

Expression levels of resistant genes affect cervical cancer prognosis

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Abstract. Tumor cells may develop multidrug resistance (MDR) to various chemotherapy regimens. Such resistance reduces the sensitivity of cells to chemotherapy drugs, leading to the failure of cervical cancer (CC) treatment and disease progression. The present study aimed to investigate the role of *MDR1*, lung resistance protein (*LRP*) and placental glutathione S-transferase π 1 (*GSTP1*) in CC and MDR, and the prognostic value of these genes. The mRNA expression levels of these resistance-associated genes were determined in 47 CC and 20 healthy cervical tissue samples. Subsequently, the data was analyzed alongside clinicopathological parameters. The mRNA expression levels of *MDR1*, *LRP* and *GSTP1* in CC were 0.57 ± 0.32 , 0.58 ± 0.29 and 0.44 ± 0.24 , respectively, whereas those in healthy cervical tissues were 0.19 ± 0.10 , 0.17 ± 0.14 and 0.18 ± 0.10 , respectively. Therefore, the expression levels of these genes were significantly greater in CC compared with healthy cervical tissue ($P < 0.05$). mRNA expression levels of *MDR1* were increased in the well differentiated group (0.68 ± 0.27) compared with the poorly differentiated group (0.38 ± 0.33 ; $P < 0.05$). No significant differences were observed between *LRP* and *GSTP1* mRNA expression levels and tumor differentiation or clinical stage of the patients ($P > 0.05$). Multivariate logistic regression indicated that the degree of differentiation and the *MDR1* gene expression levels were predictors of CC prognosis ($P < 0.05$). The survival rate of patients in the *MDR1*-negative group was significantly greater compared with the *MDR1*-positive group ($P < 0.05$). The results of the present study therefore suggested that *MDR1* gene expression is a predictor of poor survival in CC.

Introduction

Cervical cancer (CC) is a common malignancy of the female reproductive system. In China, >200,000 fatalities due to CC

occur each year, and the incidence in younger age groups is on the increase (1). Current treatment approaches to CC include surgery, chemotherapy and radiotherapy in patients with primary tumors. However, due to continuous use of various chemotherapy drugs, multidrug resistance (MDR) may develop in tumor cells. Such resistance reduces the sensitivity of tumor cells to chemotherapy, leading to failure of treatment. MDR is defined as the resistance of cancer cells to a diverse panel of structurally and functionally unassociated drugs (2). This resistance can occur naturally (inherent resistance) or acquired during the course of chemotherapy (3). To date, current research on MDR has focused on the transcriptional regulation of the *MDR* gene. The multidrug-resistance 1 (*MDR1*) gene product P-glycoprotein (P-gp) is an efflux pump that actively transports substrates, such as glucocorticoids, out of the cell (4). *LRP* is a small subcellular structure located at cytoplasmic vaults that may be in charge of subsequent exocytosis of agents from the cell. A previous *in vitro* study determined that *LRP* was associated with resistance to melphalan, cisplatin and doxorubicin (5). *GSTP1*, which has an important role in the detoxification of toxic substances, is a phase II metabolic enzyme, protects cells from anticancer drug-induced injury (6). To understand the role of the *MDR* gene in primary drug resistance, the present study detected the mRNA expression levels of *MDR1*, *LRP* and *GSTP1* in 47 cases of CC and 20 healthy cervical tissue samples. In addition, a preliminary study on the association between the expression levels of these genes and cervical pathology was conducted. The present study investigated mechanisms underlying drug resistance in CC and may aid understanding of individual responsiveness to chemotherapy and prognosis.

Materials and methods

Patients and specimens. A total of 47 fresh CC specimens were collected from February 2008 to August 2010. Patients were aged between 43 and 65 years, with a mean age of 46.7 years. Histologic cell types included 41 cases of squamous-cell carcinoma and 6 cases of adenocarcinoma. Clinical staging was performed, and was as follows: Stage I, 12 cases; stage II, 18 cases; and stage III, 17 cases. Of these cases, 10 were well differentiated, 16 were moderately differentiated and 21 were poorly differentiated carcinomas. All diagnoses were confirmed by pathology. A total of 19 cases were ≥ 4 cm in size and 28 measured < 4 cm. All patients had not received chemotherapy and radiation therapy prior to sample collection. In addition,

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20 healthy cervical tissue samples were collected (cancerous tissue from the same cervical margin tissue surgery measures >2 cm from the edge, as the tumor edge was not clear, an additional 27 cases were not collected). Samples were stored in liquid nitrogen for future use. The integrity of clinical data was maintained, and the patients were monitored for 6 to 60 months.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues using TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The concentration and purity of total RNA was determined by measuring the absorbance with a NanoDrop-2000 spectrophotometer at wavelengths of 260 and 280 nm. cDNA was synthesized from total RNA using the Reverse Transcription system (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. cDNA was utilized immediately or stored at -80°C until use. Each qPCR reaction contained 1.5 µl 2 mmol/l dNTP, 0.3 µl 5 U/µl Taq (Sangon Biotech Co., Ltd., Shanghai, China), 2.5 µl 25 mmol/l Mg²⁺, 3 µl 10X buffer, 2 µl forward primer, 2 µl reverse primer, 5 µl cDNA (0.5 µg/µl), 1.0 µl 10X SYBR®-Green I (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The total volume of each reaction was adjusted to 30 µl with sterile water. Cycling conditions were as follows: An initial predenaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. Subsequently, dissociation curve analysis was performed, and the reaction products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide for confirmation of the PCR products. The mRNA expression levels of *MDR1*, *LRP* and *GSTP1* were expressed as a ratio relative to *GAPDH* in each sample, the level of genes expression were normalized to that of the internal (*GAPDH*) and were determined by the 2^{-ΔΔC_q} method (7). Primer pairs were as follows: Forward, 5'-CCC ATC ATT GCA ATA GCA GG-3' and reverse, 5'-TGT TCA AAC TTC TGC TCC TGA-3' for human *MDR1*; forward, 5'-CCA GAA CCA GGG AGG CAA GA-3' and reverse, 5'-GAG GCG CCC CAC ATA TGC T-3' for human *GSTP1*; forward, 5'-GTC TTC GGG CCT GAG CTG GTG TCG-3' and reverse, 5'-CTT GGC CGT CTC TTG GGG GTC CTT-3' for human *LRP*; and forward, 5'-GAA GGT GAA GGT CGG AGT C-3' and reverse, 5'-GAA GAT GGT GAT GGG ATT TC-3' for human *GAPDH*. *MDR1*, *LRP*, *GSTP1* and *GAPDH* primers yielded products of 158, 325, 240 and 226 bp, respectively.

Statistical analysis. Statistical calculations were performed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA), and P<0.05 was considered to indicate a statistically significant difference. The measured data were expressed as the mean ± standard deviation. A Student's t-test, two-tailed chi-squared test and multivariate logistic regression analysis were used to compare groups. Survival curves were compared using the two-sided data log-rank method.

Results

mRNA expression levels of *MDR1*, *LRP* and *GSTP1* in tissue samples. Of CC tissues, 63.8% were positive for *MDR1*, 76.6%

were positive for *LRP* and 59.6% were positive for *GSTP1*. Of healthy tissues, 10% were positive for *MDR1*, 15.0% were positive for *LRP* and 5% were positive for *GSTP1*, compared with the healthy tissues, if the levels were ≥ 0.4, the expression was deemed to be positive; otherwise, the expression was negative. Therefore, a greater percentage of CC tissues expressed these genes compared with healthy tissues (P<0.05). The relative mRNA expression levels of the resistance genes *MDR1*, *LRP* and *GSTP1* in cancer tissues were 0.57±0.32, 0.58±0.29 and 0.44±0.24, respectively. The relative mRNA expression levels of *MDR1*, *LRP* and *GSTP1* in normal cervical tissues were 0.19±0.10, 0.17±0.14 and 0.18±0.10, respectively. Therefore, the mRNA expression levels of all three genes were greater in cancer compared with healthy cervical tissues, and this was statistically significant (P<0.001). In all cancer samples, the expression of all three genes was detected in 14 cases, expression of two genes was detected in 29 cases and expression of one gene was detected in 37 cases. *MDR1*, *LRP* and *GSTP1* mRNA expression levels are presented in Fig. 1.

mRNA expression levels of *MDR1*, *LRP* and *GSTP1* and clinicopathological data. No significant differences were identified between the mRNA expression levels of *LRP* or *GSTP1*, and tumor size, histological type or clinical stage (P>0.05; Table I). mRNA expression levels of *MDR1* were significantly greater in poorly-differentiated compared with well and moderately differentiated carcinomas (P<0.05; Table I); however, no significant differences were identified between *MDR1* mRNA expression and the other clinicopathological factors assessed.

mRNA expression levels of MDR-associated genes and survival rate of patients. Multivariate logistic regression analysis revealed that the risk factors of CC were associated with high mRNA expression levels of *MDR1* and the state of differentiation; however, there was no association between the mRNA expression levels of *LRP* or *GSTP1* (data not shown), and tumor size or stage (Table II). The cancer samples were categorized into *MDR1*-positive (n=32) and -negative (n=15) groups. The survival rate was significantly greater in the *MDR1*-negative group compared with the *MDR1*-positive group (Table II; Fig. 2; P<0.05).

Discussion

MDR may be divided into primary drug resistance that exists prior to chemotherapy treatment, and acquired drug resistance that may develop during chemotherapy. MDR was identified as an important factor that contributes to the failure of cancer treatment.

MDR is frequently associated with overexpression of the *MDR1* gene, which encodes the drug transporter, P-glycoprotein (P-gp). P-gp is one of the ABC transporter proteins that have similar trans-membrane domains that may pump chemotherapeutic drugs out of cancer cells against a concentration gradient in an ATP energy-dependent manner; therefore, reducing intracellular accumulation of chemotherapeutic agents and protecting cancer cells from toxicity (8). *MDR1* genes and/or increased expression of P-gp have been associated with drug resistance (9-11). The glutathione (GSH) system is responsible for the detoxification of reactive oxygen

Table I. Association between *MDR1*, *LRP* and *GSTP1* mRNA expression levels and clinicopathological parameters in cervical cancer.

Study groups	n	<i>MDR1</i>	<i>t</i>	P-value	<i>LRP</i>	<i>t</i>	P-value	<i>GSTP1</i>	<i>t</i>	P-value
Size (cm)										
≥4	19	0.48±0.27	-1.29	0.21	0.51±0.33	-1.34	0.19	0.42±0.25	-0.49	0.63
<4	28	0.62±0.34			0.63±0.26			0.46±0.24		
Histological type										
Squamous carcinoma	41	0.62±0.31	1.72	0.1	0.60±0.29	0.85	0.40	0.45±0.23	0.14	0.89
Adenocarcinoma	6	0.47±0.39			0.49±0.30			0.43±0.33		
FIGO stages										
I+II	30	0.62±0.31	1.53	0.13	0.58±0.31	-0.069	0.94	0.47±0.24	1.27	0.21
III	17	0.46±0.32			0.58±0.28			0.38±0.25		
Differentiation										
Well+moderately	26	0.68±0.27	3.16	0.003	0.61±0.29	0.71	0.49	0.47±0.25	0.70	0.50
Poorly	21	0.38±0.33			0.54±0.29			0.42±0.23		

Gene expression data are expressed as the mean ± standard deviation. MDR, multidrug resistance; LRP, lung resistance protein; GSTP1, placental glutathione S-transferase π 1; FIGO, The International Federation of Gynecology and Obstetrics.

Table II. Multivariate logistics analysis on factors that influence cervical cancer prognosis.

Covariates	Deceased patients/total	B	Exp (B)	95% CI	P-value
Differentiation					
Well+moderately	6/26	2.83	16.89	1.71-166.48	0.015
Poorly	8/21				
MDR1					
Positive	12/32	3.65	38.53	3.03-490.35	0.005
Negative	2/15				

MDR1, multidrug resistance 1; B, regression coefficient; Exp (B), odds ratio; CI, confidence interval.

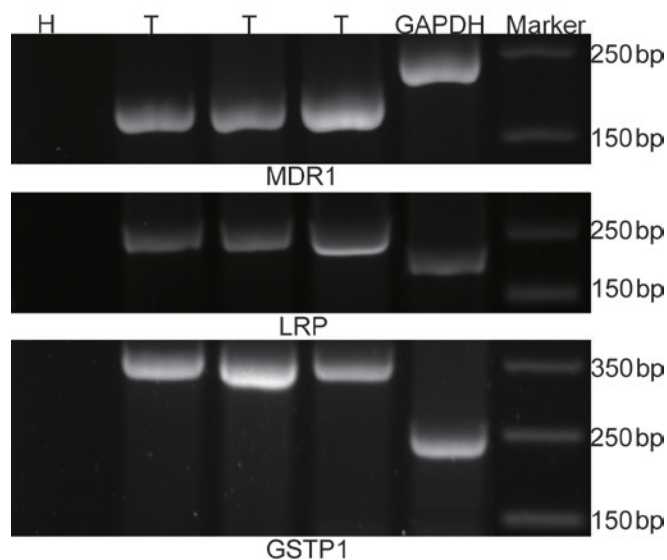


Figure 1. mRNA expression levels of *MDR1*, *LRP* and *GSTP1* in normal and cervical cancer tissue specimens. *MDR1*, multidrug resistance 1; *LRP*, lung resistance protein; *GSTP1*, placental glutathione S-transferase π 1; H, healthy; T, cervical cancer tissue.

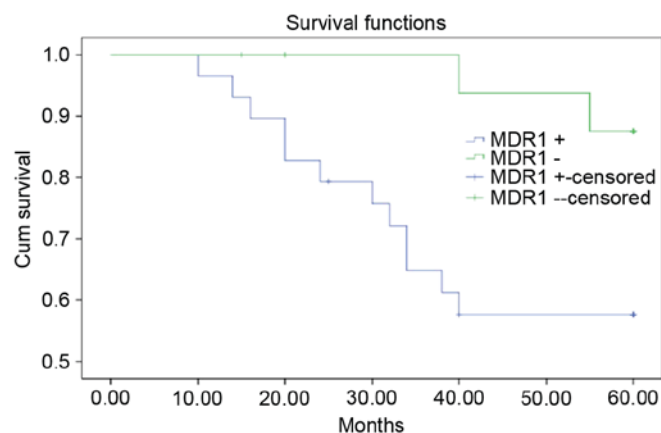


Figure 2. Survival curves of *MDR1*-positive (n=32) and *MDR1*-negative (n=15) patients. $P<0.05$. *MDR1*, multidrug resistance 1.

and nitrogen species in cells. Tumor cells that express glutathione s-transferase (GST) may catalyze the conjugation of GSH to anticancer drugs, which are subsequently removed

from the cell by transporters. This protects cells from anti-cancer drug-induced injury. GST expression is primarily induced by alkylating agents, including platinum compounds and mitomycin-c (12). LRP serves a role in drug resistance in breast cancer (13), ovarian cancer (14), lung cancer (15) and various other tumor types. Enhanced expression levels of LRP may cause tumor cells to become resistant to traditional chemotherapy drugs, including doxorubicin, cisplatin, vincristine and mitomycin (16). Therefore, findings regarding the expression of LRP revealed that cervical cancer has an intrinsic resistance of cisplatin (17). In the present study, the mRNA expression levels of the resistance genes *MDR1*, *LRP* and *GSTP1* were measured in cancer tissue specimens and compared with clinicopathological data, to investigate their role in primary drug resistance in cancer. Tolerance of tumor cells to drugs is associated with the expression levels of drug resistance genes in cells. Therefore, the expression levels of drug resistance genes indirectly reflect the resistance of tumor cells to drugs. The present study demonstrated that the mRNA expression levels of *MDR1*, *LRP* and *GSTP1* in CC tissue specimens were greater compared with healthy cervical tissues. Patients had not received chemotherapy or radiation therapy. This suggested that primary drug resistance was associated with CC.

In the present study, not all tumors expressed the MDR-associated genes; 29.8% of tumors expressed *MDR1*, *LRP* and *GSTP1*, and 61.7% expressed two of these genes. This suggested that the expression levels of these genes in CC are regulated by signaling pathways that contribute to MDR, which are likely exhibit a high degree of complexity and interconnectivity.

The mRNA expression levels of *MDR1* were greater in well and moderately differentiated carcinomas compared with poorly differentiated carcinomas, which suggested that the greater the degree of cancer differentiation, the greater the resistance to chemotherapeutic drugs. However, the mRNA expression levels of *LRP* and *GSTP1* in the tumor tissues did not exhibit a significant association with the clinicopathological features of the patients. Greater numbers of specimens would be necessary to confirm this finding. High mRNA expression levels of *LRP* and *GSTP1* may mediate drug resistance of tumor cells to cisplatin, suggesting that primary MDR of CC to cisplatin may be associated with the expression levels of *LRP* and *GSTP1* genes (18).

Sato *et al* (19) demonstrated that high expression levels of P-gp were detected in lymph node metastasis of the colon. In a follow-up study of 112 patients with CC, Nagai *et al* (20) revealed that the five-year survival rate of P-gp-positive patients was significantly reduced compared with patients with P-gp-negative tumors. In the present study, high *MDR1* mRNA expression levels were associated with poor CC survival rates, suggesting that *MDR1* may be a high-risk prognostic factor.

Reducing the expression levels of genes associated with drug resistance is likely to enhance the efficacy of therapy. The expression levels of the *MDR1* gene and its corresponding protein, P-gp, were significantly reduced in cancer cells transfected with small interfering RNA targeting *MDR1* (21). *In vitro* experiments and RNA interference technology may effectively reverse drug resistance in ovarian cancer cells (22).

Studies that aim to reverse drug resistance in solid tumors warrant further investigation.

In conclusion, *MDR1*, *LRP* and *GSTP1* genes may be involved in mechanisms underlying primary MDR in CC. Detection of *MDR1* expression levels may be utilized to predict patient response to chemotherapy. Reversal of CC drug resistance may strengthen the efficacy of chemotherapy and prolong the survival of patients with CC.

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