 cells with Runx3 siRNA vector constructed with the first pair of synthesized fragments significantly decreased Runx3 expression in the respective cells (Fig. 2). The second siRNA construct had only a weak inhibitory effect on Runx3 expression (data not shown), so cells transfected with the first siRNA construct were used in the following study. We examined the expression of the other 2 members of RUNX family and found no significant differences in Runx1 and Runx2 expression, which suggested that the siRNA construct was specific for Runx3.

MTT assay was performed to evaluate the effects of Runx3 on drug sensitivity of the cells. As shown in Table III, IC50 values of SGC7901/Runx3 cells to etopside, cisplatin, mitomycin, adriamycin and vincristine decreased significantly as compared with SGC7901/c cells (p < 0.05). Inhibition of Runx3 expression in SGC7901 or GES-1 cells by Runx3 siRNA constructs conferred the cells resistance to etopside, cispatin, mitomycin, adriamycin and vincristine. IC50 values to these drugs increased significantly (p < 0.05). By flow cytometry, the intracellular accumulation and retention of the adriamycin were examined. As shown in
Analysis of the MDR-1 and MRP-1 revealed putative RUNX binding sites located at -92 to -87 in the MDR-1 promoter and -143 to -138 in the MRP-1 promoter. Previous study suggested that Bcl-2 promoter also contained RUNX binding sites. EMSA and supershift EMSA were performed to determine whether Runx3 could bind to these putative binding sites. Nuclear extract from SGC7901 cells caused significant retardation of the labeled probes of Bcl-2, MDR-1 and MRP-1 promoter (lanes 7–9, Fig. 4). Addition of Runx3 antibody into the reaction caused supershift of the labeled probes (lanes 4–6, Fig. 4), while the control normal rabbit serum did not affect the shifted bands of the probes (lanes 10–12, Fig. 4). Our finding suggested that Runx3 did bind to the putative RUNX binding sites in Bcl-2, MDR-1 and MRP-1 gene promoters.

To elucidate the regulatory effects of Runx3 on the promoter activity of MDR-1 and MRP-1, luciferase reporter assays were performed. As shown in Figure 5, the intensity of luciferase luminescence in cells cotransfected with pcDNA3.1/Runx3 was significantly lower than controls, suggesting that Runx3 could inhibit the promoter activity of MDR-1 and MRP-1.

Discussion

Runx domain transcription factors are heterodimeric proteins composed of α-subunit and β-subunit. The α-subunit is a homolog of the product of Drosophila segmentation gene runt. The α-subunit contains a 128 amino acid domain known as the runt domain, which is required for DNA binding and heterodimerization with the β-subunit. Runx domain proteins regulate specific gene transcription in the development and differentiation pathways by binding to the consensus sequence TGT/CCTG in the promoter or enhancer of the gene. Mammalian Runx1 and Runx2 play important roles in the definitive hematopoiesis and osteogenesis, respectively. Runx3 regulates development and survival of TrkC dorsal root ganglia (DRG) neurons as well as axonal projection and pathfinding of subtypes of DRG neurons and is required for thymic T-cell differentiation and development. Li et al. suggested that Runx3 was a tumor suppressor gene and inhibited the growth and malignant transformation of gastric epithelial cells. They found that loss of Runx3 expression due to heterozygous deletion and hypermethylation of Runx3 promoter correlated with progress of gastric cancer; reactivating Runx3 expression in the gastric cancer cell inhibited its tumorogenicity and growth both in vitro and in vivo. Other studies suggested that hypermethylation of Runx3 promoter could be found in tumors or precancerous lesions of various tissues such as larynx, lung, liver, biliary duct, pancreas, colon, stomach, breast, prostate, endometrium, uterine cervix and infant yolk sac. Hypermethylation of Runx3 promoter in cancer and premalignant lesions suggested that Runx3 might function as a tumor suppressor gene in a wide range of tissues.

In this study, we demonstrated that overexpression of Runx3 in gastric cancer cells sensitized the cells to chemotherapeutic drugs, while blocking Runx3 expression in immortalized gastric epithelial cells or gastric cancer cells conferred the cells multi-drug resistance. We found that SGC7901 cells transfected with Runx3 eukaryotic expression vector had a significantly weaker expression of Bcl-2, MDR-1 and MRP-1 compared to SGC7901/c, while SGC7901/siRunx3 and GES/siRunx3 cells had a significantly decreased fluorescence than their control cells.

To investigate the mechanism underlying the effects of Runx3 on drug sensitivity, semiquantitative RT-PCR and Western blot were used to examine expression of related molecules in the cells. We found that SGC7901/Runx3 had significantly weaker expression of Bcl-2, MDR-1 and MRP-1 compared to SGC7901/c; higher expression of these molecules was observed in SGC7901/siRunx3 and GES/siRunx3 cells as compared with their respective control cells. No significant difference in the expression of Bax was detected (Fig. 3).

Table IV, fluorescence intensity in SGC7901/Runx3 cells was significantly higher than the control cells, SGC7901/c, while SGC7901/siRunx3 and GES/siRunx3 cells had a significantly decreased fluorescence than their control cells.

In this study, we demonstrated that overexpression of Runx3 in gastric cancer cells sensitized the cells to chemotherapeutic drugs, while blocking Runx3 expression in immortalized gastric epithelial cells or gastric cancer cells conferred the cells multi-drug resistance. We found that SGC7901 cells transfected with Runx3 eukaryotic expression vector had a significantly weaker expression of Bcl-2, while GES/siRunx3 and GES/siRunx3 cells transfected with Runx3 siRNA had an increased Bcl-2 expression. EMSA and supershift assay suggested that Runx3 could bind Bcl-2 promoter probe, but it did not necessarily mean that Runx3 was a transcription repressor of Bcl-2. Previous study has found that Bcl-2 promoter activity was unaffected by cotransfection with Runx1, which also bound to the same putative binding sites. Moreover, as an integrated molecule in TGF-β signal transduction pathway. Indeed, TGF-β was found to regulate Bcl-2
expression. Hence, it is not clear whether the regulation of Bcl-2 expression by Runx3 observed in the present study was due to direct repression of the gene transcription or disturbance of TGF-β signaling pathway. Further work is needed to clarify the regulatory effects of Runx3 on Bcl-2 expression.

Flow cytometry examination suggested that Runx3 could increase the adriamycin accumulation and retention in the cells; RT-PCR and Western blot suggested that P-gp and MRP-1 expression decreased in 7901/Runx3 and increased in 7901/siRunx3 and GES/siRunx3 cells compared with their respective control cells. Previous study found that Runx3 could inhibit transcription from the MDR-1 promoter in leukemic cells. Analysis of MDR-1 and MRP-1 core promoters revealed putative RUNX binding sites localized at −92 to −87 and −153 to −148, respectively, in these 2 promoters. EMSA and super EMSA assay suggested that Runx3 could interact with the MDR-1 and MRP-1 promoter probes containing the putative RUNX binding sites. Luciferase reporter assay demonstrated that Runx3 could inhibit the transcription of luciferase driven by MDR-1 and MRP-1 promoter. Therefore, we believed that Runx3 inhibited the MDR-1 and MRP-1 expression in the gastric cancer cells by directly repressing their promoter activity.

In conclusion, this study provided multiple lines of evidence indicating that Runx3 sensitizes gastric cancer cells to chemotherapeutic drugs by inhibiting Bcl-2, MDR-1 and MRP-1, suggesting that Runx3 could serve as a good therapeutic target for gastric cancer. However, whether loss of Runx3 is involved in the development of multidrug resistance in clinical context remains open for discussion. This aspect of the work is underway in our laboratory.

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