P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• CLINICAL RESEARCH •

Correlation of P-glycoprotein expression with poor vascularization in human gallbladder carcinomas

Yu Tian, Li-Li Zhu, Ren-Xuan Guo, Chui-Feng Fan

Yu Tian, **Ren-Xuan Guo**, Department of General Surgery, First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China
Li-Li Zhu, Department of General Surgery, Second Affiliated Hospital, China Medical University, Shenyang 110004, Liaoning Province, China
Chui-Feng Fan, Department of Pathology, China Medical University, Shenyang 110001, Liaoning Province, China
Correspondence to: Dr. Yu Tian, Department of General Surgery, First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, China. tianyu5460@21cn.com
Telephone: +86-24-23256666-6237 **Fax:** +86-24-23896804
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Abstract

AIM: To investigate the relationship between the expression of P-glycoprotein (P-gp) and the degree of vascularization in gallbladder carcinomas.

METHODS: P-gp was stained with streptavidin-peroxidase complex immunohistochemical method in routine paraffin-embedded sections of gallbladder carcinomas. Microvessel counts (MVC) were determined using factor-VIII-related antigens.

RESULTS: The average MVC in 32 cases of gallbladder carcinomas was (34±10)/HP. The value of MVC was closely correlated with Nevin staging and tumor differentiation ($P<0.01$ and $P<0.05$). The total expression rate of P-gp was 62.5 %. The P-gp expression rate in cases of Nevin staging S1-S3 (78.6 %) was higher than that of S4-S5 (50.0 %) with no statistical significance. The P-gp expression rate was not correlated with tumor differentiation or pathologic types. The value of MVC in P-gp (+) cases was markedly lower than that in P-gp (-) cases ($P<0.01$). The positive rate of P-gp was significantly higher in cases of smaller MVC than those of bigger MVC ($P<0.05$).

CONCLUSION: MVC may be used as one of the important parameters to reflect the biological behaviors of gallbladder carcinomas. As a major cause of drug resistance, the overexpression of P-gp is closely correlated with the poor vascularization in gallbladder carcinomas.

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INTRODUCTION

Because the rate of neovascularization (angiogenesis) frequently fails to keep pace with tumor growth, tumor vasculature is often insufficient in supplying the tumor mass, therefore many solid tumors contain subpopulation of hypoxic cells. Some researches showed that the drug resistance was partially due to the poor tumor vascularization in reducing the influx of cytotoxic agents. Additionally, the hypoxic environment due to poor vascularization could also inhibit tumor cell proliferation, yet noncycling cells would be expected to be less sensitive to many agents. In recent years, some biochemical mechanisms of drug resistance have been identified; one of them is the overexpression of transmembrane transport protein and P-glycoprotein. We therefore linked angiogenesis assessed by microvessel counts with the expression of P-gp in human gallbladder carcinomas. The aim of the present study was to investigate whether MVC could be used as an important parameter to reflect the biological behaviors of gallbladder carcinomas and to illustrate the relationship between P-gp expression and vascularization.

MATERIALS AND METHODS

Clinical materials

Thirty-two cases of gallbladder carcinomas were randomly selected and diagnosed histologically. All the patients were treated surgically in our hospital. No chemotherapy or anti-angiogenesis therapy was used prior to surgery. There were 17 males and 15 females with an average age of 56 years. Histological types included 4 cases of papillary adenocarcinoma (12.5 %), 25 cases of tubal adenocarcinoma (78.1 %) and 3 cases of mucous adenocarcinoma (9.4 %). Twelve cases had well-differentiated gallbladder carcinomas (37.5 %), 9 cases moderate-differentiated gallbladder carcinomas (28.1 %) and 11 cases poor-differentiated gallbladder carcinomas (34.4 %). The Nevin staging (Table 1) was determined based on clinical features: 14 cases at stages S1, S2 and S3, and 18 cases at stages S4 and S5. All available hematoxylin and eosin-stained sections in each case were reviewed.

Table 1 Nevin staging system for gallbladder cancer[1]

Stage	Definition
1	Tumor invades mucosa only
2	Tumor invades muscularis and mucosa
3	Tumor invades subserosa, muscularis and mucosa
4	Tumor invades all layers of gallbladder wall plus cystic lymph node
5	Tumor extends into liver bed or distant spread

Immunohistochemical stains

Four micrometer-thick sections from formalin-fixed and paraffin-embedded tissues were placed on poly-L-lysine-coated slides for immunohistochemistry study. The expression of P-gp was assessed by SP immunohistochemical method using a mouse-anti-human P-gp monoclonal antibody (JSB1) and a UltraSensitive™ S-P kit (kit 9710). Blood vessels were highlighted by staining endothelial cells for factor VIII-related antigens. The deparaffinized sections were boiled in citrate buffer at high temperature and high pressure for antigen retrieval for staining of P-gp, pepsin digestion for factor VIII-related antigen staining, and then incubated with each antibody

at 4 ℃ overnight. Immunohistochemical staining was then performed according to the UltraSensitive™ S-P kit manual. All reagents were supplied by Maixin-Bio Co, Fuzhou, China. The cells with brown-yellow granules in cytoplasms or on cytomembranes were considered as positive for P-gp expression.

Immunostaining

P-gp Stained slide was examined by two independent observers and scored semi-quantitatively. Staining intensity was assessed in comparison with positive slide of colon cancer, supplied by Maixin-Bio Co, Fuzhou, China. The staining intensity was scored as none (0), weak (1), moderate (2) and strong (3). The slides were classified as negative (0), positive (1), strong positive (2) and strongest positive (3) with corresponding rates of positive cells at <10 %, 10-20 %, 20-40 %, and >40 %, respectively. When the mean score in each group was 3 or more, the slide was considered as positive. Negative controls were stained without primary antibody.

Microvessel counts

MVCs were assessed according to Weidner *et al*[2]. The hot spots were selected under a microscope (40x), then individual counts were made under 200x field (Olympus BH-2 microscope,0.74 mm^2 per field). The average counts in 5 fields were recorded. Any single highlighted endothelial cell or endothelial cell cluster clearly separated from adjacent microvessel, and distinct clusters of brown-staining endothelial cells were counted as separate microvessels. Vessel lumens were not the sole criteria in identifying a microvessel.

Statistical analysis

Statistical analysis was performed using the Chi-square test and *t* test with SPSS software (Ver.10.0). $P<0.01$ or $P<0.05$ was considered as significant.

RESULTS

Expression of P-gp and MVC

The microvessels in malignant tissues were heterogeneously distributed. These highly neovascularized areas distributed within the tumor and dominated around the tumor margins (Figure 1). The P-gp was stained in cytoplasms and on the cytomembranes of gallbladder carcinoma cells (Figure 2).

Clinicopathologic characteristics of MVC and P-gp expressions

The average MVC in 32 cases of gallbladder carcinoma was (34±10)/HP. The number of MVC was markedly higher in cases of Nevin stages S4-S5 than in those of Nevin stages S1-S3 ($t=2.833$, $P=0.008$). MVC in moderately or poorly differentiated group was higher than that in well-differentiated group ($t=2.581$, $P=0.015$). The differences of MVC among the different pathologic types were not statistically significant ($P=0.313$, 0.822, 0.168) (Table 2).

The P-gp expression rate was 62.5 % in these 32 cases. The positive rate of P-gp was higher in cases of Nevin stages S1-S3 (78.6 %) than in those of Nevin stages S4-S5 (50.0 %) with no statistical significance ($\chi^2=2.743$, $P>0.05$). The expression rate of P-gp was not correlated with tumor differentiation or pathologic types ($P>0.05$) (Table 3).

Relationship between expression of P-gp and MVC

The value of MVC in P-gp (+) cases was 30±9/HP which was significantly lower than that in P-gp (-) cases (40±8/HP) ($t=2.987$, $P=0.006$). The P-gp expression rate was significantly higher in cases with less median MVC (33.6/HP) than in those with MVC over median MVC (81.3 % *vs* 43.8 %, $\chi^2=4.800$, $P<0.05$).

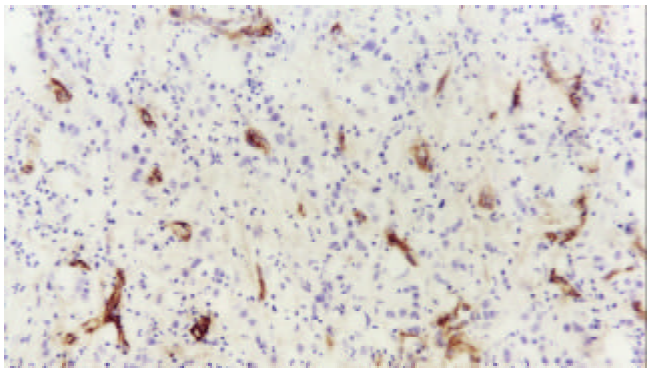

Figure 1 Distribution of microvessels in section of gallbladder carcinoma (S-P ×200).

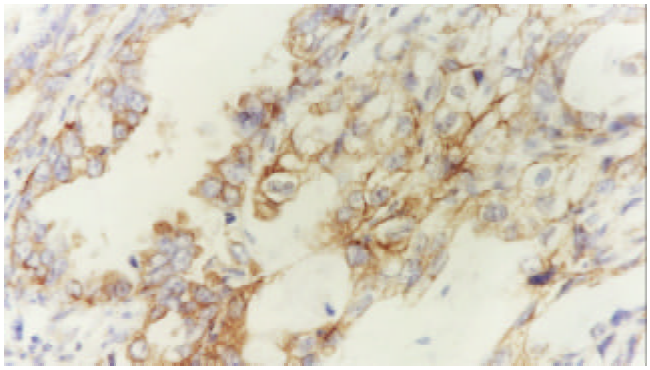

Figure 2 Expressed P-gp in gallbladder carcinoma (S-P ×400).

Table 2 Characteristics of MVC in gallbladder carcinoma

Characteristics	*n*	MVC
Pathologic types		
Papillary adenocacinoma	4	27±8[a]
Tubal adenocarcinoma	25	35±10[a]
Mucous adenocarcinoma	3	32±10[a]
Tumor differentiation		
Well	12	28±9[b]
Moderate-poor	20	37±9[b]
Nevin staging		
S1, S2, S3	14	28±10[c]
S4, S5	18	38±8[c]

[a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$.

Table 3 Characteristics of P-gp expression in gallbladder carcinoma

Characteristics	*n*	+	%
Pathologic types			
Papillary adenocacinoma	4	3	75.0[a]
Tubal adenocarcinoma	25	15	60.0[a]
Mucous adenocarcinoma	3	2	66.7[a]
Tumor differentiation			
Well	12	9	75.0[b]
Moderate-poor	20	11	55.0[b]
Nevin staging			
S1, S2, S3	14	11	78.6[c]
S4,S5	18	9	50.0[c]

[a]$P>0.05$, [b]$P>0.05$, [c]$P>0.05$.

DISCUSSION

In 1971, Folkman proposed that tumor growth be dependent on angiogenesis, and then considerable evidences showed that tumor growth was angiogenesis dependent, and the neovascularization was closely associated with the growth, invasion, metastasis, staging and prognosis of tumors[2-15]. Our study indicated that MVC was correlated to Nevin staging and tumor differentiation. The case at later stage and with poorer differentiation had higher level of MVC in gallbladder carcinomas. MVC might be one of the most important parameters in reflecting the biologic behaviors of gallbladder carcinomas.

Though tumor growth depends on the angiogenesis, its rate often fails to keep pace with tumor growth, as tumor vasculature is inadequate for the tumor mass. Therefore, many solid tumors have subpopulations of hypoxic cells. Studies showed that the hypoxic tumor cells were relatively resistant to certain cytotoxic drugs[16]. In the past, authors proposed that drug resistance be partly caused by poor tumor vascularization in reducing the influx of cytotoxic agents. Additionally, the hypoxic environment due to poor vascularization inhibited proliferation of tumor cells, yet noncycling cells would be expected to be less sensitive to many agents. In recent years, the identified biochemical mechanism of drug resistance was the overexpression of the transmembrane transport protein, P-glycoprotein (P-gp). P-gp is an ATP-binding-cassette transporter that is ubiquitously expressed, and often has high concentrations on plasma membrane of cancer cells, where it causes multidrug resistance by pumping lipophilic drugs out of the cell. The expression of P-gp influenced the efficacy of postoperative chemotherapy[17-24]. In our study, P-gp expression rate was 62.5 % which was similar to the result of another report on hepatocellular carcinoma[25]. Our result showed that overexpression of P-gp in gallbladder carcinoma tissue might be an important cause of drug resistance.

Recent studies showed that hypoxia-induced resistance to doxorubicin and methotrexate was attributed to an amplification of the P-gp gene and the dihydrofolate reductase gene[26-29]. Recently, it has also been shown that poor vascularization in lung carcinomas correlated with an up-regulation of drug-resistance enzymes, such as glutathione S-transferase-Ï, metallothionein and thymidylate synthase[30]. In another study on rectal cancer, poor angiogenesis was also linked to an expression of glutathione S-transferase and metallothionein[31]. Moreover, lung tumors with low vessel density and low VEGF expression have been found to be more frequently resistant to doxorubicin *in vitro* than tumors with high vessel counts and high expression of VEGF[32]. In our study, the value of MVC was markedly lower in P-gp (+) cases than in P-gp (-) cases. The positive rate of P-gp was significantly higher in cases with small MVC than in cases with big MVC.

In conclusion, the finding that poor vascularization links to overexpression of the most important multidrug resistance enzyme—P-gp provides us an additional insight into drug resistance in gallbladder carcinomas.

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