

Nested polymerase chain reaction technique for the detection of *Gpc3* and *Afp* mRNA in liver cancer micrometastases

J. Luo¹, K. Yang¹ and Y.G. Wen²

¹Department of Hepatobiliary and Intestinal Surgery, Hunan Cancer Hospital, The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha, Hunan, China

²The Second Department of General Surgery, Third Xiangya Hospital, Xiangya School of Medicine, Central South University, Changsha, Hunan, China

Corresponding author: Y.G. Wen
E-mail: yangangwen221@sina.com

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ABSTRACT. The incidence of liver cancer has gradually risen to a high level in China, and tumor metastasis occurs via multiple pathways. Alpha fetal protein (AFP) is the main biomarker of liver cancer micrometastases. A recent study showed that glypican-3 (GPC3), which is abundant in hepatoma cells, has promising specificity and could be used to determine the presence of malignant cells. The nested polymerase chain reaction (PCR) technique is superior in experimental sensitivity. Using rat models of liver cancer in the current study, we utilized nested PCR to detect *Gpc3* and *Afp* mRNA to determine their relationship with liver cancer micrometastases. The aim was to provide an experimental basis for clinical examination. We randomly assigned male Sprague-Dawley rats to sham and experimental groups. The experimental group constituted a liver cancer model induced by diethylnitrosamine, whereas

the sham group was administered with an equivalent volume of normal saline. *Gpc3* and *Afp* mRNA was detected using nested PCR. Analysis was performed to determine statistical significance. Compared with the sham group, the rates of occurrence of *Gpc3* and *Afp* mRNA were significantly higher in the experimental group ($P < 0.05$). Compared with the total positive ratio of hepatoma cells examined by joint detection, the rates of occurrence of *Gpc3* and *Afp* mRNA increased significantly in the four subgroups of the experimental group ($P < 0.05$). The use of nested PCR significantly improved sensitivity for the detection of *Gpc3* and *Afp* mRNA in liver cancer micrometastases.

Key words: Nested PCR; Liver cancer micrometastases; *GPC3* mRNA; *AFP* mRNA

INTRODUCTION

The incidence of liver cancer in China has gradually increased. The rate of liver cancer recurrence is still high despite various procedures and therapies (Lemoine et al., 1997). Research has shown that the 5-year recurrence rate has increased to 50%, and the 5-year survival rate after liver transplantation is only 24-32% (Louha et al., 1999). The biological characteristics of liver cancer are uncontrolled cell proliferation, abnormal cell differentiation, and high invasive capability. Micrometastasis mainly refers to the diffusion of non-blood system malignancies via the blood or lymphatic systems. Liver cancer micrometastases are characterized by tiny lesions, small cell volume, and a lack of specific features, and they are difficult to diagnose by routine clinical examination (Wong et al., 1997). The nested polymerase chain reaction (PCR) technique shows promising sensitivity for the detection of malignant tumor cells (Mou et al., 2002). The mRNAs of alpha fetal protein (AFP) and albumin are the main biomarkers of liver cancer micrometastases, but AFP and albumin are also expressed in patients with viral hepatitis, and there is controversy about their suitability for the diagnosis of liver cancer (Yvamoto et al., 2015). Research indicates that gene expression in metastases differs from that in primary liver cancer lesions, and single tumor markers are prone to false-negative results. Glypican-3 (GPC3) exhibits a high level of expression in hepatoma cells and has promising specificity because it is not expressed in liver cells or liver inflammatory cells. Accordingly, GPC3 has been used as a novel tumor marker (Chen et al., 2000; Yu et al., 2015). We hypothesized that nested PCR could further improve sensitivity to liver cancer micrometastases when used to detect *GPC3* and *AFP* mRNA (Chu et al., 2011).

Using rat models of liver cancer in the current study, we utilized nested PCR to detect *Gpc3* and *Afp* mRNA to determine their relationship with liver cancer micrometastases. The aim was to provide an experimental basis for clinical examination.

MATERIAL AND METHODS

Experimental animals

Clean male Sprague-Dawley rats (N = 120), weighing 210-280 g, were provided by the Central South University [accession number: SCXK (ning) 2015-0006]. The rats were raised in separate cages maintained at 20°-25°C and 50-60% relative humidity. The rats were used

for all experiments; all procedures adhered to the guidelines provided by the Animal Ethics Infolink and were approved by the Animal Ethics Committee of Hunan Cancer Hospital.

Reagents and equipment

Diethylnitrosamine was purchased from Nanjing Haisen Chemical Limited (Nanjing, China), phosphate-buffered saline (PBS) was from Beijing Zhongshan Biotechnological Company (Beijing), TriPure Isolation Reagent was from Sigma-Aldrich (St. Louis, MO, USA), PCR primer from Nanjing Plant Industry Company, ultracentrifuge (BIOSOURCE, USA), gel imaging system (Shanghai Shida Biotechnological Company), Ficoll-Hypaque solution (Sigma-Aldrich), Eppendorf tubes (Brilliant, USA), real-time fluorescent quantitative PCR apparatus (Shanghai Lisi Biotechnological Company), and a transmission electron microscope (Nanjing Kangrui Biotechnological Company).

Experimental model and grouping

The 120 rats were randomly assigned to an experimental group (100) and a sham (control) group (20). A rat liver cancer model was established in the experimental group as follows (Kim et al., 2016): 0.9 mL 2% diethylnitrosamine was administered by oral intubation for 5 days every week from Week 1 to Week 12, and for 3 days every week from Week 13 to Week 20. The rats in the sham group were injected with an equivalent dosage of normal saline.

According to the number of liver cancer nodules detected, the experimental group was divided into four subgroups: a single-nodule subgroup, a 2-5-nodule subgroup, a ≥ 5 -nodule subgroup, and a distant metastasis subgroup.

Index detection

Sample collection

Sample collection began 1 day after the final gavage at Weeks 10 to 12; at Weeks 12, 14, 16, 18, and 20, the peripheral venous blood of the experimental rats was collected, and heparin was used to prevent coagulation. The ratio of the whole blood to heparin (125 U/mL) was 10:1. The entire sampling process was conducted under aseptic conditions.

Peripheral blood mononuclear cell separation and RNA extraction

Mononuclear cells were separated using Ficoll-Hypaque solution. Peripheral blood diluted with heparin solution was added to the Ficoll-Hypaque solution and centrifuged at 3000 rpm at room temperature for 30 min. The white cell membrane interface layer (which can contain cancer cells) was then aspirated using a capillary pipette. The cells were then washed twice with PBS and centrifuged at 3000 rpm at room temperature for 30 min. After removing the supernatant, the mononuclear cells remained.

The total RNA of the mononuclear cells was extracted according to the TriPure Isolation Reagent manufacturer instructions (De Luca et al., 2000). The extracted mononuclear cells were added to TriPure Isolation Reagent and the cells were lysed at room temperature. They were then transferred to Eppendorf tubes, incubated for 10 min at 25°C, and 0.2 mL chloroform

was added. The cells were further incubated for 10 min at room temperature after agitation for 30 s. The cells were then centrifuged for 15 min at 1000 rpm and the upper colorless solution was transferred to Eppendorf tubes. Finally, isopropanol (0.5 mL) was added, the solution was agitated again for 30 s and incubated at 25°C for 10 min, and RNA was precipitated.

***Afp* and *Gpc3* mRNA semi-nested PCR detection in peripheral blood**

cDNA synthesis of the first chain took place using a reaction mixture comprising: 1 µL dithiothreitol, 4 µL 5X buffer, 200 U (1 µL) Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 18-20 (0.45 µL) RNase, 4 µL (2.5 mM) dNTPs, 0.2 mg (0.4 µL) random primer, and approximately 4 µL total RNA sample or 8 mg mRNA made up to a total volume of 20 µL. The mixture was reacted for 60 min at 25°C, and the reverse transcriptase was inactivated for 5 min at 95°C (Miyamoto et al., 2000).

The PCR amplification mixture comprised: 2.4 µL (2.5 mM) dNTPs, the reverse transcripts mentioned above (2 µL), 3 µL 10X PCR buffer, 20 pmol = 0.8 µL (25 M) forward primer, 20 pmol = 0.8 µL (2 M) reverse primer, 0.5 U Taq enzyme, and 20.8 µL diethylpyrocarbonate (DEPC). Mineral oil (30 µL) was added followed by mixing. The reaction mixture was then subjected to 30 cycles of denaturing for 40 s at 94°C, annealing for 4 s at 54°C, and elongation for 45 s at 72°C.

Electrophoresis was carried out on 2% agarose gel at 100 V for 15-20 min. The bromophenol blue marker ran to 2-3 cm, and the results were imaged using an ultraviolet lamp. The *Afp* and *Gpc3* PCR primers are shown in Table 1.

Table 1. *Afp* and *Gpc3* mRNA polymerase chain reaction primers.

Gene	Sequence	Length (bp)
<i>Afp</i> mRNA	Forward 5'-GCGTTGGCTGCAATGAAG-3'	228
	Reverse 5'-CTGGAAAGTGGAAAGGGTG-3'	
<i>Gpc3</i> mRNA	Forward 5'-GCAAGGCTGTATGGCAGGTG-3'	776
	Reverse 5'-GCTIGTTCCTGGAGCATCG-3'	
β-actin	Forward 5'-CAAGGGCCAGGTCACCAA-3'	450
	Reverse 5'-CCCCAACCCATCTTCGTA-3'	

Statistical analysis

The results of this study were summarized and analyzed using the SPSS 17.0 software. The *t*-test was used for measured data and the chi-square test was used for enumerated data. A *P* value <0.05 was considered statistically significant.

RESULTS

Rat models of induced liver cancer

The rats in the experimental group were treated with diethylnitrosamine to establish an induced liver cancer model; 92 rats were successful rat models. Therefore, the success rate of the induced liver cancer model was 92% and the model mortality was 8%. The detailed data for the four subgroups were as follow: there were 26 rats in the single-nodule group, in which most nodules were in the right lobe of the liver; there were 25 rats in the 2-5-nodule

group, with uniform distribution of nodules in the two liver lobes; there were 23 rats in the ≥ 5 -nodule group, which was equivalent to diffuse liver cancer; and there were 18 rats in the distant metastasis group, with multiple-metastasis lesions in the abdominal cavity. No tumor lesions were detected in the sham group.

Nested PCR results for liver cancer micrometastases

Analysis of *Afp* mRNA using nested PCR

The peripheral blood test results revealed that the rate of occurrence of *Afp* mRNA in the sham group was 10% (2/20). The detailed rates of occurrence for the four experimental subgroups were as follows: 26.9% (7/26) for the single-nodule group, 36.0% (9/25) for the 2-5-nodule group, 60.9% (14/23) for the ≥ 5 -nodule group, and 77.8% (14/18) for the distant metastasis group. There was a significant difference between the sham and experimental groups ($P < 0.05$). The rate of occurrence of *Afp* mRNA increased significantly with increasing nodule numbers and the possibility of metastasis. All the results described above indicate that *Afp* mRNA can be used as a biomarker for liver cancer micrometastases (Table 2 and Figure 1).

Table 2. Incidence of *Afp* mRNA in the various groups.

Group	Samples	Incidence of <i>Afp</i> mRNA	χ^2	P value
Sham group	20	10.0% (2/20)	8.546	P < 0.05
Single-nodule group	24	26.9% (7/26)		
2-5-nodule group	22	36.0% (9/25)		
≥ 5 -nodule group	21	60.9% (14/23)		
Distant metastasis group	14	77.8% (14/18)		

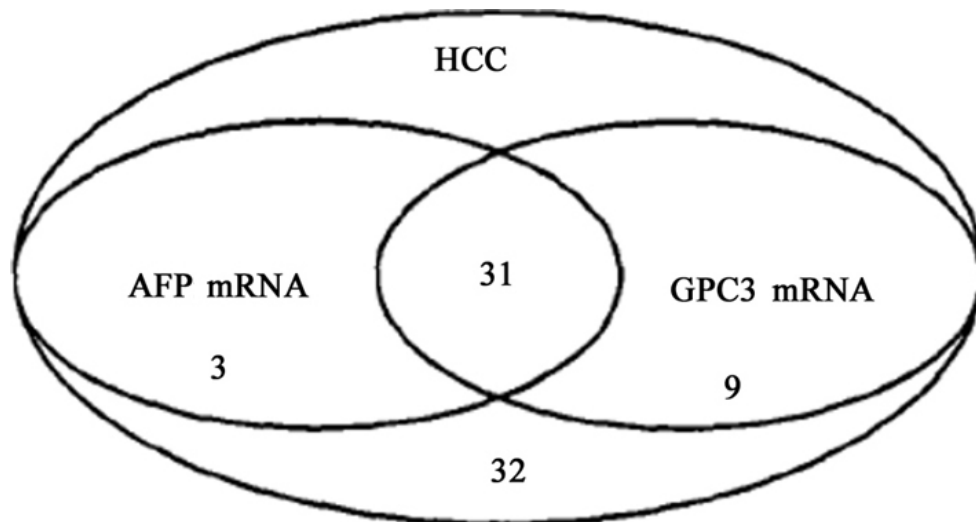


Figure 1. Venn diagram representing the relationship between *Afp* and *Gpc3* mRNA.

Analysis of *Gpc3* mRNA using nested PCR

The peripheral blood test results revealed that the rate of occurrence of *Gpc3* mRNA in the sham group was 0% (0/20). The detailed rates of occurrence for the four experimental subgroups were as follows: 34.6% (9/26) for the single-nodule group, 48.0% (12/25) for the 2-5-nodule group, 73.9% (17/23) for the ≥ 5 -nodule group, and 83.3% (15/18) for the distant metastasis group. There was a significant difference between the sham and experimental groups ($P < 0.05$). The rate of occurrence of *Gpc3* mRNA increased significantly with increasing nodule numbers and the possibility of metastasis. All the results described above indicate that *Gpc3* mRNA can be used as a biomarker for liver cancer micrometastases (Table 3 and Figure 1).

Table 3. Incidence of *Gpc3* mRNA in the various groups.

Group	Samples	Incidence of <i>Gpc3</i> mRNA	χ^2	P value
Sham group	20	0% (0/20)	8.546	P < 0.05
Single-nodule group	26	34.6% (9/26)		
2-5-nodule group	25	48.0% (12/25)		
≥ 5 -nodule group	23	73.9% (17/23)		
Distant metastasis group	18	83.3% (15/18)		

Nested PCR joint detection result

Compared with the total positive ratio of hepatoma cells examined by joint detection, the rates of occurrence of *Gpc3* and *Afp* mRNA increased significantly in the four experimental subgroups ($P < 0.05$). Such results indicate that nested PCR joint detection of *Gpc3* and *Afp* mRNA can compensate for errors (Table 4 and Figure 2).

Table 4. Rates of occurrence of *Afp* and *Gpc3* mRNA in the various combinations of nested polymerase chain reaction data.

Group	A+G-	A-G+	A+G+	Incidence of <i>Afp</i> mRNA	Incidence of <i>Gpc3</i> mRNA	Total incidence
Sham group	2	0	0	10% (2/20)	0% (0/20)	10%
Single-nodule group	1	2	6	26.9% (7/26)	34.6% (9/26)	37.5%*
2-5-nodule group	0	2	8	36.0% (9/25)	48.0% (12/25)	45.5%*
≥ 5 -nodule group	2	4	11	60.9% (14/23)	73.9% (17/23)	76.1%*
Distant metastasis group	1	2	10	77.8% (14/18)	83.3% (15/18)	92.9%*

Asterisks indicate statistical significance ($P < 0.05$).

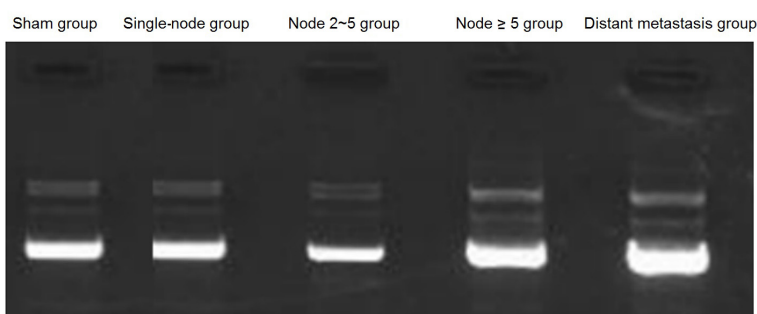


Figure 2. Peripheral blood test results for *Afp* and *Gpc3* mRNA. Groups from left to right are: the sham group, the single-nodule group, the 2-5-nodule group, the ≥ 5 -nodule group, and the distant metastasis group.

DISCUSSION

The incidence of liver cancer is gradually increasing in China, and the rate of liver cancer metastasis remains high despite the development of multiple therapies for its treatment. Liver cancer micrometastases are mainly caused by the diffusion of non-blood system malignancies via the blood or lymphatic systems. Liver cancer micrometastases are characterized by tiny lesions, small cell volume, and a lack of specific features, and they are difficult to diagnose by routine clinical examination (Martin et al., 2010). Liver cancer micrometastases mainly occur in the early stages of tumors or in transient recurrence. The detection of liver cancer micrometastases is the focus of intensive research in the field of liver cancer treatment, and should be improved with the development of novel technology (Hackl et al., 2016).

Nested PCR has been extensively applied to the examination of liver cancer micrometastases. RNA is free and degradation-resistant in peripheral blood. Accordingly, the detection of liver cancer-specific RNA can be used to identify hepatoma cells in the peripheral blood. In other words, mRNA can be used as a biomarker for liver cancer micrometastases (Kelly et al., 2012). Research has shown that nested PCR has a high success rate when it is used to detect liver cancer; for example, it can detect one cancer cell in 107 normal cells, indicating promising sensitivity (Chen et al., 2008; Hori et al., 2013). Therefore, our study explored the potential role of nested PCR in liver cancer examination.

Some researchers have found that *AFP* mRNA can be used to detect liver cancer micrometastases. AFP is highly expressed in the fetal yolk sac, but is hardly expressed in healthy adults. However, AFP is expressed during liver cancer and can be used as a biomarker for it (Mearini et al., 2009; Ouyang et al., 2009; Filella and Giménez, 2013). Our study showed that the rate of occurrence of *Afp* mRNA in the peripheral blood of rats with liver cancer was 48.1% (39/81), and increased significantly with cancer progression, indicating an association between *Afp* mRNA levels and the number of hepatoma cells. Moreover, compared with the single-nodule subgroup, the other subgroups with not less than 2 nodules had higher rates of occurrence of *Afp* mRNA. We also detected *Afp* mRNA in two of the normal (sham) rats, suggesting that there was a possibility of false-positive results for Afp. To remedy this potential failing, *Gpc3* mRNA was also investigated as a potential biomarker. Research has proved that, like *AFP*, *GPC3* is a proto-oncogene of fetal origin, and is highly expressed in fetal malignant tumors. Therefore, *GPC3* is a tumor biomarker with promising specificity (Rigau et al., 2010, 2011). Our results indicated that the rate of occurrence of *Gpc3* mRNA significantly increased with cancer progression. Moreover, there were no false-positive results from the examination of *Gpc3* mRNA. Further analysis showed that joint detection of *Afp* and *Gpc3* mRNA resulted in a higher rate of liver cancer detection. Taken together, these results prove that nested PCR joint detection of *Gpc3* and *Afp* mRNA improves the accuracy of liver cancer micrometastases detection.

In conclusion, the investigation of *Gpc3* and *Afp* mRNA using nested PCR significantly improved the specificity and sensitivity of liver cancer micrometastases detection.

Conflicts of interest

The authors declare no conflict of interest.

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