Clinical significance of mrp gene in primary hepatocellular carcinoma

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OBJECTIVE: To study the relations among the expression of the multidrug resistance associated-protein (mrp) gene and clinicopathologic features, the influence of α-fetoprotein (AFP), and prognosis of patients who received adjuvant chemotherapy after resection of primary hepatocellular carcinoma (HCC).

METHODS: The expression of the mrp gene encoding MRP and mRNAmrp was determined in tissues from 54 untreated patients with HCC, adjacent tissues from 24 patients with HCC and archival paraffin-embedded tissues from 12 patients with posthepatitic cirrhosis. The relationship between the mrp gene expression and the change level of AFP was analyzed in the 24 postoperative HCC patients whose AFP level was measured after 2 weeks. All of the HCC patients were followed up.

RESULTS: The percentage of positive expressions of MRP and mRNAmrp in the three kinds of tissues was 57.40%, 25.00%, 16.67%, and 72.22%, 37.50%, 33.33% respectively. Significant difference was noted in the untreated HCC tissue, compared to the other two tissues ($P<0.05$). No difference existed between the mrp gene expression and such clinicopathologic findings, as age, sex, and tumor size ($P>0.05$), but the expression was related to the degree of differentiation of HCC ($P<0.05$). The effective rate of AFP in the mrp gene positive expression group or postoperative chemotherapeutic patients was lower than that in the negative group ($P<0.05$). Although no difference was seen in the 1-, 3-, 5-year survival rates of HCC patients ($P>0.05$), the mean survival time of postoperative HCC patients or the negative mrp gene expression group was longer than that of the positive group ($P<0.05$).

CONCLUSIONS: Multidrug resistance (MDR) of HCC is related to mrp gene expression and initiates the intrinsic MDR. Detection of mrp gene expression is of great significance in accessing chemotherapeutic resistance of HCC, which provides evidence for reversing MDR in HCC. The mrp gene may be a useful marker in detecting prognosis of HCC patients because its expression is correlated with tumor differention and mean survival time of the patients.

(*HBPD Int 2003; 2: 397-403*)

Key words: hepatocellular carcinoma; gene; mrp; MRP; mrp RNA/messenger

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Introduction

Human hepatocellular cancer (HCC) is one of the most common malignancies worldwide.[1] The increased incidence of HCC during the past decade is considered the first cause of death among patients with cirrhosis and one of the ten most prevalent neoplasms worldwide.[2,3] Although advances in diagnosis and treatment have improved survival rates,[1,4] HCC recurs frequently even after curative surgical treatment, both within a short period and after several years.[5-8] The resection rate is about 20% in patients undergoing radical removal of hepatocellular carcinoma.[9]

Chemotherapy of HCC appears to be of importance, but the effective rate is about 20%. Drug resistance is one of the major factors explaining failure of chemotherapy in patients with cancer.[10] In the past years, the molecular mechanisms resulting in tumor cell resistance to a variety of chemotherapeutic drugs have been widely studied. Multidrug resistance (MDR) is a serious obstacle to cancer eradication by limiting the efficacy of chemotherapy. The most common MDR mechanism in cancer cells is the expression of an energy-dependent efflux pump (P-glycoprotein). Drug resistance is due to the overexpression of the human MDR[11-13] and probably to the multidrug associated protein (MRP).[14]

In this study immunohistochemistry S-P method and in situ polymerase chain reaction (IS-PCR) were used to detect the expression of the mrp gene in HCC. HCC patients were given postoperative chemotherapy to observe the relationship between α-fetoprotein (AFP) and mrp gene expression, the relationship between the mrp gene with some clinicopathologic features and the influence on AFP, and the correlation with the prognosis of HCC patients who received chemotherapy after operation. The aims of this study are to analyze the MRP gene expression and observe the relationship between the MRP gene expression and the effectiveness of chemotherapy in HCC.

Methods

Clinical data and sections

Archival paraffin-embedded HCC tissues were surgically removed from 54 patients (46 men and 8 women) aged from 21 to 72 years (median 44 years) who had not undergone preoperative chemotherapy, and adjacent tissues from 24 HCC patients, and biopsy tissues from 12 patients with posthepatitic cirrhosis. All patients were treated at our hospital from 1995 through 1997 and the specimens were routinely stained with haematoxylin-eosin (HE) for pathological examination. AFP was found positive in 38 patients and negative in the other 16; it was kept positive in 24 patients 2 weeks after the operation (Table 3). All of these patients were subjected to chemotherapy by a pump implanted subcutaneously, with a catheter being placed in the hepatic artery during the operation for those who had not undergone chemotherapy. Fourty-six of the 54 patients underwent local resection, and 8, cryotherapy during the operation (more than 2 neoplasms). Follow-up was carried out by revisit to our out-patient department or by telephone.

Main reagents

S-P immune test-kit and multidrug resistance associated protein (MRP)-antibody were purchased from Beijing Zhongshan Company, Beijing, China. dNTPs, RNasin, AMV reverse transcriptase, Taq enzyme and DNA enzyme were purchased from Sigma Company, USA. Protease K, 3-amino-9-ethylcarbozole (AEC) and diaminobenzidine (DAB) stains were purchased from Huamei Biological Products Company, China. Hybridization solution and magnesium chloride were produced by Beijing Biosynthesis Company, Beijing, China.

Project and synthesis of primers and probes

Following the principle of primer project, primers and probes were projected by PCGENE software according to one part of the mrp gene sequence which was cited from GeneBank, and was given isogeneic comparison. Mrp primer sequence 454–785 (331bp) included sense: 5'-AGCCA-GTGGAAGCAAGATA-3'; antisense: 5'-TTTGG-TATGAGGCTTGCTGTA-3'; probe: ATTTAGGT-GTATCTTGCTTCC.

There was no complementary pair-matching phenomenon between or among the above primers. The mrp gene length was 331bp. Primers and probes were produced by Shanghai Biosynthesis Co., Shanghai, China.

S-P immunohistochemical staining

SP-immunohistochemistry (SP-IHC) was performed according to the manufacture's instructions for SP kit. Sections were stained with DAB and restained with haematoxylin for visulization of nuclei. Paraffin sections of stomach carcinoma served as positive controls. For negative controls, primary antibodies were substituted by phosphate buffered saline (PBS) instead of MRP antibodies.

Detection of in situ reverse transcription polymerase chain reaction (ISRT-PCR)

Paraffin sections were treated by DEPC water, roasted for 4 hours under 55℃, deparaffinized and hydrated as usual.

Pretreatment: protease K was digested, endogenous peroxidase activity quenched with 3% hydrogen peroxide, hydrochloric acid acidized to block intergenic alkaline phosphatase and neutralize alkaline protein, and washed by PBS.

Removal of genome DNA: slide samples were air dried after gradient dehydrated by alcohol, treated by DNAase free RNAase, and stayed over at room temperature.

Reverse transcription reaction: 30 μl reverse transcription solution containing 6 μl of 1:5 buffer, 3 μl 3 μm/μl of each of sense and antisense specific primers (sense: 5'-AGCCAGTGGAAGCAAGATA-3', antisense: 5'-TTTGGTATGAGGCTTGCTGTA-3', designed by gene Fisher 1.3, produced by Shanghai Biosynthesis Co., Shanghai, China), 6 μl of 250 μm/μl dNTPs (sigma), 0.75 μl RNasin 1 u/μl and 3 μl of 1:10 reverse transcription enzyme AMV were dropwised into samples and put them into wet boxes for 1 hour. The gradient was dehydrated by alcohol.

In situ amplification: 60 μl of amplification solution was added containing 6 μl of 10 × PCR buffer, 15 μm magnesium chloride, 1.2 μl of 250 μm/μl dNTPs (sigma), 1 μm/μl of 0.6 μl the sense and antisense specific primers (sense: 5'-AGCCAGTGGAAGCAAGATA-3'; antisense: 5'-TTTGGTATGAGGCTTGCTGTA-3', with an expected 331bp product designed by Gene Fisher 1.3, produced by Shanghai Biosynthesis Co., Shanghai, China), 1.2 μl Taq polymerase 4 u/50 μl, 2.4 μl bovine serum albumin 3 mg/μl and 43 μl PBS dropwised in samples. The samples were put in a situ PCR system instrument degenerated at 94 ℃ for 5 min before circulation under the following conditions: 2 min at 94 ℃, 2 min at 55 ℃, 3 min at 72 ℃, repeated for 35 times, extended at 72 ℃ for 7 min and washed with PBS, then fixed by alcohol and air-dry.

In situ hybridization: the sections of the samples were put into PBS, water bathed 10 minutes under 80 ℃, ice bathed, and added mrp gene probe cross-liquid containing biotin label overnight in a bake oven at 52 ℃. The probe content of cross-liquid was 2.5 μg/ml.

Washing after crossing: the sections were placed into 2 × SSC – 50% formamide solution at 37 ℃ 15 min for 2 times, and 1 × SSC, 0.5 × SSC, and 0.2 × SSC in turn respectively 5 min for 2 times.

Detection after crossing: the samples were washed by PBS while adding S-A/HRP (SP-9002) antibody, second antibody and third antibody at 37 ℃ for 20 min. The samples were coloured under microscopy with AEC before being put on slide and covered.

Experiment control: negative control: A, reverse transcription enzyme control: adding nothing reverse transcription enzyme into reverse transcription amplification solution, and other steps were as same as routine operation; B, primer control: adding nothing primer into amplification reaction solution, and other steps were the same; C, Taq enzyme control: adding nothing Taq enzyme into amplification reaction solutions, and other steps were the same; D, probe control: adding nothing probe into hybridization solution; E, detective system control: adding nothing anti DTG-AP when detected; positive control: the same steps as immunohistochemistry.

Postoperative chemotherapy for HCC and mrp gene expression and evaluation of AFP validity after chemotherapy

According to the mrp gene expression, the 24 patients with positive AFP 2 weeks after operation were divided into 2 groups of mrp positive and negative and subjected to chemotherapy by the pump. The dose of postoperative chemotherapy consisted of 5-Fu 1000 mg, cisplatin (DDP) 40 mg and mutamycin (MMC) 20 mg. In a month for a course of treatment (2-3 courses on average), the level of AFP decreases to 50% or normal was considered valid.

Statistical analysis

Staining brown in cell membrane and/or cytoplasm under a light microscope by SP-immunohistochemistry (HIC) was defined positive, which was the same as staining red granule in cytoplasm by IS-PCR. Double-blind method was used to count staining cells by two pathologists to observe ten high visual fields. The intensity and proportion of the cells stained were assessed: 1) no staining cells defined as negative; 2) weak positive staining and positive cells less than 10%; 3) moderate positive staining twice and positive cells from 10% to 29%; 4) intense positive staining thrice and positive cells from 30% to 100%.

Categorical variables were analyzed with the chi-square contingency test and exact probability test. A P value less than 0.05 was considered significant.

Results

Location and distribution of mrp gene expression

MRP expression was located in the cell membrane and/or cytoplasm of HCC and normal liver cells stained in brown dots, but not in bile canaliculus (Fig. 1). mRNAmrp expression was located mainly in the cytoplasm of HCC and normal liver cells like intense red parcels distributed in sheets (Fig. 2). The mrp gene expression positive rate was significantly higher in HCC than in adjacent HCC liver tissue cells and hepatic cirrhosis tissue cells ($P < 0.05$, Table 1).

The mrp gene expression and clinicopathologic features of HCC

The mrp gene expression was related to the

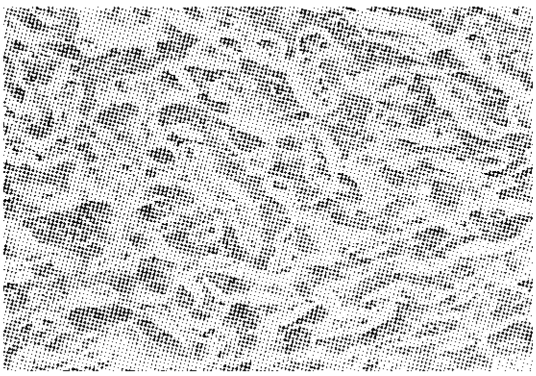

Fig. 1. Immunohistochemical staining of MRP in HCC. The cell membrane and cytoplasm were moderately-positive stained (++) into brown by DAB and the nuclei slightly stained by haematoxylin (original magnification ×200).

Fig. 2. In situ PCR, the positive strong expression of mRNAmrp in HCC was stained in the tumor cell cytoplasm (+++) which was shown to be red granule by AEC and the nuclei were slightly stained by haematoxylin (original magnification ×200).

Table 1. The expression of mrp gene in HCC tissue, adjacent liver tissue of HCC and cirrhosis tissue

Tissue type	Number	MRP				Positive rate (%)	$P<$	mRNAmrp				Positive rate (%)	$P<$
		-	+	++	+++			-	+	++	+++		
Adjacent tissue of HCC	24	18	4	2	0	25.00	0.05	15	5	3	1	37.50	0.05
HCC	54	23	24	6	1	57.41	0.05	15	5	12	22	72.22	0.05
Cirrhosis	12	10	1	1	0	16.67	0.05	8	1	1	2	33.33	0.05

Table 2. The relationship between the mrp gene expression and clinicopathologic features in HCC patients

Clinical data	Number	MRP				Positive rate (%)	*P* value	mRNAmrp				Positive rate (%)	*P* value
		−	+	++	+++			−	+	++	+++		
Sex													
Male	46	19	22	4	1	58.70	>0.05	13	2	10	21	71.74	>0.05
Female	8	4	2	2	0	50.00		2	3	2	1	75.00	
Age (year)													
>40	30	13	13	4	0	56.67	>0.05	8	1	8	13	73.33	>0.05
≤40	24	10	11	2	1	58.33		7	4	4	9	70.83	
HBsAg													
Positive	38	16	16	5	1	57.89	>0.05	11	3	10	14	71.05	>0.05
Negative	16	7	8	1	0	56.25		4	2	2	8	75.00	
AFP													
Positive	38	17	17	3	1	55.26	>0.05	11	4	9	14	69.23	>0.05
Negative	16	6	7	3	0	62.50		4	1	3	8	75.00	
Tumor location													
Left liver	28	12	14	1	1	57.14	>0.05	8	2	7	11	71.43	>0.05
Right liver	18	8	8	2	0	55.56	>0.05	5	2	3	8	72.22	>0.05
Left and right liver	8	3	2	3	0	62.50		2	1	2	3	75.00	
Tumor diameter (cm)													
>5	20	8	10	1	1	60.00	>0.05	6	2	5	7	70.00	>0.05
≤5	34	15	14	5	0	55.88		9	3	7	15	73.53	
Number													
One tumor	46	20	22	3	1	56.32	>0.05	13	4	11	18	71.74	>0.05
Multiple tumor	8	3	2	3	0	62.50		2	1	1	4	75.00	
Growth style													
No infiltration	30	13	13	4	0	56.67	>0.05	8	3	6	13	73.33	>0.05
Infiltration	24	10	11	2	1	58.33		7	2	6	9	70.83	
Cell differentiation (Edmondson)													
Ⅰ~Ⅱ	28	16	11	1	0	42.86	<0.05	12	2	4	10	57.14	<0.05
Ⅲ~Ⅳ	26	7	13	5	1	73.08		3	3	8	12	88.46	

Table 3. The relationship between the mrp gene expression and AFP in postoperative chemotherapeutic HCC patients

Mrp gene expression	Number	AFP curative effective (%)	AFP curative ineffective (%)	*P* <
MRP				
Positive	14	3(21.43)	11(78.57)	0.05
Negative	10	8(80.00)	2(20.00)	0.05
mRNAmrp				
Positive	17	4(23.53)	13(76.47)	0.05
Negative	7	6(85.71)	1(14.29)	0.05

The expression of AFP was positive 2 weeks postoperation in 24 HCC patients who received chemotherapy with a pump implanted subcutaneously with a catheter placed in the hepatic artery, on average 2–3 courses, once a month.

Table 4. The relationship between the expression of mrp gene and postoperative survival time in HCC patients (mon)

Tissue type	Number	Survival time (mean ± SD)	*P* <
mRNAmrp			
Positive	39	24.79 ± 9.72	0.05
Negative	15	72.35 ± 11.76	
MRP			
Positive	31	22.03 ± 9.81	0.05
Negative	23	59.52 ± 10.93	

grade of HCC ($P < 0.05$), but not to patient age, tumor size or tumor growth style ($P > 0.05$, Table 2).

Table 5. The relationship between the mrp gene expression and the 1-, 3-, 5-year survival rates in postoperative HCC patients

Mrp gene expression	Number	The number of survival rates (%) and *P* value					
		1-	*P* >	3-	*P* >	5-	*P* >
MRP							
Positive	31	23(74.19)	0.05	19(61.30)	0.05	9(29.03)	0.05
Negative	23	18(78.26)		15(65.22)		8(34.78)	
mRNAmrp							
Positive	39	29(74.36)	0.05	24(61.54)	0.05	12(30.79)	0.05
Negative	15	12(80.00)		10(66.67)		5(33.33)	

The mrp gene expression and AFP level after postoperative chemotherapy

Twenty-four HCC patients showed AFP positive 2 weeks after the operation (Table 3). They received chemotherapy through a pump being implanted subcutaneously with a catheter placed in the hepatic artery during the operation. The effective rate of AFP in the positive mrp gene expression group was obviously lower than in the negative group ($P < 0.05$).

The mrp gene expression and postoperative survival time and 1-, 3-, 5-year survival rates of HCC patients

The average survival time of the mrp gene expression positive group was shorter than that of the negative group ($P < 0.05$, Table 4). However, the 1-, 3-, 5-year survival rates between the two groups were not significantly different ($P > 0.05$, Table 5).

Discussion

At present, chemotherapy is one of the major treatments for HCC. One reason of chemotherapy handicap is that the tumor cells are resistant to many chemodrugs. The multidrug resistance associated-protein gene mrp was found to media tumor cells drug resistance by transporting GS. X compound to participate in vehicle translocation.[15]

The mrp gene is widely expressed in tumor tissues.[16] In this study, it was expressed to some extent in HCC tissues undergoing preoperative chemotherapy, adjacent tissues and biopsy tissues posthepatitic cirrhosis. The mrp gene expression was significantly higher in HCC than in the other two tissues ($P < 0.05$, Table 1). It is clearly indicated that HCC multidrug resistance (MDR) is correlated with mrp gene expression and HCC MDR is endogenous. By analyzing the mrp gene expression in patients receiving postoperative chemotherapy and the effective rate of AFP, we found the rate is significantly lower in the mrp gene expression positive group than in the negative group ($P < 0.05$). These results showed that the mrp gene could direct the chemotherapy of HCC patients. Detecting mrp gene expression in HCC patients may help to evaluate drug resistance, fault chemotherapy, and possible curative effect.

In this study, we found that the degree of mrp gene expression in HCC tissues is not related to some clinicopathologic features such as age, tumor size and growth style. However, mrp gene expression is enhanced after the differentiation of tumor cells ($P < 0.05$), showing that the mrp gene is related to some biological features of HCC.

Debates exist on whether mrp serves as a prognosis indicator of tumor patient.[17,18] Norris et al[17] found MRP could be a useful prognosis indicator of neuroma patients. But Shima et al[18] found it could not be used as a prognotic indicator for patients with carcinoma of colon metastasized to the liver. In the HCC patients of our group, though the 1-, 3-, 5-year survival rates were not significantly different between the positive and negative mrp gene expression groups ($P > 0.05$), the average survival time of the negative mrp gene expression group was longer than that of the positive group ($P < 0.05$). The positive rate of mrp gene expression was increased as the degree of HCC differentiation was decreased ($P<0.05$). The fact shows that poor differentiation, malignant and worse prognosis are consistent with chemotherapeutical drug resistance of HCC. Hence mrp gene is expected to be an indicator of clinical prognosis.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Received November 29, 2002
Accepted after revision May 19, 2003