·Colerectal Cancer-related Research·

Correlation of chemosensitivity tested using histoculture drug response assay to expression of multidrug resistance genes and proteins in colorectal cancer tissues

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[Abstract] **Background and Objective**: The therapeutic effects of chemotherapy for malignant neoplasms are still unsatisfactory. This study was to evaluate the chemosensitivity of colorectal cancer tissues to therapeutic agents using histoculture drug response assay (HDRA), and explore the correlation of chemosensitivity to the expression levels of multidrug resistance (MDR) genes and proteins. **Methods**: Twenty-two specimens of colorectal cancer were collected. The inhibition rates of single agents, including epirubicin, cisplatin (DDP), oxaliplatin, 5-FU, taxetere, irinotecan, and combinations of these agents, including 5-FU +epirubicin +DDP, 5-FU + irinotecan, 5-FU+oxaliplatin, 5-FU+taxetere+ DDP on colorectal cancer tissues were evaluated by HDRA. The agent whose inhibition rate was greater than 30% was considered sensitive, and the sensitivity was calculated. mRNA and protein levels of MDR genes and proteins in colorectal cancer tissues were measured by RT-PCR and immunohistochemistry. **Results**: Among the single agents, the inhibition rate of oxaliplatin (17.5%) and sensitivity of cancer tissues to 5-FU (36.4%) were the highest. In the combination groups of agents, the inhibition rate of 5-FU + oxaliplatin (54.1%), and sensitivity of cancer tissues to 5-FU+epirubicin+DDP (71.4%) and to 5-FU+taxetere+DDP (71.4%) were the highest. The inhibition rates of and sensitivity of cancer tissues to combined agents were higher than those of single agents (P <0.05). Expressions of MDR1, multidrug resistance protein-1 (MRP1), ABC-binding cassette transporter superfamily-G-2 (ABCG2) mRNA were detected in 88.9%, 55.6% and 55.6% of specimens respectively; while those of MDR1, MRP1 and ABCG2 proteins were detected in 55.6%, 33.3%, and 50.0% of specimens respectively. Expressions of mRNA and proteins had no correlation in MDR1, MRP1 and ABCG2 (P >0.05). High expression of ABCG2 protein was correlated to the resistance of colorectal cancer cells to epirubicin (P<0.05). **Conclusions**: Expressions of MDR proteins are correlated to chemosensitivity of colorectal cancer to some extents. By combining HDRA with measurement of MDR genes and proteins, chemosensitivity of individual tumors may be predicted to guide selection of effective chemotherapeutic agents.

Key words: colorectal cancer, tissue culture, drug sensitivity test, multidrug resistance, MDR1, MRP1, ABCG2

Colorectal cancer has always been the primary threat to the health of population in Western world. There were 153,760 new cases reported in 2007 and 52,180 patients died of this disease, which took up 10% of all mortalities of cancers.[1] In China, colorectal cancer is among the top five among malignant tumors. In each year, there are approximately 130,000 reports of intestinal cancer and the incidence rate is increasing at a pace of 2% to 4% per year. Surgery is still the most effective treatment for colorectal cancer, where early radical resection of colorectal cancer can yield a five-year survival rate of more than 90%. However, the rate of early diagnosis is low and thus, about 60% of patients of initial onset or recurrence requires systemic chemotherapy.[2] The heterogeneity and existing multi-drug resistance (MDR) in tumors can greatly influence the therapeutic outcome of chemotherapy. Inappropriate chemotherapy can result in great toxic side effects and may even induce MDR that ultimately results in failure. According to the tumor type, clinical staging, and previous treatment history, drug response assay in combination with personalized chemotherapy is suggested to replace traditional treatment to achieve the best results.[3] This study used histoculture drug response assay (HDRA) to measure the chemosensitivity of colorectal cancer, as well as analyze its correlation to the levels of multi-drug resistance genes and proteins, which would hopefully provide references for individualized chemotherapy.

Materials and Methods

General information. Twenty-two patients of colorectal cancer undergoing surgical treatment at Sun-Yet Sun University Cancer Center from November 2007 to June 2008 were selected. These patients were pathologically confirmed before the surgery. There were 13 males and nine females. Their ages were between 27 and 77-year-old, with a medium age of 60.5 years. The maximum diameters of tumors were between 1cm and 14cm, with a medium value of 4cm. Among these cases, there were 10 cases of colon cancer, 12 cases of rectal cancer, one case of highly differentiated adenocarcinoma, 14 cases of moderately differentiated adenocarcinoma, three cases of poorly differentiated adenocarcinoma, and four cases of mucoid adenocarcinoma. Five cases were in stage I, eight cases in stage II, seven cases in stage III, and two cases in stage IV (AJCC/UICC 2002).

Samples for the drug response test came from surgically removed fresh tumor tissues of all cases. RT-PCR and immunohistochemical analysis were performed on frozen tissues and paraffin block tissues of the same batch.

in vitro HDRA. Once fresh tissues had been isolated from the body, samples were obtained from the side close to the plasma membrane, where tumor tissues of approximately 1cm^3 were acquired. It was placed in RPMI-1640 culture media containing antibiotics without serum and sent to laboratory within 2 h for further analysis. In addition, tumor tissues were obtained and stored in a clean sample cup with RNA preserving solution, before they were stored in sample storage within 1h. Tumor tissues were washed three times and 10 min per trial with disinfecting solution, while residual blood stains were also washed off. Under a relatively sterile environment and a microscope of low magnification, fibrous, lipids, blood vessels, and necrotic tissues were removed, before tumor tissues were sliced into small blocks with the size of 0.5 to 1.0mm^3. Small tissue blocks were inoculated onto a 24-well filter paper, where each well was filled with four to six tissue blocks. Sterile culture media were added, before the whole setup was placed in the incubator at 37℃ and 5% CO_2. On the next day, the imaging analysis instrument was used to test the area of tissue blocks in each well (area, A). Each drug (10L) was added into each well and each type of the drugs was added to four wells, while a control well was established. The drug concentration for chemotherapy was prepared accordingly to the peak value of the effective drug concentration in human body: 2.3g/ml epiadriamycin (EADM), 2.5g/ml cisplatin (DDP), 5g/ml oxaliplatin, 60g/ml 5-fluorouracil (5-FU), 20g/ml taxotere, and 10g/ml irinotecan. The test groups were the above single drug administration group and the combinations of 5-FU+EADM+DDP, 5-FU+irinotecan, 5-FU+oxaliplatin, and

5-FU+taxotere+DDP. After four days of culture, 25l MTT was added to each well and proceeded with another 3h of culture. The imaging analysis instrument was used again to measure the area of tissue blocks, which was stained by formazan (blue area, BA). The imaging analysis instrument was assembled by Sun-Yet Sun University Cancer Center, which included a CCD-equipped video camcorder, a micro-computer with a video card, a monitor, and a printer. The imaging analysis software in binary code was self-programmed.

Based on the following equation, the inhibitory rate on cells by each drug was calculated: inhibitory rate = $[1-(BA_{treated}/A_{treated})/(BA_{control}/A_{control})]\times 100\%$, where BA was the blue stained area of the tissue block by formazan and A was the area of tissue block. The average inhibitory rate from four wells with the same tested agent was be considered as the final inhibitory rate. The inhibitory rate of greater than or equal to 30% was defined as sensitive to the drug, where the rate of greater than and equal to 50% was defined as highly sensitive. The rate of less than 30% was defined as drug tolerance.

The expression levels of mRNAs of multi-drug resistant genes measured by RT-PCR. One-step Trizole method was used to extract total RNA. Agarose gel electrophoresis (2%) was performed to test the completeness of total RNA, while the photospectrometer was used for OD-value. The purity and the concentration of RNA were calculated. RNA (0.5g) was used for reverse transcription. A 10l system included 2l $MgCl_2$, 1l RT buffer, 3.75l RNase free dH_2O, 1l dNTP mixture (10mmol/L for each), 0.25l RNase inhibitor, 0.5l AMV reverse transcriptase, 0.5l random9 mers, and 1l experimental sample RNA. Reaction conditions were the followings: one cycle of 30℃ for 10 min, 48℃ for 30 min, 99℃ for 5 min, and 5℃ for 5 min. In 10l of reverse transcription products, 10l 5xPCR buffer was added, as well as 28.75l sterile distilled water, 0.25l Ex Taq enzyme, 0.5l upstream PCR primer, and 0.5l downstream PCR primer, yielding a system of 50l for PCR (TaKaRa Company, Japan). Primers and amplification conditions were based on the references.[4-6] Primers were synthesized by Invitrogen Company. The lengths of amplification products of mRNAs of MDR1, MRP1, ABCG2, and -actin were 237 bp, 561 bp, 342 bp, and 619 bp, respectively. Agarose gel electrophoresis (2%) was run to test amplification products. The imaging analysis system, Quantity One, was used for grayscale scan, where the ratio was used to express relative levels of mRNAs of multi-drug resistance genes-MDR1, MRP1, and ABCG2. When the ratio of multi-drug resistance gene to -actin was greater than or equal to 0.6, it meant high expression. When the ratio was between 0.3 and 0.6, it signified moderate expression. When such ratio was between 0.1 and 0.3, it indicated low expression. When the ratio was below 0.1, it was considered as a negative result.

The protein expression levels of multi-drug resistance genes measured by immunohistochemical assay. Double-step immunohistochemical test with non-biotin was performed to measure the protein expression levels of MDR1-encoding protein P-glycoprotein (P-gp), MRP1, and ABCG2 (rats anti-human monoclonal antibodies by US Abcam Company). P-gp is located on the cell membrane and is primarily in the glandular type of cells. MRP1 and ABCG2 are expressed on the cell membrane and in plasma. PBS was used to replace the primary antibody as control. Slides of hepatic cancer (P-gp and MRP1) and breast cancer (ABCG2) were used as negative control.

The percentage of positive cells in five cancer regions under 400x magnifications was counted. The score was assigned according to the following rules: one point for the average number of positive cells of less than 25%, two points for the average from 25% to 49%, three points for the average from 50% to 74%, and four points for the average greater than and equal to 75%. Based on the staining intensity of tumor cells, the score was calculated accordingly: one point for light yellow, two points for yellow, and three for brownish yellow. For each sample, the product of the scores from two categories was the total score: (-) for 0 to 4 points, (+) for 5 to 8 points, and (++) for 9 to 12 points. The staining

status of each tissue block on slides was assessed and recorded by experienced pathologists under light microscopy.

Statistical analysis. SPSS 13.0 for Windows software was used for statistical analysis. The Kruskal-Walis H. method of non-parameter was performed to analyze the variation between the inhibitory rate and the sensitivity in each pharmaceutical group. Spearman correlation analysis was used to distinguish the relationship between the chemosensitivity of tumor and the expressions of multi-drug resistance proteins, as well as the relationship between the expression of protein and expression of mRNA of multi-drug resistance genes. Paired test was performed and = 0.05.

Results

The inhibitory rate and the sensitivity rate of colorectal cancer tissues to each drug. From high to low in order, the inhibitory rates of the single drug administration groups were: oxaliplatin, 5-FU, taxotere, cisplatin, irinotecan, and epiadriamycin. The order, from high to low, of the inhibitory rates for the combinatory drug administration groups were: 5-FU+oxaliplatin, 5-FU+epiadriamycin+cisplatin, 5-FU+taxotere+cisplatin, and 5-FU+irinotecan.

In order of high to low, the sensitivity rates of the single drug administration groups were: 5-FU, oxaliplatin=cisplatin=taxotere, irinotecan, and epiadriamycin. The order, from high to low, of the sensitivity rates for the combinatory drug administration groups were: 5-FU+taxotere+cisplatin=5-FU+epiadriamycin+cisplatin, 5-FU+oxaliplatin, and 5-FU+irinotecan (Table 1).

There were 12 patients who were tolerant to all single drug administrations, in which four of them were also resistant to single drug administration and combinations. There were two patients who responded well to all single drug administrations, while there were nine patients who responded well to all combinatory drug administrations.

mRNAs and protein expressions of multi-drug resistance genes in colorectal cancer tissues and their correlation analysis. The expression rates of mRNAs of MDR1, MRP1, and ABCG2 in colorectal cancer tissues were 88.9%, 55.6%, and 55.6%, respectively. The expression rates of proteins of P-gp, MRP1, and ABCG2 in colorectal cancer tissues were 55.6%, 33.3%, and 50.0%, respectively (Table 2, Figures 1 and 2).

Correlation study suggested that there was no relationship between the multi-drug resistance genes and the expression of their proteins (correlation index r = 0.377, p = 0.123; r = 0.332, p = 0.179; r = 0.304, p = 0.220).

The correlation analysis on the drug response of tumor and the expression of multi-drug resistance proteins. In tumor tissues, the expression of ABCG2 protein was obviously in negative correlation to the drug resistance to epiadriamycin in tissues (r = -0.536, p = 0.022). Expressions of other multi-drug resistance proteins were not related to the sensitivity of tumors to drugs as measured by HDRA (p > 0.05).

Table 1 Inhibition rates of and sensitivity to different chemotherapeutic agents in colorectal cancer tissues

Agent	Cases	Resistant (cases)	Sensitive (cases)	Highly-sensitive (cases)	Sensitivity (%)	Inhibition rate(%)
						Median
EADM	22	18	2	2	18.2	11.4
DDP	22	16	4	2	27.3	13.0
Oxaliplatin	22	16	3	3	27.3	17.5[a]
5-FU	22	14	5	3	36.4[a]	16.8
Taxotere	22	16	5	1	27.3	16.8
Irinotecan	22	17	5	0	22.7	11.4
FED	21	6	3	12	71.4[a]	51.4
FI	21	11	6	4	47.6	22.9
FO	21	9	1	11	57.1	54.1[a]
FTD	21	6	6	9	71.4[a]	39.9

EADM, epiadriamycin; DDP, cisplatin; 5-FU, 5-flurouracil; FED, 5-FU+epiadriamycin+cisplatin; FI, 5-FU+irinotecan; FO, 5-FU+oxaliplatin; FTD, 5-FU+taxotere+cisplatin.

a: The sensitivity to and inhibition rate of different single agents have no significant difference (χ^2=2.045, 2.702, respectively, P>0.05). The sensitivity of colorectal cancer tissues to different combined agents have no significant difference (χ^2=3.591, P>0.05) except the inhibition rate of that does (χ^2=11.012, P<0.05). The sensitivity to and inhibition rate of combined agents are significantly higher than those of single agents (χ^2=31.811, 61.281 respectively, P<0.05).

Table 2 mRNA and protein expressions of multidrug resistance genes in colorectal cancer tissues

Gene	Cases	mRNA expression					Protein expression			
		(−) (cases)	(+) (cases)	(++) (cases)	(+++) (cases)	Positive rate (%)	(−) (cases)	(+) (cases)	(++) (cases)	Positive rate (%)
MDR1	18	2	9	7	0	88.9	8	8	2	55.6
MRP1	18	8	7	3	0	55.6	12	5	1	33.3
ABCG2	18	8	9	0	1	55.6	9	8	1	50.0

MDR1, multidrug resitance-1; MRP1, multidrug related protein-1; ABCG-2, ATP-binding cassette transporter superfamily-G-2.

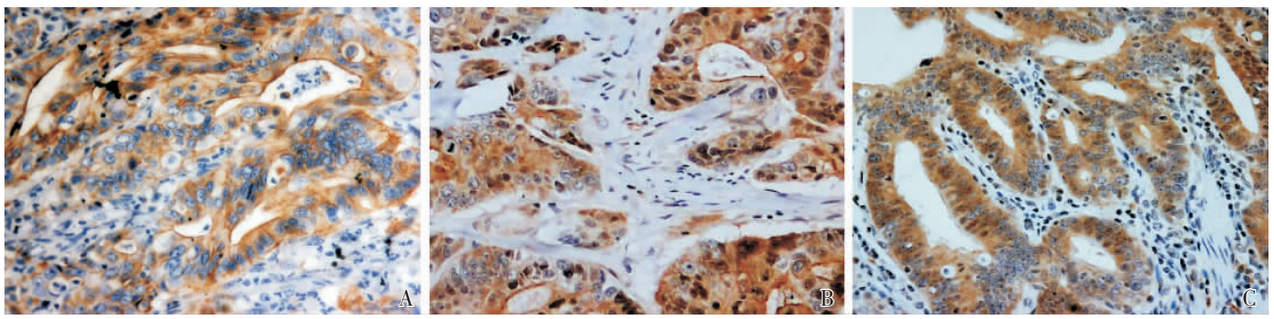

Figure 1 Protein expression of multidrug resistance genes in colorectal cancer detected by immunohistochemistry (SP ×400)
A: P-glycoprotein (P-gp) (yellow) is expressed on the membrane of colorectal cancer cells; B: Multidrug resistance protein-1 (MRP1) (dark yellow) is expressed in cytoplasm of colorectal cancer cells; C: ABC-binding cassette transporter superfamily-G-2 (ABCG2) (yellow) is expressed in cytoplasm of colorectal cancer cells.

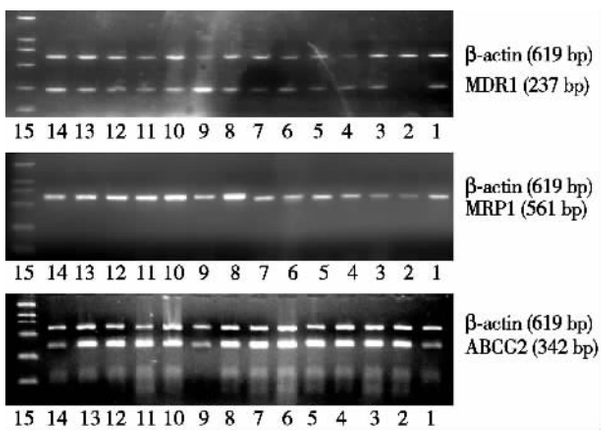

Figure 2 mRNA expression of multidrug resistance genes in colorectal cancer detected by RT-PCR

Discussion

Clinical tests prove that assisting chemotherapy can lower the risk of recurrence of colorectal cancer after radical surgery and it can prolong a non-cancerous life, while it improves the overall survival period.[7,8] Current therapeutic strategies against cancer relay on clinical tests of large populations, which does not grant a basis for personalized treatment. Also, these strategies fail to be effective in promoting clinical application of cytotoxical or molecular targeting drugs.[9] For postoperative patients who do not have an observable lesion site, the in vitro drug response test on the tumor sample can be used to predict chemosensitivity, where it can serve as a feedback to guide clinical treatment, which would enhance the targeting capability of chemotherapy.

The HDRA in this study has significant advantages over the traditional single-layered drug response test. Because tumor tissues are able to maintain growth in three dimensions, as well as possess other intrinsic properties, such as histological structures, cellular heterogeneity, and carcinogenesis, it can mimic several mechanisms such as extracellular matrix, intercellular adhesion, and signal transduction, which give them a more realistic, physical and clinical condition.[10] There were references available on its application in ovarian cancer and hepatic carcinoma,[11,12] where its value in prediction has been verified. Colorectal cancer tissues can be easily contaminated by bacteria and thus, it is

hard to culture. Due to this fact, there are few references so far. In this study, during sample acquisition, certain effective measures were taken, such as closure of intestine by clamps after sample isolation to prevent contamination by stools, following strict sterilization protocols, acquisition of samples from serous membrane, thin slide acquisition, and gradual inward probing, which guaranteed the quality of samples and avoided the contamination. Correct sampling technique is the key to successful culturing, because the subsequent procedures are rather easier. The total experimental time is short and would not affect the actual clinical treatment. The assessment of results is objective, which was based on an easy, convenient, repetitive, and relatively low cost technique. Furukawa et. al.[13] reported the predictive value of HDRA for progressive gastric carcinoma and intestinal cancer. They applied different drugs such as MMC, adriamycin, 5-FU, and DDP. In 38 cases with assessable lesion sites, 29 patients were all tolerant to insensitive drugs for chemotherapy. Three out of nine patients who were treated by sensitive drugs for chemotherapy developed tolerance. The accuracy rate had reached 92.1% (35/38). For patient who used HDRA-sensitive drugs for chemotherapy, his or her survival rate was improved significantly over others who used insensitive drugs.

The research results demonstrated that single administration of 5-FU had a relatively higher inhibitory rate and sensitivity for colorectal cancer tissues, where the sensitivity rate reached 36.4%, similar to the reported effective rate (21-43%).[14,15] There is no doubt that 5-FU should be the basic first-line choice of chemotherapeutic drugs. This study also observed that sensitivity rates of single drug administration for oxaliplatin, cisplatin, and taxotere were relatively high, even reached 27.3%. Clinically, the use of a single drug, oxaliplatin, has limited effectiveness. However, it has a synergic effect with 5-FU, and the combination of the two is a common regimen to treat colorectal cancer. This study also suggested that the inhibitory rate on tumor by 5-FU and oxaliplatin was the highest at a rate of 54.1%, which corresponded to clinical reports. In addition, in the process of exploring chemotherapeutic drugs for colon cancer, cisplatin used to be an important drug, too, where its effectiveness is similar to oxaliplatin. It was only replaced by oxaliplatin because of many of its side effects.[16] On the other hand, taxotere, in clinical testing, has been proven to inhibit gastrointestinal carcinoma. In the 90s, it was used in the stage II clinical trail. Unfortunately, it had never been noted to have effectiveness in clinical treatment for colorectal cancer and thus, it was slowly discarded. Its in vivo and in vitro results are not the same and the underlying mechanism was still not known, which requires further research.[17]

In the drug response test for the single drug administration groups, the inhibitory rate and the sensitivity rate for epiadriamycin were relatively low, which corresponded well with clinical practice. It was, historically, used in ECF proposal for chemotherapy on colorectal cancer. Many reports confirmed that it is not beneficial to survival, but rather would bring many toxic side effects.[18,19] Also, the inhibitory rate and the sensitivity rate for irinotecan were low, which was inconsistent with clinical reports. This is possibly due to its unique pharmacokinetic mechanism. Irinotecan is a precursor drug, which requires the hydrolytic effect by carboxylesterase in liver to be converted into an active metabolic product, SN-38. Its anti-tumor ability is 100 to 1,000 times of the precursor drug.[20] It is obvious that this metabolism cannot be simulated outside the body. Therefore, in vitro drug response test has limited capability to understand the predicative value of these drugs.

Drug response test showed the inhibitory rate and the sensitivity rate for the combinatory drug administration groups were much higher than the single drug administration groups. When we select appropriate chemotherapy proposal in clinical practice, we can use 5-FU as the foundation drug and under the prerequisite of reducing side effect, we can combine other sensitive drugs for combinatory chemotherapy, in order to improve the therapeutic effect.

Research reported that the primary drug tolerance developed in tumor is actually a

molecular event, concerning the cellular biochemistry and pharmacokinetics in host, in which any imbalance would affect tumors growth, proliferation, differentiation, or apoptosis. Multi-drug resistance is an important mechanism and a cause for the development of tolerance to chemotherapy in clinical practice. It is primarily mediated by several multi-drug resistance proteins of the ABC superfamily, where P-gp, MRP1, and ABCG2 are the mostly studied members of the group.

In hepatic carcinoma, pulmonary carcinoma, ovarian cancer, and gastrointestinal cancer, it is generally believed that mRNAs of MDR1 and P-gp are moderately or highly expressed, and they are directly related to the poor therapeutic effect of chemotherapy on the tumor.[21, 22] Additionally, MRP1 and P-gp are very similar in the structure and functions, which results in crossed drug tolerance to vinblastine analogues, adriamycin, and etoposide.[23] The expression is closely related to the tolerance and the prognosis of breast cancer, gastric carcinoma, retinoblastoma, and lung cancer.[24, 25] Salud et. al. 26 reported that the positive rate of P-gp in colorectal cancer was 57%. Jiang et al.[27] reported the positive rate of MDR1 mRNA as 72% . References showed the expression rate for MRP1 proteins was 41.3% to 64.4% [28,29] and the expression rate of its mRNA was 39.5% to 68.3% .[30,31] This study, by using immunohistochemical analysis, measured the positive rate of P-gp in colorectal cancer as 55.6%, which was moderately expressed, while the mRNA of MDR1 was highly expressed at 88.9%. The positive rates of MRP1 proteins and mRNA were 33.3% and 55.6%, respectively. These were lower than the positive rates of MDR1 proteins and mRNA.

The study showed no correlation in expressions of mRNA and proteins of MDR1 and MRP1. This is possibly because that the mRNA expression of MDR gene in tumor is heterogeneous, which suggests that only a small population of tumor cells is expressed this gene. As a result, mRNA in acquired tumor samples could not accurately reflect the mRNA level in that tumor.[32] Some studies also believe that he phenotypic expression of MDR gene is over-expressive of MDR gene with or without gene amplification. In cell strain of low drug resistance, there may no MDR gene amplification, but only the increased expression of MDR gene (enhanced the functions of MDR protein). At this point, the improvement on functions of MDR protein in cell with low drug resistance is possibly due to the increased transcription level of mRNA, but not by the gene duplication.[33] The effects of this post-transcriptional mechanism include the change in stability of mRNA and/or in the regulatory change after translation.[34] This research did not observe the correlation between the drug response test and the expressions of proteins of MDR1 and MRP1. It is necessary to increase studied subjects for further verification by in vivo experiment. It also suggested that there were other participating proteins in mediating drug tolerance of colorectal cancer to chemotherapy, such as subtypes of MRP and ABCG2 as described below.

ABCG2 represents the newest member in the ABC transmembranous superfamily. It only has a binding site for ATP/GTP and a hydrophobic transmembranous domain. It is also called half-transport protein. Tumor cells with hyperactive expression of ABCG2 will develop cross-tolerance to mitoxantrone, adriamycin, daunorubicin, etoposide, topotecan, and SN-38, while it is not crossed-tolerant to taxol and vincristine.[35] Gupta et al.[36] discovered down-regulation in the expression of protein and mRNA of ABCG2 in colorectal cancer and cervical cancer. Currently, reports on ABCG2 are about the measurement of cell strains, and few reports are available for the expression status in surgically removed tumor samples. The positive rates of mRNA and protein of ABCG2 in colorectal cancer in this study were 55.6% and 50.0%, respectively. Also, the expression of ABCG2 protein was clearly in negative correlation to the inhibitory rate of its important substrate epiadriamycin, indicating tolerance to epiadriamycin in patients with high expression of ABCG2 proteins, as shown by the drug response test.

In colorectal cancer, HDRA can predict the chemosensitivity of tumor, which is highly accurate and assessable. However, it is necessary to expand the study population and to conduct in vivo experiments to increase the credibility of this study. The measurement of expression products of multi-drug resistance genes could observe a negative correlation between the expression of ABCG2 proteins and the inhibitory rate of epiadriamycin, suggesting that the tolerant protein of ABCG2 might participate in the mediation of tolerance to epiadriamycin. The effect of mediating tolerance to epiadriamycin by MDR1 and MRP1 would need further research verification. Combined HDRA and measurement of proteins to multi-drug resistance genes can provide a prediction to primary drug tolerance in tumor, where more sensitive drugs can be screened out for the optimal chemotherapy.

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