

Original Article

Relationship between the expression of MDR1 in hepatocellular cancer and its biological behaviors

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Abstract: Objective: By the detection of HBV infection, AFP and AST, the targets of biological behavior and the gene expression of multi-drug resistance gene 1 (MDR1) in hepatocellular carcinoma (HCC), we investigate characteristics of the expression of MDR1 in HCC and its relationship with HCC biological behavior. Methods: Using real-time fluorescence quantitative PCR (FQ-PCR) to detect the expressions of MDR1 in 102 samples of HCC tissue and 20 samples of non-cancerous tissue, we analyze the relationship between expressions of MDR1 and biological characteristics of HCC. Results: The expression of MDR1 in HCC is 0.55 ± 0.27 , and in normal liver tissues is 0.23 ± 0.10 , respectively. The expression in HCC is higher than it in normal liver tissue, the difference is statistically significant ($P<0.05$) and the difference between the expression and the HCC envelopes is statistically significant, and the expression increases along with the increase of Edmondson classification ($P<0.05$). HBV infection, AFP positive, the rise of AST, all these factors have positive correlations with the expression ($r=0.463, 0.473, 0.299$). In MDR1 expressions of HCC patients, the survival curve of the negative is higher than that of the positive, but the difference is not statistically significant. Conclusion: There are drug resistance phenomena in HCC, MDR1 expression may play an important role in primary HCC drug resistance. HBV infection can be detected as a reference indicator of HCC chemotherapy resistance, plasma levels of AFP, AST can be used as a reference index change dynamic monitoring of MDR1 expression.

Keywords: MDR1 gene, hepatocellular carcinoma, HBV infection, AFP, expression

Introduction

Liver cancer is one of the major cancer threats to human life, the mortality and morbidity rank third in malignancies. At present, the main treatment for liver cancer is surgery, chemotherapy, radiation therapy, biological therapy and cryotherapy and other methods. As an important means of comprehensive treatment of liver cancer, chemotherapy catches more and more attention, but the emergence of multi-drug resistance has greatly reduced the efficacy of chemotherapy, causing the failure of chemotherapy. Liver cancer multi-drug resistance phenomenon is one of the main reasons to reduce the chemotherapy efficacy [1]. MDR's current research focuses on the transcriptional

regulation of MDR genes. HBV, alpha-fetoprotein (AFP) is major biological indicators of liver cancer. HBV infection and liver cancer occurrence and development are closely related, and the AFP is recognized liver cancer diagnosis and screening indicators for liver cancer is relatively specific tumor markers, which predict changes in liver cancer chemotherapy and prognosis has important reference value [2]. Therefore, a clear HBV infection, the relationship between drug-resistant tumor biological behavior AFP, AST and other indicators arising from changes in liver cancer multi-drug for clinical understanding of drug resistance, in order to develop a scientific and effective "individualized". There is certain chemotherapy guiding significance.

Relevant indicators and expression of MDR1 in HCC

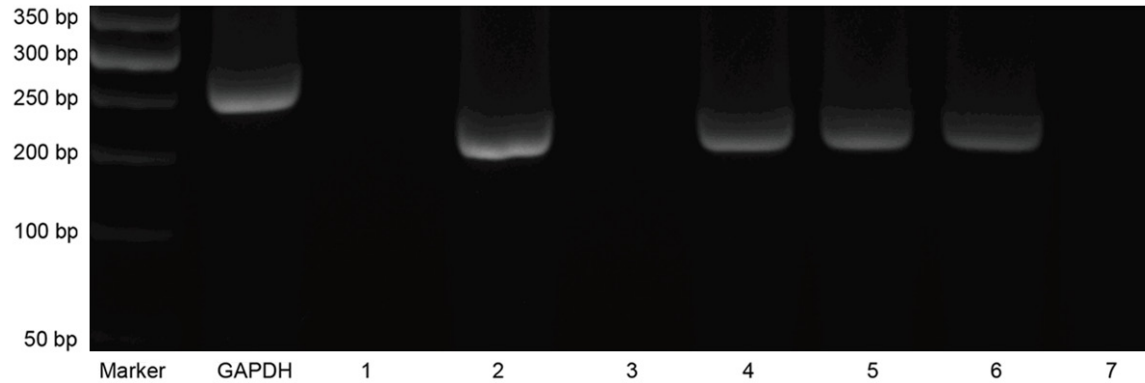


Figure 1. Expression of mRNA for MDR1 and GAPDH in HCC tissues and non-cancerous tissue(1: GAPDH; 1~6: HCC tissues; 7: non-cancerous tissue).

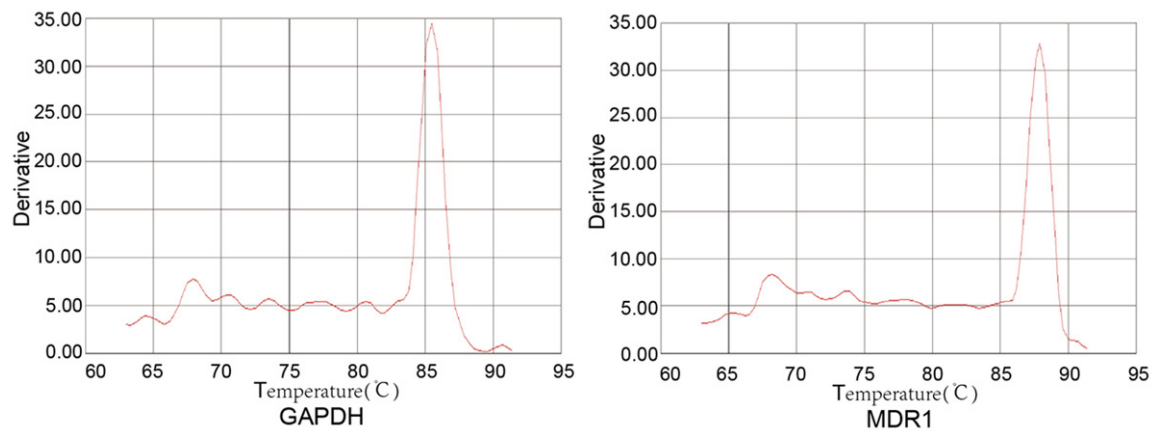


Figure 2. The melting curve of MDR1, GAPDH.

Materials and methods

Patients and specimens

Collection of Hubei University of Medicine Affiliated Taihe Hospital in September 2009-August 2010 fresh specimens of 102 cases of hepatocellular carcinoma, and 20 cases of normal liver tissues (>5 cm distance to the resection margin), were confirmed by pathology. Male 70, female 32; aged 45 to 67 years, mean age was 52.6 years; press Edmondson taxonomy classification: Class I 34 cases, II grade 26 cases, grade III 30, grade IV 12 cases; 48 cases of patients with portal vein thrombosis in patients with radiologic examination or surgical procedure described in presence of tumor thrombus found prevail; 16 cases enveloped. All patients had not received chemotherapy and radiation therapy. And collect patient heparin anticoagulant 5 ml, after

centrifugation retains plasma, all specimens collected in liquid nitrogen and stored at. All patients with complete clinical data, patients were followed up from 6 to 36 months.

RNA extraction and cDNA synthesis

Total RNA was also extracted using TRIzol® Reagent (InterGen Discovery Products, Purchase, NY, USA) according to the manufacture's protocol. RNA concentration was estimated by spectrophotometric method (BioRad Smart-SpecTMPlus Spectrophotometer, CA, USA). The concentration and purity of total RNA was determined by measuring the absorption with NanoDrop-2000 (Thermo Fisher) at 260 nm and 280 nm. First-strand cDNA was prepared from total RNA using Promega reverse transcription system (Promega, WI, USA) based on the manufacturer's instructions. cDNA was used immediately or stored at -80°C until use.

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Table 1. mRNA expression of MDR1 in different clinical pathological data in HCC patients

	N	MDR1	t	P
Age				
≥50 y	80	0.57±0.28	0.68	0.51
<50 y	22	0.50±0.28		
Size (cm)				
≥5 cm	40	0.50±0.27	-1.15	0.25
<5 cm	62	0.59±0.27		
Edmondson				
I~II	60	0.48±0.27	-2.20	0.03
III~IV	42	0.65±0.25		
Envelope				
Yes	32	0.44±0.27	-2.05	0.04
No	70	0.60±0.26		
Embolus				
Yes	48	0.48±0.27	-1.81	0.07
No	54	0.62±0.27		

Table 2. MDR1 gene expression with HBV infection, the relationship between the levels of AFP

Indicator	N	MDR1 Expression	t	P
HBSAg				
+	84	0.62±0.24	4.60	<0.001
-	18	0.23±0.17		
AFP				
+	66	0.63±0.22	2.84	0.006
-	36	0.42±0.31		
AST				
Normal	48	0.45±0.28	2.78	0.008
Rising	54	0.65±0.24		

Real-time PCR detects mRNA expression of MDR1 and GAPDH

The primers were used for the polymerase chain reactions (PCR) as follows: 5'-CCC ATC ATT GCA ATA GCA GG-3' (forward), 5'-TGT TCA AAC TTC TGC TCC TGA-3' (reverse) of the MDR1 gene [3]; and 5'-GAA GGT GAA GGT CGG AGT C-3' (forward), 5'-GAA GAT GGT GAT GGG ATT TC-3' (reverse) of the GAPDH gene. The MDR1, GAPDH primers yielded products of 158 bp, 226 bp, respectively.

The PCR reactions were performed using the SYBR Green PCR Core Reagents kit (Takara Bio Inc, Shiga, Japan). Real-time detection of the emission intensity of SYBR Green bound to

double-stranded DNAs was performed using the ABI 7500 Sequence Detection System (ABI Biosystems). The quantitative PCR reactions were performed in triplicate for each sample-primer set, and the mean of the three experiments was used as the relative quantification value. To accurately determine the starting copy number regardless of the precise amount and qualities of input DNA, we also quantified internal control genes (GAPDH) in each single reaction and normalized the target genes to them. The ABI 7500 sequence detecting system software automatically determines the Ct value and refers the starting copy numbers in each sample. The PCR reaction mixture included 1 µL of cDNA, 10× Buffer 3 µL, 2 mmol/L dNTP 1.5 µL, 2 µL each of 5' and 3' primer, 5 U/µL Taq DNA polymerase 0.3 µL (Sangon Biotech, Shanghai, China), and 10× SYBR-Green I 1.0 µL (Gene). For diminishing noise signal of primer dimer, the thermal profiles were modified according to Ball's method by altering the PCR strategy to take advantage of the ABI 7500's ability to measure fluorescence at a temperature greater than the melting point of primer dimers. The resulting measurements determine fluorescence of only the desired PCR product and can eliminate the fluorescence induced by primer dimers and obtain accurate product quantitation.

A total of 40 cycles were used; at the end point of 40 PCR cycles, dissociation curve analysis was performed, the reaction products were separated electrophoretically on a 2% agarose gel and stained with ethidium bromide for further confirmation of the PCR products. The level of mRNA for the MDR1 was expressed as a ratio relative to the GAPDH mRNA in each sample. The levels of mRNA for each MDR1 in cancerous was compared with the mean level in the corresponding non-cancerous tissue.

Detection of markers associated with HBV infection (HBSAg), AFP, AST in blood

HBSAg detection using enzyme-linked immunosorbent assay (ELISA) principle, in accordance with the kit (Shanghai KHB) instructions; AFP were measured to >20 µg/L as positive with COBAS 6000 (Roche); AST using automatic biochemical analyzer (AU2700, Beckman) were measured to 0~40 U/L as normal, >40 U/L is increased.

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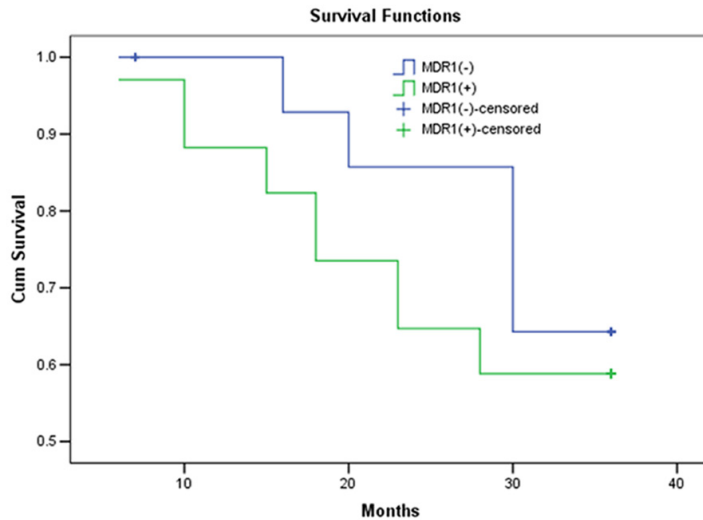


Figure 3. Survival curves of MDR1 mRNA-positive and negative patients.

Statistical analysis

Results are presented as the means \pm SD. The two-sided, two independent sample *t*-test was used throughout to assess the statistical significance of differences between two sets of data. All count data using χ^2 test, the closeness of relationships between variables using Spearman correlation analysis was used to compare survival curves data Log-rank method to bilateral $P < 0.05$ indicates a statistically significant difference. Difference was considered as significant if the probability was less than 0.05 ($P < 0.05$).

Results

mRNA expression of MDR1 in all samples

Following quantitative PCR, examination of the agarose gels confirmed that specific products of approximately 158 bp, 226 bp were obtained upon amplification of MDR1, GAPDH in **Figure 1**. And the melting curve of MDR1, GAPDH were shown in **Figure 2**.

MDR1 in normal tissues is low or no expression status, expression was 20% (8/40), in HCC high expression status, expression was 68.6% (70/102), the difference was statistically significance ($\chi^2=8.28$, $P < 0.05$). MDR1 mRNA in cancer tissue gene expression relative quantitative value of 0.55 ± 0.27 , normal liver tissue gene expression relative quantification were 0.23 ± 0.10 , the expression of resistance genes in cancer tissue compared with normal control

liver tissue, the difference is statistically significance ($t=6.38$, $P < 0.01$).

mRNA expression of MDR1 in different clinical pathological data of HCC

Statistically, the difference between whether the envelope MDR1 mRNA expression in hepatocellular carcinoma was statistically significant, and its expression with increasing grade and increased Edmondson, but not with tumor size, age, with portal vein thrombosis nothing to do. Relationship with clinicopathological features of MDR1 expression shown in **Table 1**.

MDR1 gene expression relationship with HBV infection, AFP levels, AST levels

According to HBV infection, as measured AFP levels, AST values of all cancer tissue specimens were divided into six groups, namely HBSAg (+), HBSAg (-), AFP (+), AFP (-), AST (normal), AST (rise). HBSAg positive group and the expression levels of AFP-positive group and the group AST increased MDR1 gene is significantly higher than the negative group. The relationship between the four is shown in **Table 2**. MDR1 expression HBSAg (+), AFP (+) AST elevated group and the positive rate was respectively 94.3%, 80.0%, 57.1%, MDR1 expression with HBV infection, AFP level was between AST levels were significantly positively correlated ($r=0.463$, 0.473 , 0.299 , $P=0.001$, 0.000 , 0.033).

MDR1 mRNA expression of positive and negative groups survival curves in comparison

The average survival time of positive group was 28.4 months, 95% confidence interval, 25.0 to 31.8 months, with an average survival time of negative group was 32.1 months, 95% CI bit 28.8~35.4 months, MDR1 expression negative group survival curve is higher than the positive group, but the difference was not statistically significant ($P > 0.05$), shown in **Figure 3**.

Discussion

MDR is a tumor cell resistant to a different structure, function and effect of the drug killing

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mechanisms [4]. HCC important reason is the failure of chemotherapy on tumor cells with one or more chemotherapy drugs produce resistance. MDR1 gene encoding P-gp glycoprotein, it is a pharmaceutical carrier can be formed ATP dependent membrane pumps in the cell membrane, and upon binding of anti-cancer drugs can be combined again with the ATP site on the nucleotide by intracellular ATP can provide concentration gradient anticancer drugs transported out of the cell, the effective intracellular concentration of the drug decreased leading to drug resistance. The current study suggests that P-gp is a sign of MDR, the MDR-mediated resistance to classical pathway [5], MDR1 over-expression in renal cell carcinoma [6], ovarian cancer cell lines [7], liver cancer [8] may lead to resistant to chemotherapy drugs.

Since the expression of tumor cells to tolerate intensive chemotherapy with the drug resistance gene in cells related, it can indirectly reflect tumor cells to chemotherapeutic drug resistance by detecting the expression of resistance genes. This study used a fluorescent dye SYBR-Green I binding expression by RT-PCR to detect resistance genes in normal liver tissue and cancer tissue, through a combination of dyes and purpose of the PCR products, real-time monitoring of fluorescence intensity and improve the detection sensitivity, and thus gene expression be well quantified and more accurate determination of its expression in vivo. In this study, the expression of MDR1 gene in HCC tissue compared with normal liver tissue, which is consistent research with Zhong et al. [9]. And before all patients who had not received chemotherapy drawn, indicating a high resistance gene MDR1 gene expression may play an important role in the formation of HCC's inherent resistance to the process, which may also be the beginning of chemotherapy, most patients with liver cancer a lack of sensitivity to chemotherapy one of the reasons. MDR1 expression and liver Edmondson grade also has some relevance, and increased with the grade and higher, which is clinically significant low grade match against HCC tumor treatment. Probably because of the high expression of MDR1 gene, making the encoded glycoprotein P-gp in combination with chemotherapy drugs, by the ATP-dependent membrane pump chemotherapy drugs transported to the extracellular leaving intracellular drug effectively

reduce the concentration of lead chemotherapy failed.

The experimental detection of different HCC resistance gene MDR1 gene expression by FQ-PCR, the patient may express resistance genes in the body for more accurate quantitative analysis. Since the MDR1 gene in normal tissues is relatively low expression state, and when there will be resistance to liver cells express relatively high, then we pre-treatment of HCC patients or treatment can be detected by the machine body MDR1 gene expression, and thus the resistance genes more accurate quantitative analysis, so not only can understand the reasons for resistance to be targeted clinical formulation of effective chemotherapy regimen, try to avoid using the drug resistant, timely Change chemotherapy, thereby obtaining a more satisfactory therapeutic effect.

HCC as viral hepatitis "trilogy", the final step, HBV infection is one of the main reasons. In this study, 51 cases of liver cancer patients with HBV infection rate was 82.4% (42/51), the expression of MDR1 gene of HBV infection was significantly higher than HBV-negative patients, HBV and liver cancer drug showed that there is some connection. And is not all patients had chemotherapy, but the incidence of HBV infection in the liver requires a lengthy process, but also shows HBV infection in patients with hepatocellular carcinoma, the body's resistance should give more consideration to the possibility of the existence of primary resistance. These results were consistent with You J [10] that the hepatitis B virus infection can induce multidrug resistant hepatocellular carcinoma. Doong SL et al. [11] found that HBx protein is able to trans-activate the MDR1, leading to tumor cell resistance. Guan J et al. [12] that can upregulate the expression of HBx gene MDR through ERK/MAPK pathway, leading to liver tumor cells resistant. But in the end is the activity of MAPK pathway which factors affected thereby changing the expression of MDR1 gene and whether to change a variety of MDR genes (such as MRP, LRP, TOP-II, GST- π) expression leading to MDR produce also requires us to do further study. However, detection of HBV infected liver cancer chemotherapy drug can be used as a reference in particular is one of the primary indicators of drug resistance. AFP, AST commonly used to check for liver cancer indicators for the diagnosis and prognosis of liver cancer

have a certain reference value. This study found that the expression of AFP, AST increased with the resistance gene MDR1 positive correlation and these findings indicate plasma using AFP, AST levels are relatively high expression of MDR1 gene risk factors. And MDR1 gene mediated resistance mechanisms of resistance to the classic, so we can dynamically monitored by AFP MDR1 gene expression in vivo, the levels of AST indirectly reflects the machine, so as to achieve the purpose of changing the dynamic monitoring of drug resistance, and more conducive to clinical development of "individualized" chemotherapy, to improve the effect of chemotherapy, improve patient quality of life is important.

Studies have shown that, MDR1 gene expression-positive breast cancer, colon cancer patients than generating negative expression of disease-free time is shorter [13, 14], but in this study the expression of MDR1-negative group, although the survival curve is higher than the positive group, but the difference was not statistically significant. This may be related to our follow-up time is short, small study sample size has a certain relationship, and thus insufficient to show the difference between the two.

In summary, HBV infection induced expression of MDR1 gene may be an important mechanism leading primary liver cancer drug, for the HCC patients infected with HBV, we should take full account of their primary drug resistance exist, thus chemotherapy for patients to develop a reasonable choice and chemotherapy. AFP levels, changes in the peripheral blood plasma AST can be used as a reference index dynamic monitoring of MDR1 expression.

Disclosure of conflict of interest

None.

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