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Original Article

Correlation between chemosensitivity to anticancer drugs and Bcl-2 expression in gastric cancer

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Abstract: Objective: To investigate chemoresistance of human gastric cancer to chemotherapeutic drugs in vitro and explore the relationship with Bcl-2 protein expression. Methods: Single-cell suspensions were prepared from freshly excised samples of primary gastric cancer, and were separately exposed to taxol (TAX), cisplatin (CDDP), 5-fluorouracil (5-FU), adriamycin (ADM) and mitomycin (MMC) for 48 h. The induction of cell death was confirmed by microscopic analysis of cell morphology. Metabolic activity and the inhibitory rate (IR) of cells were evaluated by MTT assay. Expression of Bcl-2 was determined by immunohistochemistry of gastric cancer tissue samples. Results: The IRs of cancer cells exposed to different chemotherapeutic drugs varied as follows: the IRs for TAX, CDDP and 5-FU were significantly higher than those for ADM and MMC ($P < 0.01$). Poorly differentiated gastric cancer cells were more sensitive than well-differentiated cells ($P = 0.021$). The positive rate of Bcl-2 expression was 80%, and Bcl-2 expression was significantly associated with chemoresistance to 5-FU ($r_s = 0.265$, $P = 0.041$), ADM ($r_s = 0.425$, $P = 0.001$) and MMC ($r_s = 0.40$, $P = 0.002$). Furthermore, Bcl-2 expression was strongly associated with lymph node metastasis in gastric cancer ($P = 0.009$). Conclusion: Overexpression of Bcl-2 may predict a loss of the efficacy of the chemotherapy drugs 5-FU, ADM and MMC in patients with gastric cancer.

Keywords: Gastric cancer, Bcl-2, chemosensitivity, chemotherapeutic drugs, inhibitory rate

Introduction

Gastric cancer is the fourth most common malignancy and the second leading cause of cancer deaths worldwide. In 2008, it was estimated that half of all gastric cancer cases, and the highest mortality rates, occurred in Eastern Asia, with the majority in China (GLOBOCAN 2008, http://globocan.iarc.fr/). Chemotherapy is the primary treatment for patients with advanced gastric cancer and plays an important role in preoperative and postoperative adjuvant therapy. As such, resistance to chemotherapy is a major impediment to the success of treatment. A principle cause of chemoresistance is aberrant expression of genes involved in drug resistance and apoptosis; therefore, identification of the factors associated with chemoresistance and sensitivity is urgent.

The B-cell lymphoma 2 (Bcl-2) gene is overexpressed in most cancers. Although the gene is involved in the regulation of multiple molecular pathways, its main function is in the mediation of anti-apoptotic activity. Tumor cells that overexpress Bcl-2 are less able to undergo apoptosis, resulting in resistance to chemotherapeutic agents and physiological mediators of cell death [1]. Studies in several types of cancers have shown that Bcl-2 antagonists can enhance the efficacy of anticancer drugs, making them promising candidates for combination therapies [2].

In this study, we cultured gastric cancer cells obtained from freshly resected primary gastric cancer tissues and assessed their sensitivity to five widely used first-line chemotherapy drugs, namely, Taxol (TAX), cisplatin (CDDP), 5-fluorouracil (5-FU), adriamycin (ADM) and mitomycin (MMC). We also analyzed their Bcl-2 protein levels and explored the relationship between chemoresistance and Bcl-2 expression in order to advance our understanding of the factors underlying drug resistance in gastric cancer.

Materials and methods

Patient characteristics and clinicopathological features

A total of 68 patients with primary gastric cancer were enrolled in this study. The patients had all undergone gastrectomy at the General Hospital of Jinan Military Command (Jinan, Shandong, China) between January 2007 and December 2008. The age range was 33-75 years, and the ratio of males to females was 36:32. The tumor tissue samples were immediately collected following surgery. There was no evidence of other malignancies, and none of the patients had received chemotherapy or radiotherapy prior to surgery. The diagnoses were histopathologically confirmed by independent experienced pathologist. The pathological type and grade of the tumors were determined according to WHO criteria and were as follows: papillary adenocarcinoma, 20 cases; tubular adenocarcinoma, 20 cases; mucinous adenocarcinoma, 16 cases; and signet ring cell carcinoma, 12 cases. This study received approval from the ethics committee of General Hospital of Jinan Military Command, and all patients provided written informed consent.

MTT chemosensitivity assay

The drug sensitivities of the primary gastric cancer tissue samples were evaluated *in vitro* by the MTT colorimetric assay [3]. Resected specimens were stored in Hank's balanced salt solution supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Gibco, Gaithersburg, MD, USA). Single-cell suspensions were prepared enzymatically by incubating the specimens for 30 min with 0.5 mg/ml Pronase, 0.2 mg/ml collagenase type I and 0.2 mg/ml DNase (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 1000 rpm for 5 min and filtration through a 200 mesh copper filter, the cells were suspended to a dilution of 1×10^5 cells/ml in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Approximately 10^4 cells were plated per well into 96-well microplates (Gibco) by adding 100-μl aliquots of the cell suspension. The drug solutions were dissolved in RPMI-1640 medium supplemented with 10% FBS, and 100-μl aliquots were added to each well to give final concentrations of 5.0 μg/ml TAX (Taiji Pharmaceutical Co., Sichuang, China), 3.0 μg/ml CDDP (Qilu Pharmaceutical Co., Shandong, China), 1.0 μg/ml MMC (Huangshi Co., Hubei, China), 50 μg/ml 5-FU (Hualian Co., Shanghai, China) or 4 μg/ml ADM (Xinhua Co., Shandong, China). Three duplicate wells were plated for each specimen. The control wells contained 100 μl of cell suspension plus 100 μl RPMI-1640 with 10% FBS; blank controls contained 200 μl RPMI-1640 with 10% FBS. The microplates were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2. Following incubation, 20 μl of 0.4% MTT (Sigma) and 0.1 M sodium succinate, both dissolved in 10 μl phosphate-buffered saline (PBS) and filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, USA), were added to the wells, and the plates were incubated for a further 4 h at 37°C. After the final incubation, the MTT-formazan salt was dissolved by adding 150 μl dimethyl sulfoxide (Gibco) to each well, and the microplates were mechanically shaken on a mixer for 10 min. The optical density of each well was determined using an SM-3 easy reader (Tianshi, Beijing, China) at 570 nm. The inhibitory rate (IR) was calculated using the formula $(A_c - A_d)/(A_c - A_b) \times 100\%$, where A_d, A_c and A_b represent the mean absorbance of the drug-treated, control and blank wells, respectively. The results were defined as follows: highly sensitive, IR > 50%; moderately sensitive, IR 30%-50%; and resistant, IR < 30%. The morphology of the cells was observed under an inverted microscope.

Immunohistochemistry analysis of Bcl-2 protein expression in tissue samples

Immunohistochemical (IHC) staining for Bcl-2 protein was performed on formalin-fixed, paraffin-embedded (FFPE) gastric cancer tissue sections, using the Ventana BenchMark XT system (Ventana, Oro Valley, AZ, USA) [4]. Samples were stained with mouse anti-human Bcl-2 monoclonal primary antibody (Maixin Biotechnology Co., Fuzhou, China). Blank controls were prepared by replacing the primary antibody with PBS. Bcl-2 protein appeared as cytoplasmic brown-yellow staining and was compared to known positive controls. The results were evaluated as previously described [5], by counting 100 cells per field in 10 random fields under microscopy at high magnification (400 ×, Olympus BX53). Positive staining was defined > 10% and negative staining as ≤ 10%.

Table 1. Summary of drug sensitivity of primary gastric cancer cells by MTT assay

Drug	Sensitivity			Mean IR (% ± S)
	High (%)	Moderate (%)	Low (%)	
TAX	6 (10.0)	17 (28.3)	37 (61.7)	40.6 ± 9.9*
CDDP	5 (8.3)	16 (26.7)	39 (65.0)	38.4 ± 7.8*
5-FU	5 (8.3)	15 (25.0)	40 (66.7)	38.9 ± 9.2*
ADM	2 (3.3)	15 (25.0)	43 (71.7)	31.6 ± 8.5
MMC	1 (1.7)	17 (28.3)	42 (70.0)	28.9 ± 9.8

Notes: *$P < 0.05$ compared to ADM or MMC.

Statistical analysis

Statistical analysis was carried out using SPSS v. 17.0 for Windows. Quantitative results were expressed as mean ± standard error of the mean. Significant differences were determined using the Friedman test, Fisher protected least squares difference (PLSD) test or the chi-square test. Associations were determined using the Spearman rank correlation test. r_s represented the correlation coefficient. *P*-values were two-sided, and $P < 0.05$ was considered significant.

Results

Morphological changes in gastric cancer cells post-chemotherapy

The MTT colorimetric assays were successfully carried out on 60 of the 68 (88.2%) gastric cancer tissue samples. Following 48 h exposure to the different chemotherapeutic drugs, a decrease in the number of cells and loss of cellular activity was observed under microscopy, indicating inhibition of the tumor cells. Drug-sensitive cells lost their ability to adhere and showed increased intracytoplasmic vacuoles. Further damage to morphological integrity with a large amount of cellular debris was observed in the most severe cases. The anticancer drugs induced different levels of inhibition in the tumor cells by (**Table 1**): TAX, CDDP and 5-FU induced similar IRs in the cells, which were significantly higher than the IRs induced by ADM and MMC ($P < 0.01$).

Relationship between histological type and chemosensitivity in gastric cancer

The degree of chemosensitivity varied with the histological type of gastric cancer, decreasing from signet ring cell carcinoma to tubular adenocarcinoma, mucinous adenocarcinoma and papillary adenocarcinoma (**Table 2**). The difference was significant between signet ring cell carcinoma and papillary adenocarcinoma ($P < 0.01$).

Each chemotherapy drug induced different degrees of chemosensitivity in the various histological types of gastric cancer. This difference was significant with 5-FU ($\chi^2 = 8.750$, $P = 0.033$). However, the differences in chemosensitivity to other chemotherapy drugs were not significant within different histological type (**Table 2**).

Expression of Bcl-2 in gastric cancer tissue

Bcl-2 expression in the gastric cancer tissue samples was determined by IHC. Our observations showed that staining of Bcl-2 protein, indicated by brown-yellow granules, was mainly distributed in the cytoplasm. Positive expression of Bcl-2 was observed in 48/60 (80%) of the tissue samples. Bcl-2 expression was significantly higher in well-differentiated gastric cancer cells than in poorly differentiated cells ($P = 0.021$). In addition, Bcl-2 expression was strongly associated with lymph node metastasis in gastric cancer ($P = 0.009$), suggesting that patients with negative Bcl-2 expression may have a higher risk of lymph node metastasis (**Table 3**). However, there was no significant correlation between the expression of Bcl-2 and the different histological types of gastric cancer ($P = 0.964$).

Correlation between Bcl-2 expression and chemosensitivity

As stated in the methods, the tumor cells were considered to be chemosensitive if the IR was ≥ 30% and chemoresistant if the IR < 30%. In the gastric cancer tissue samples that were resistant to 5-FU, ADM and MMC, the Bcl-2 expression rates were 87.5%, 90.1% and 90.5%, respectively (**Table 4**), indicating that Bcl-2 expression was significantly correlated with resistance to these drugs. Furthermore, the IRs with these drugs were significantly lower in gastric cancer tissue samples showing positive expression of Bcl-2 protein than in those showing negative Bcl-2 expression ($P < 0.05$). In contrast, there was no significant correlation

Table 2. Relationship between tumor histological type and chemosensitivity

Histological type	Cases	TAX (%)	CDDP (%)	5-FU (%)	ADM (%)	MMC (%)
Papillary adenocarcinoma	18	6 (33.3)	3 (33.3)	2 (11.1)	3 (16.7)	5 (27.8)
Tubular adenocarcinoma	18	7 (38.9)	5 (27.8)	8 (44.4)	5 (27.8)	6 (33.3)
Mucinous adenocarcinoma	12	4 (33.3)	5 (41.7)	3 (25.0)	4 (33.3)	4 (33.3)
Signet ring cell cancer	12	6 (50.0)	5 (41.7)	7 (58.3)	5 (41.7)	3 (25.0)*

Notes: *$P < 0.05$ compared to papillary adenocarcinoma.

Table 3. Relationship between Bcl-2 expression and clinicopathological features of gastric cancer

	Cases	Positive	Negative	Positive rate (%)	χ^2	P
Histological type					0.278	0.964
Papillary adenocarcinoma	18	14	4	77.8		
Tubular adenocarcinoma	18	14	4	77.8		
Mucinous adenocarcinoma	12	10	2	83.3		
Signet ring cell carcinoma	12	10	2	83.3		
Differentiation					7.734	0.021
High	16	15	1	93.3		
Moderate	20	18	2	90.0		
Low	24	15	9	62.5		
Lymph node metastasis					6.857	0.009
Absent	25	24	1	96.0		
Present	35	4	11	68.6		

between *in vitro* Bcl-2 protein expression in the gastric cancer samples and resistance to TAX or CDDP ($P > 0.05$).

Discussion

Drug sensitivity tests carried out *in vitro* can provide sensitivity profiles of tumor cells to various chemotherapeutic drugs. Studies have shown that the correlation between *in vitro* chemosensitivity tests and the efficacy of chemotherapy drugs *in vivo* is approximately 90% [6]. Nakamura *et al.* assessed the postoperative therapeutic effects of medication in patients and compared these with the results of *in vitro* chemosensitivity tests, clinical experience and outcomes in patients receiving no medication. They determined that the survival rate of patients was significantly higher when the medication was selected in accordance with the chemosensitivity test results instead of the latter two approaches [7]. Our study found that patients with same histological type of gastric cancer, with the same differentiation status, showed various degrees of chemosensitivity to the same chemotherapeutics. Therefore, using MTT assays to screen tissues samples for sensitivity to different drugs may provide an objective basis for selecting the optimum chemotherapeutic program for patients with gastric cancer.

Our study also found that the efficacy of chemotherapeutic drugs was related to the degree of differentiation of the gastric cancer. The chemosensitivity of poorly differentiated cells was significantly higher than that of well-differentiated cells. A possible reason is that poorly differentiated tumors have a higher proportion of cells in the proliferation phase (S phase and G2 phase); therefore, as the majority of chemotherapeutic drugs act on proliferating cells, their effects will be greater in poorly differentiated gastric cancer.

Bcl-2 is the principle member of the Bcl-2 gene family, which inhibits apoptosis in a variety of tissues. Wang *et al*. reported a trend of increased Bcl-2 protein expression in atrophic gastritis, gastric intestinal metaplasia, dysplasia and gastric cancer [8]. Our IHC results showed that expression of Bcl-2 protein was also significantly higher in well-differentiated gastric cancer cells than in poorly differentiated cells, and was also remarkably higher in tissues from patients without lymph node metas-

Table 4. Relationship between Bcl-2 expression and chemosensitivity of gastric cancer

Drug	Chemosensitivity	Bcl-2 expression (cases)		r_s	*P*
		Positive	Negative		
TAX	sensitive	17	6	-0.120	0.361
	resistance	31	6		
CDDP	sensitive	14	7	-0.245	0.060
	resistance	34	5		
5-FU	sensitive	13	7	-0.265	0.041
	resistance	35	5		
ADM	sensitive	9	8	-0.425	0.001
	resistance	39	4		
MMC	sensitive	10	8	-0.400	0.002
	resistance	38	4		

tasis than in those from patients with lymph node metastasis. This suggested that overexpression of the Bcl-2 gene may inhibit apoptosis and promote mutant cell survival during the formation of the tumor. During tumor progression, Bcl-2 expression may decrease, leading to a reduction in the inhibition of apoptosis and other regulatory pathways involved in the abnormal proliferation of tumor cells.

Recently there has been an increasing number of reports on the range of the roles of the Bcl-2 gene and protein. These reports have shown that overexpression or phosphorylation of Bcl-2 may not be limited to the regulation of apoptosis and proliferation but may also play key roles in multidrug resistance in tumor cells [9]. As such, Bcl-2 has been a candidate target in experimental therapies in multiple carcinomas [10]. The majority of anticancer drugs induce cell death in tumors by promoting apoptosis. In contrast, Bcl-2 opposes this effect by inhibiting apoptosis, thereby increasing drug resistance. The mechanism by which Bcl-2 promotes drug resistance is different from the mechanisms mediated by many other resistance genes, in that it does not prevent intracellular drug accumulation or alter drug-induced DNA damage. Studies have confirmed that tumor cells treated with anticancer drugs that induce cell cycle arrest, fail to undergo normal apoptosis if Bcl-2 protein levels are high [11]. Jie *et al*. demonstrated that miR-21 increased chemoresistance to gemcitabine, inhibited apoptosis and promoted proliferation in cancer cells by upregulating Bcl-2 expression [12]. Our study indicated that high expression of Bcl-2 protein was significantly associated with resistance to 5-FU, MMC and ADM but was not related with resistance to CDDP and TAX. Lv *et al*. demonstrated that 5-FU-induced apoptosis could be promoted by targeting Bcl-2-triggered mitochondrial pathways [13], supporting the role of Bcl-2 in multidrug resistance. These observations suggest that Bcl-2 expression may mediate multidrug resistance in gastric cancer through anti-apoptotic activity.

Conclusion

Determining the sensitivity of tumor cells to chemotherapy drugs is a major challenge in adjuvant therapy for gastric cancer. Our study has demonstrated a correlation between Bcl-2 expression and chemosensitivity in gastric cancer to five widely used anticancer drugs; however, further data will be required to give a more accurate review. Our findings suggest that measurement of Bcl-2 expression levels may offer an alternative strategy for selecting effective chemotherapeutic agents in the absence of chemosensitivity assays. Bcl-2 overexpression in gastric cancer tumors could predict a loss of efficacy of chemotherapies based on 5-FU, MMC or ADM. Furthermore, negative Bcl-2 expression may predict a higher risk of lymph node metastasis in patients with gastric cancer. This study has demonstrated the potential clinical relevance of Bcl-2 assessment in patients with gastric cancer prior to induction of chemotherapy.

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Disclosure of conflict of interest

The authors declare no conflicts of interest.

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