Abstract. The development of multidrug resistance (MDR) is a crucial cause of therapy failure in gastric cancer, which results in disease recurrence and metastasis. Long non-coding RNAs (lncRNAs) have been proven to be critical in carcinogenesis and metastasis of gastric cancer. However, little is known about the roles of ANRIL (antisense non-coding RNA in the INK4 locus) in gastric cancer MDR. The aim of our study is to identify the biological function of ANRIL in gastric cancer MDR. In our results, ANRIL was highly expressed in gastric cancer tissues of cisplatin-resistant and 5-fluorouracil (5-FU)-resistant patients, and the same upregulation trends were observed in cisplatin-resistant cells (BGC823/DDP) and 5-FU-resistant cells (BGC823/5-FU). In addition, BGC823/DDP and BGC823/5-FU cells transfected with ANRIL siRNA and treated with cisplatin or 5-FU, respectively, exhibited significant lower survival rate, decreased invasion capability, and high percentage of apoptotic tumor cells. The influence of ANRIL knockdown on MDR was assessed by measuring IC50 of BGC823/DDP and BGC823/5-FU cells to cisplatin and 5-FU, the result showed that silencing ANRIL decreased the IC50 values in gastric cancer cells. Moreover, qRT-PCR and western blotting revealed that ANRIL knockdown decreased the expression of MDR1 and MRP1, both of which are MDR related genes; regression analysis showed that the expression of ANRIL positively correlated with the expression of MDR1 and MRP1, respectively. In summary, knockdown of lncRNA ANRIL in gastric cancer cells inhibits the development of MDR, suggesting an efficacious target for reversing MDR in gastric cancer therapy.

Introduction

Gastric cancer is the fourth most common type of cancer and the second leading cause of cancer-related deaths in the world (1). Chemotherapy plays an important role in the treatment of gastric cancer both in adjuvant and advanced settings. It is known that MDR involves a large number of molecules and complex mechanisms. Classical drug-resistant molecules, such as multidrug resistance gene 1 (MDR1) (2) and multi-drug resistance protein (MRP1) (3), have been found to play important roles in mediating MDR in some gastric cancers. However, the efficacy of chemotherapy for gastric cancer is limited due to insensitivity and the development of MDR (4).

LncRNAs with lengths of more than 200 nt are abundant in the human genome and have attracted increasing scientific interest (5). Despite being initially regarded as ‘transcriptional noise’, accumulating evidence has found that lncRNAs can manipulate local or global gene expression via transcriptional, post-transcriptional and epigenetic regulation (6). The lncRNAs that have been characterized are implicated in diverse physiological and pathological processes, such as X-chromosome inactivation (7), stem cell pluripotency (8), development (9) and cancer metastasis (10). However, little is known about the function of lncRNAs in gastric cancer MDR.

INK4b-ARF-INK4a encodes 3 tumor-suppressor proteins, p15 (INK4b), p14 (ARF), and p16 (INK4a), and its transcription is a key requirement for replicative or oncogene-induced senescence and constitutes an important barrier for tumor growth (11). Long non-coding RNA ANRIL was identified by Pasmant et al (12), it is transcribed as a 3.8-kb lncRNA in the opposite direction from the INK4B-ARF-INK4A gene cluster (13). In a previous study, Aguiló et al reported that both polycomb repressive complex-1 (PRC1) and PRC2 interact with lncRNA ANRIL to form heterochromatin surrounding the INK4b-ARF-INK4a locus, leading to its repression (14).

Correspondence to: Dr Dian-Hong Xu, Department of Oncology, Affiliated Hospital of Binzhou Medical University, 661 Yellow River Second Road, Binzhou, Shandong 256603, P.R. China
E-mail: byfuhl_2015@163.com

Key words: long non-coding RNA, ANRIL, multidrug resistance, gastric cancer
damage (16). However, the role of ANRIL in gastric cancer MDR remains largely unexplored.

In the present study, we explored the role of IncRNA ANRIL in cisplatin-resistant and 5-FU-resistant gastric cancer and further investigated the biological function of ANRIL on BGC823/DDP and BGC823/5-FU cells. Furthermore, we evaluated the effects of silencing ANRIL on the expression of genes associated with multidrug resistant, including MDR1 and MRP1.

Materials and methods

Patient characteristics and clinicopathological features. A total of 83 patients with primary gastric cancer were enrolled in this study. The patients received cisplatin-based (n=47) or 5-FU-based (n=36) palliative chemotherapy without surgery. Obvious primary tumor shrinkage and reduction of malignant pleural effusions were considered effective treatment. Cisplatin-resistant or 5-FU-resistant cases were distinguished when primary tumor enlarged, pleural effusions increased or new metastasis occurred within 12 months, otherwise cisplatin-sensitive (n=11) and 5-FU-sensitive (n=8) case was defined. The tumor tissue samples were collected from these patients. This study received approval from the Ethics Committee of Affiliated Hospital of Binzhou Medical University, and all patients provided written informed consent.

Cell lines and culture. Human gastric cancer cell lines BGC823 were purchased from the Beijing Zhongyuan Biotech (Beijing, China). The cells were grown at 37°C in a humidified incubator with 5% CO2. Cisplatin and 5-FU were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cisplatin-resistant BGC823/DDP and 5-FU-resistant were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cisplatin-resistant BGC823/DDP cells were developed from the parental BGC823 cells that were subjected to persistent gradient exposure to cisplatin for about 12 months, through increasing cisplatin concentration from 0.05 mg/ml until the cells acquired resistance to 1 mg/ml. The 5-FU-resistant BGC823/5-FU cells were obtained in the same way.

siRNA and transfection. siRNA specially targeting ANRIL were purchased from GenePharma Biotech (Shanghai, China), the sequence was as follows: sense, 5'-GGGCCAGAGUCACA GAUUUAUU-3' and antisense, 5'-UAAUCUUGACUCUGG CCCUUU-3'. BGC823 control cells, BGC823/DDP and BGC823/5-FU cells were seeded in 6-well plates, after 24-h incubation, cells were transfected with either 50 nM siRNAs targeting ANRIL (si-ANRIL) or scrambled negative controls (si-NC) using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the instructions provided by the manufacturer.

Quantitative real-time PCR. Total RNA (500 ng) was used for cDNA synthesis by Superscript III First Strand synthesis (Invitrogen, USA). SYBR Premix Taq (BioRad, USA) was used for real-time PCR assays. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected and the mRNA levels were used as endogenous controls. Primer pairs used for quantitative RT-PCR were from Sigma-Aldrich and their sequences are: ANRIL sense, 5'-TTTGGGTGCAATTTCACTCTG-3' and antisense, 5'-CTCACCGATGCCAATGTTT-3'; GAPDH sense, 5'-CTCACCGATGCCAATGTTT-3' and antisense, 5'-CCATTGCTCAATGTCACT-3'; MDR1 sense, 5'-TTTGGGTGCAATTTCACTCTG-3' and antisense, 5'-CCAAAAATGAGTAGCCAGCCTT-3'; and MRP1 sense, 5'-GGTGATGTTTTATGGAAG-3' and antisense, 5'-GGATAGCCAGCCTT-3'.

Cytotoxicity assay. The cytotoxicity of gene transfection was determined by Cell Counting Kit-8 (CCK-8) assay. In 96-well plates, cells were seeded in 100-µl PRMI-1640 medium supplemented with 10% FBS at 5x104 cells/well. Then chemotherapy agents were added in normal growth medium supplemented with FBS. After 48 h incubation, 10 µl CCK-8 was added and culture was continued for 1 h in humidified atmosphere containing 5% CO2. Absorbances at 450 nm were measured by microplate reader (Bio-Tech Company). The relative drug resistance folds were analyzed by comparison with IC50.

Western blot assays and antibodies. Cell lysates were prepared in a buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 1 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride, and 1% (v/v) NP-40. After protein quantitation using the Lowery protein assay, equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the semi-dry blotting method. The membranes were incubated with a dilution of primary antibody (anti-MDR1, sc-55510, 1:500 dilution; anti-MRP1, sc-365635, 1:500 dilution; anti-GAPDH, sc-25778, 1:1,000 dilution), overnight at 4°C. The membrane was washed with TBST and incubated with a peroxidase-conjugated secondary antibody (1:2,000; Zhongshan Jinqiao Biotech, Beijing, China) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce, Rockford, IL, USA), according to the manufacturer's recommendations.

Cell proliferation assays. Cell viability of gastric cancer cells transfected with si-ANRIL or si-NC was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 48 h after transfection, 20 µl MTT solution (Sigma-Aldrich) was added into the culture medium for 4 h incubation. Then, 150 µl DMSO (Sigma-Aldrich) was added into each well to dissolve the crystals. The absorbance of each sample was recorded at 490 nm. All experiments were performed in quadruplicate. For each treatment group, wells were assessed in triplicate.

Wound-healing assay. Approximately 5x104 cells were seeded in 24-well plates and cultured until 70-80% confluent. Wounds were established using a 20-µl pipette tip and the cells were allowed 24 h to migrate into the wounds. To assess the migration of the cells across the artificial wound, a total of five optical fields were randomly selected and analyzed using a Leica DMI 4000B inverted microscope with the Leica application suite software (Leica Microsystems).

Cell invasion assay. The invasive potential of cells was measured in 12-well Matrigel-coated invasion chambers (BD Biosciences, Bedford, MA, USA). The lower chambers were filled with 0.75 ml of RPMI-1640 medium containing 10% fetal
bovine serum (FBS). A cell suspension of $2.5 \times 10^4$ in 0.5 ml RPMI-1640 medium was added into each well of the upper chamber. After the cells were incubated for 20 h at 37˚C in a humidified incubator with 5% CO$_2$, the non-invading cells that remained on the upper surface of the membrane were removed by gentle scraping. The invasive cells attached to the lower surface of the membrane insert were fixed in 10% formalin at room temperature for 30 min and stained with 0.05% crystal violet. The number of invasive cells on the lower surface of the membrane was then counted under a microscope.

**Cell apoptosis assay.** Cells transfected with si-ANRIL, or si-NC were harvested 48 h and then collected. After the double staining with FITC Annexin V and PI was done using the FITC Annexin V apoptosis detection kit (BD Biosciences) according to the manufacturer's protocol, the cells were analyzed with a flow cytometry (FACScan) equipped with a CellQuest software (both from BD Biosciences). Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells, and then the relative ratio of early apoptotic cells was compared to control transfectant from each experiment.

**Statistical analysis.** All statistical analyses were performed by using SPSS 17.0 software (IBM, Chicago, IL, USA). The significance of differences between groups was estimated by the Student's t-test. Correlation between gene expressions were analyzed with regression analysis. Two-sided p-values were calculated, and differences were considered to be statistically significant at P<0.05.

**Results**

**ANRIL is upregulated in cisplatin-resistant and 5-FU-resistant gastric cancer tissues and cells.** To investigate whether ANRIL participates in the development of MDR in gastric cancer tissue, we examined the mRNA levels of ANRIL in the gastric cancer tissues of cisplatin-sensitive patients (n=36) and cisplatin-resistant patients (n=11), 5-FU-sensitive patients (n=28) and 5-FU-resistant patients (n=8). As a result, significantly elevated mRNA level of ANRIL was observed in the cancer tissues of cisplatin-resistant or 5-FU-resistant patients comparing to drug-sensitive patients (Fig. 1A and B). To verify this differential expression of ANRIL, we detected ANRIL expression in cisplatin-resistant cells BGC823/DDP and 5-FU-resistant cells BGC823/5-FU, which were both developed from the parental BGC823 cells. Consistent with the results in gastric cancer tissues, ANRIL was greatly upregulated in BGC823/DDP and BGC823/5-FU cells (Fig. 1C). These results indicated that ANRIL may be associated with the development of cisplatin and 5-FU resistance in gastric cancer.

**Knockdown of ANRIL inhibits gastric cancer cell proliferation in vitro.** To investigate the potential role of ANRIL on gastric cancer cell proliferation, ANRIL siRNA was transfected into BGC823/DDP and BGC823/5-FU cells. To ensure the efficiency of interference and avoid off-target effects, qRT-PCR assays revealed that ANRIL expression was significantly reduced after transfection with si-ANRIL, while other lncRNAs, such as HOTAIR (17), PVT1 (18), and H19 (19) showed nearly no change (Fig. 2A and B). Then MTT assay showed that knockdown of ANRIL expression significantly inhibited cell proliferation both in BGC823/DDP and BGC823/5-FU cells compared with control cells (Fig. 2C and D).

**Knockdown of ANRIL inhibits gastric cancer cell migration and invasion in vitro.** Migration and invasion are significant aspect of cancer progression, which are involved in the dissolution of extracellular matrix proteins and the migration of tumor cells into contiguous tissues. To investigate whether ANRIL had a direct functional role in cell invasion in gastric cancer, we performed wound-healing assay and Transwell assay. The results showed that inhibition of ANRIL could significantly suppress BGC823/DDP and BGC823/5-FU cell migration (Fig. 3A and B) and invasion ability (Fig. 3C and D) when compared with control cells.

**Knockdown of ANRIL promotes gastric cancer cell apoptosis in vitro.** We next examined the effect of ANRIL expression on the apoptosis of GC cells. The results of flow cytometry revealed that BGC823/DDP (Fig. 4A and B) and BGC823/5-FU
cells (Fig. 4C and D) transfected with si-ANRIL exhibited a significantly increased apoptosis index compared to the control groups (P<0.05). These data indicate that knockdown of ANRIL promotes cisplatin-induced apoptosis in BGC823/DDP and 5-FU-induced apoptosis in BGC823/5-FU cells.

**ANRIL knockdown reverses the drug resistance in cisplatin- and 5-FU-resistant GC cell lines.** On the basis of above results, we examined the effect of ANRIL knockdown on cisplatin- or 5-FU-induced cytotoxicity in BGC823/DDP and BGC823/5-FU cells. After transfected with si-ANRIL, BGC823/DDP and BGC823/5-FU cells were treated with cisplatin or 5-FU for 48 h, and CCK-8 assay was performed to detect the cytotoxicity. As shown in Tables I and II, cells transfected with si-ANRIL had a significantly lower IC_{50} values than that in si-NC group. These results indicate that si-ANRIL reverses the MDR in drug-resistant GC cell lines.

**Knockdown of endogenous ANRIL downregulates MDR-related gene expression.** To investigate the mechanisms...
underlying ANRIL induced MDR, we detected the expression of mRNA of several MDR-related genes by RT-PCR including MDR1 and MRP1 in different group. The levels of MDR1 and MRP1 mRNA were both significantly decreased in BGC823/DDP and BGC823/5-FU cells transfected with si-ANRIL compared with si-NC (Fig. 5A and B). Protein levels of MDR1 and MRP1 were also detected, and were increased in BGC823/DDP and BGC823/5-FU cells transfected with si-ANRIL compared with si-NC (Fig. 5C). These results suggested that knockdown of endogenous ANRIL could downregulate the MDR-related gene expression in BGC823/DDP and BGC823/5-FU cells.

**ANRIL expression positively correlates with the MDR-related gene expression.** To investigate whether there is a correlation between the expression of ANRIL and the multidrug resistance gene MDR1 and MRP1 in vivo, we isolated mRNA from the 64 gastric cancer tissues enrolled in this study, and analyzed the expressions of ANRIL, MDR1, and MRP1 using real-time PCR. After analyzed using the regression analysis, we found the R-square of ANRIL and MDR1 expression was 0.623 (Fig. 6A), and the R-square of ANRIL and MRP1 expression was 0.612 (Fig. 6B), which indicated their goodness-of-fit of linear regression, and changes of ANRIL expression relate to changes of MDR1 and MRP1 (P<0.05, respectively). These results strongly suggest that ANRIL may regulate the expression of MDR1 and MRP1 in gastric cancer patients.

### Discussion

The development of MDR to cancer chemotherapy is a major obstacle to the effective treatment of gastric cancer (20). MDR plays a critical role in tumor initiation and progression by promoting cell proliferation and inhibiting apoptosis (21). Various mechanisms contribute to MDR, for example, MDR1 and MRP1, both of which belong to the ATP-binding cassette (ABC) superfamily, and play important roles in MDR development (22). These transporter proteins mediate the efflux of drugs in the MDR spectrum, out of cells and thus reduce drug efficacy. In this study, cisplatin and 5-FU are selected as the model anticancer drugs, because it is a substrate of MDR1 and MRP1 (23,24). However, the mechanism of MDR remains unclear.
Recent improvements in high-throughput gene expression analysis have led to the discovery that transcription from <2% of the human genome yields many lncRNAs with limited or no protein-coding capacity because of a lack of open reading frames (25). Dysregulated expression of these lncRNAs have been revealed in several human diseases but their exact biological functions on MDR are unclear. Zhang et al showed that PVT-1 (plasmacytoma variant translocation 1) was highly expressed in gastric cancer tissues of cisplatin-resistant patients and cisplatin-resistant cells, and overexpression of PVT1 in gastric carcinoma promotes the development of MDR (26); Wang et al reported lncRNA MRUL (MDR-related and upregulated lncRNA) was significantly upregulated in two multidrug-resistant gastric cancer cell lines, and MRUL knockdown in these two cells led to increased rates of apoptosis, increased accumulation, and reduced doxorubicin release in the presence of adriamycin or vincristine (27); Jiang et al showed that a specific differentially expressed lncRNA ARA (adriamycin resistance associated) was validated in MCF-7/ADR and HepG2/ADR cells, ARA expression is significantly associated with adriamycin sensitivity in a panel of breast and liver cancer cell lines, and ARA knockdown reduced the proliferation, induced cell death, G2/M arrest and migration defects (28); Jin et al showed that lncRNA MEG3 (maternally expressed gene 3) was decreased in cisplatin-insensitive lung adenocarcinoma tissues while p53 protein levels were decreased and Bcl-xl protein levels increased; MEG3 overexpression in A549/DDP cells increased their chemosensitivity to cisplatin both in vitro and in vivo by inhibiting cell proliferation and inducing apoptosis. By contrast, MEG3 knockdown in A549 cells decreased chemosensitivity (29). Taken together, lncRNA might participate in the process of MDR in gastric cancer.

ANRIL is transcribed in anti-sense direction with respect to the primary INK4 and ARF transcripts (12). ANRIL was shown to be involved in epigenetic regulation of the INK4BARF-INK4A locus by direct binding to the INK4b transcript and recruiting the Polycomb repressor complex (PRC) to repress the transcription of genes at this locus (30). Recent studies reported that ANRIL exerts its biological function in several kinds of cancer, such as hepatocellular carcinoma, lung cancer, ovarian cancer and gastric cancer. Qiu et al reported that ANRIL was highly expressed in ovarian cancer cells, siRNA-mediated ANRIL silencing in ovarian cancer cells impaired cell migration and invasion (31); Lin et al showed that higher expression of ANRIL in non-small cell lung cancer (NSCLC) tissues was associated with higher TNM stage and advanced lymph node metastasis, moreover, knockdown of ANRIL expression inhibited NSCLC cell proliferation, migration and invasion in vitro (32); Huang et al showed that ANRIL expression was
upregulated in hepatocellular carcinoma (HCC) tissues, and the higher expression of ANRIL was significantly correlated with tumor size and Barcelona Clinic Liver Cancer (BCLC) stage, knockdown of ANRIL expression impaired cell proliferation and invasion and induced cell apoptosis both in vitro and in vivo. For the detailed mechanism, ANRIL was able to epigenetically repress KLF2 transcription in HCC cells by binding with PRC2 and recruiting it to KLF2 promoter region (33). In respect of gastric cancer, Zhang et al showed that in a cohort of 120 gastric cancer patients, the higher expression of ANRIL was significantly correlated with a higher TNM stage and tumor size, and ANRIL knockdown could significantly repress the proliferation both in vitro and in vivo. In addition, E2F1 could induce ANRIL and ANRIL-mediated growth promotion in part due to epigenetic repression of miR-99a/miR-449a in Trans (controlling the targets - mTOR and CDK6/E2F1 pathway) by binding to PRC2, thus forming a positive feedback loop, continuing to promote gastric cancer cell proliferation (34). Though there are several papers on the exploration and the function of ANRIL, the effect of ANRIL on the MDR in gastric cancer is still unclear.

In summary, the expression of ANRIL was significantly upregulated in cisplatin-resistance and 5-FU-resistance gastric cancer tissues and cells, suggesting that its overexpression may be an important factor for gastric cancer MDR. We showed that silencing ANRIL may inhibit the proliferation and invasion ability, and promote the apoptosis rate of drug-resistant gastric cancer cells, which suggested that ANRIL contributes to gastric cancer cell biological function. Further insights into the functional implications of ANRIL and its downstream genes related with MDR, which are identified as MDRI and MRPI, may contribute to the understanding of gastric cancer MDR, and facilitate the development of ANRIL therapeutics against gastric cancer.

References


