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• *BRIEF REPORTS* •

Tissue microarray for high-throughput analysis of gene expression profiles in hepatocellular carcinoma

Kai Liu, Xue-Zhong Lei, Lian-San Zhao, Hong Tang, Li Liu, Ping Feng, Bing-Jun Lei

Kai Liu, Xue-Zhong Lei, Lian-San Zhao, Hong Tang, Li Liu, Ping Feng, Bing-Jun Lei, Division of Molecular Biology of Infectious Diseases, Key Laboratory of Biotherapy of Human Disease, Ministry of Education, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China
Supported by the National Natural Science Foundation of China, No. 39670812
Correspondence to: Lian-San Zhao, Division of Molecular Biology of Infectious Diseases, Key Laboratory of Biotherapy of Human Disease, Ministry of Education, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China
Telephone: +86-28-85422635 Fax: +86-28-85422113
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Abstract

AIM: To study the expression profiles of HBsAg, HBcAg, $p21^{WAF1/CIP1}$ (p21), Rb genes in hepatocellular carcinoma (HCC) and to investigate their roles in the hepatocarcinogenesis.

METHODS: HCC tissue microarray containing 120-min tissues of 40 HCC cases was constructed. HBsAg, HBcAg, p21 and Rb proteins were immunohistochemically stained by streptavidin-peroxidase conjugated method (S-P). The expression loss of these genes in cancerous, para-cancerous tissues and adjacent normal liver tissues of 40 HCCs were comparatively examined.

RESULTS: The positive rate of HBsAg expression in cancerous tissues of 40 HCCs was 7.5%, which was lower than that in para-cancerous and adjacent normal liver tissues (χ^2 =12.774, P<0.01; χ^2 = 18.442, P<0.01). The positive rate of HBcAg expression in cancerous tissues of 40 HCCs was 20.0%, which was also lower than that in para-cancerous and adjacent normal liver tissues (χ^2 = 9.482, P<0.01; χ^2 = 14.645, P<0.01). p21 protein deletion rate in cancerous tissues of 40 HCCs was 27.5%, which was higher than that in para-cancerous and adjacent normal liver tissues (χ^2 = 7.439, P<0.01; χ^2 = 11.174, P<0.01). p21 protein deletion correlated remarkably with the pathological grade of HCC (χ^2 = 0.072, P<0.05). Rb protein deletion rate in cancerous tissues of 40 HCCs was 42.5%, which was also higher than that in para-cancerous and adjacent normal liver tissues (χ^2 = 10.551, P<0.01; χ^2 = 18.353, P<0.01). Rb protein deletion rate did not correlate remarkably with tumor size or pathological grade of HCC (χ^2 = 0.014, P>0.05; χ^2 = 0.017, P>0.05).

CONCLUSION: Expression deletion of HBsAg, HBcAg, p21 and Rb proteins in HCCs may play important roles in the carcinogenesis of HCC. Tissue microarray is an effective high-throughput technique platform for cancer research.

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Key words: Tissue microarray; Hepatocellular carcinoma; p21; Rb; HBsAg; HBcAg

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INTRODUCTION

Completion of the draft sequence of the whole human genome has provided the basic structural information on all human genes[1]. Detailed information on the sequence of genes has set the stage for the development of highly powerful expression screening tools. It is well known that alteration of multiple genes is involved in the carcinogenesis, and the next challenge is to validate, prioritize and select the best targets from thousands of candidate genes and proteins[2]. It is also important to analyze molecular targets *in situ* and assess their expression in tissues.

Unfortunately, compared with the powerful screening tools such as cDNA microarrays, which enable simultaneous analysis on expression levels of thousands of genes, most tissue-based molecular techniques are slow, cumbersome and require massive workload[3,4]. For over a century, tissue has been preserved in formalin and embedded in paraffin for sectioning before microscopic examination. This method has become the standard method of histopathologic analysis. Usually, only about 300 5-μm-thick sections can be cut from an average-sized clinical tissue specimen for use in molecular analyses such as immunohistochemistry or *in situ* hybridization. Analysis of 300 molecular targets corresponds to about 1% of the estimated 30 000 genes in the human genome. Thus, genome-scale research is almost impossible using conventional molecular pathology techniques.

In the present study, we constructed hepatocellular carcinoma (HCC) tissue microarray that enables simultaneous analysis of molecular alternations in as many as 120 min tissues (including cancerous, para-cancerous and adjacent normal liver tissues) from 40 HCCs. By the tissue microarray, we studied the expression of HBsAg, HBcAg, $p21^{WAF1/CIP1}$ (p21) and Rb proteins in HCC and tried to clarify their

roles in the hepatocarcinogenesis.

MATERIALS AND METHODS

Tissues and tissue microarray

Tissues of 40 primary HCCs including cancerous, corresponding para-cancerous and adjacent normal liver tissues were obtained from patients who underwent hepatectomy at the West China Hospital of Sichuan University. The surgically resected tissues were fixed in 40 g/L formaldehyde formalin, and embedded in paraffin. Five-micrometer thick sections were cut from each paraffin block (donor block) and stained with hematoxylin-eosin. Histopathological diagnosis and classification were performed by the same pathologist. Cylindrical minute tissues (0.8 mm×0.8 mm×4.0 mm) were punched from morphologically representative sites of donor blocks and wrapped in a special semi-fluid medium, arrayed in the bottom of custom-made embedding box, embedded in paraffin again. Paraffin block containing 120 min tissues (recipient block) was cut into 5-μm-thick sections to generate tissue microarray slides.

Immunohistochemical staining

The S-P method was used for immunohistochemical staining; the first antibodies were mouse anti-human monoclonal HBsAg, HBcAg (DAKO, dilution 1∶100), p21 and Rb antibody (Santa Cruz, dilution 1∶50). S-P kit was purchased from DAKO, Glostrup, Denmark. DAB was used for color development. Already known positive sections were taken as positive controls. Negative mouse serum and PBS were used instead of first antibody as negative control and blank control, respectively.

Assessment of staining reaction

Dark brown granules in cytoplasm were taken as positive reaction and immunoreactivity was registered semiquantitatively. The intensity of immunostaining was scored according to the percentage of positive cells: (-) no positive cells, (+) positive cells <30%, (++) positive cells ≥30-60%, (+++) positive cells >60%.

Statistical analysis

The difference between variables was assessed with χ^2 and Fisher's exact tests. $P<0.05$ was considered statistically significant.

RESULTS

HBsAg and HBcAg expression in HCC

The positive rate of HBsAg expression in cancerous, para-cancerous and adjacent normal liver tissues of 40 HCCs were 7.5, 40 and 45%, respectively. The positive rate of HBsAg expression in cancerous tissues was lower than that in para-cancerous and adjacent normal liver tissues ($P<0.01$). The positive rate of HBcAg expression in cancerous, para-cancerous and adjacent normal liver tissues of 40 HCCs was 20.0, 52.5 and 67.5%, respectively. The positive rate of HBcAg expression in cancerous tissues was lower than that in para-cancerous and adjacent normal liver tissues ($P<0.01$).

p21 and Rb proteins expression in HCC

The deletion rate of p21 proteins in cancerous, para-cancerous and adjacent normal liver tissues was 27.5, 5.0 and 2.5%, respectively. The deletion rate of p21 proteins in cancerous tissues was higher than that in para-cancerous and adjacent normal liver tissues ($P<0.01$). p21 protein deletion had no statistical relation with the age, gender and tumor size of HCC, but correlated with the pathological grade of the tumor. p21 deletion rate was 13.6% in 22 well-differentiated HCC cases and 44.4% in 18 poorly differentiated HCC cases. Poorly differentiated HCC cancerous tissues has higher p21 deletion rate than that of well-differentiated cases ($P<0.05$) (Table 1).

The deletion rate of Rb proteins in cancerous, para-cancerous and adjacent normal liver tissues was 42.5, 10.0 and 2.5%, respectively. The deletion rate of Rb proteins in cancerous tissues was higher than that in para-cancerous and adjacent normal liver tissues ($P<0.01$). Rb protein deletion had no statistical relation with the age, gender and tumor size of HCC cases. However, there was no relation between the deletion rate of Rb protein and pathological grade of the tumor ($P>0.05$) (Table 2).

Table 1 Expression of p21 in patients with HCC

	n	Negative	Deletion rate (%)	χ^2	*P*
Histology					
Cancerous tissues	40	11	27.5		
Para-cancerous tissues	40	2	5.0	7.439	<0.01
Adjacent normal liver tissues	40	1	2.5	11.174	<0.01
Grade					
Well-differentiated	22	3	13.6		
Poorly-differentiated	18	8	44.4	4.713	<0.05
Tumor size (diameter)					
<2 cm	8	2	25.0		
2–5 cm	15	4	26.7		
>5 cm	17	5	29.4	0.072	>0.05

Table 2 Expression of Rb in patients with HCC

	n	Negative	Deletion rate (%)	χ^2	*P*
Histology					
Cancerous tissues	40	17	42.5		
Para-cancerous tissues	40	4	10.0	10.551	<0.01
Adjacent normal liver tissues	40	1	2.5	18.353	<0.01
Grade					
Well-differentiated	22	10	45.5		
Poorly-differentiated	18	7	38.9	0.014	>0.05
Tumor size (diameter)					
<2 cm	8	3	37.5		
2–5 cm	15	6	40.0		
>5 cm	17	8	47.1	0.017	>0.05

DISCUSSION

HCC is one of the most prevalent human tumors throughout the world and particularly in certain areas of Asia and Africa. The majority of HCCs are related to chronic liver diseases such as hepatitis B or C[5]. Previous studies showed that hepatocarcinogenesis is a multi-step process which involves

complicated molecular mechanisms including activation of oncogenes, function loss of tumor suppressor genes, deficiency of anti-tumor immunization, *etc*.[6-9].

Uncontrolled cell proliferation is the hallmark of cancer including HCC. In cell cycle, the period from late G1 to S phase is the most important for cell proliferation. Several studies have shown that various types of alterations in cell cycle regulators are found in many kinds of cancer[10-13].

p21 is one of these cell cycle regulators and belongs to a family of genes that negatively regulate the cell cycle, thereby inhibiting cell proliferation. The cell cycle is positively controlled by a complex of cyclin and cyclin-dependent kinases (CDK)[14,15]. Thus, this complex can accelerate cell proliferation. The main substrate of cyclin and CDK is the rentinoblastoma (Rb) gene. Rb gene, located at chromosome 13q14, is a negative regulator of cell proliferation and plays a critical role in cell proliferation control. Rb suppresses the G1 to S transition[16,17]. The anti-proliferate activity of Rb is mediated by its ability to inhibit the transcription of genes that are required for cell cycle progression. Phosphorylation of Rb by the cyclin/CDK complex allows progression of the cell cycle. p21, by binding to cyclin/CDK, abrogates their activity. Consequently, Rb is not phosphorylated and cell cycle progression is arrested. Though p21 is a small protein, by inhibiting the cyclin/CDK complex, p21 has the ability to halt the cell cycle proliferation and to function as a tumor growth suppressor[18-21]. Loss or decreased expression of p21 may facilitate the proliferation of cancer cells[22-24]. Our study showed that the deletion rate of p21 in HCCs was higher than that in para-cancerous and adjacent normal liver tissues, suggesting that the loss of p21 protein may play an important role in the hepatocarcinogenesis. What is more, poorly differentiated cancerous tissues showed a higher deletion rate than well-differentiated cancerous tissues. Therefore, p21 may be useful predicting the overall survival of patients with HCC.

Previous studies have shown that Rb is deleted or mutated in a wide range of human cancers including HCC[25-28]. However, over expression of Rb in HCC tissues is also reported[29]. In the present study, we evaluated the Rb protein expression in a series of specimens (cancerous, para-cancerous and adjacent normal liver tissues) that corresponded to the different stages of multi-step hepatocarcinogenesis. Tissue microarray technology enabled us to study the molecular alterations in as many as 120 of tissue specimens. We observed that almost all the normal liver tissues showed normal Rb expression, whereas remarkable deletion rate was found in HCC tissues. Thus, whether there is a relationship between over expression of Rb and progression of HCC needs to be documented.

Original HCC cells probably express HBV antigens. As the tumor gets larger, immune response against HBV antigens is elicited and reacts with HCC cells bearing HBV antigens[30]. During this process, inactivation of viral gene expression through HBV gene or excision of HBV may occur. HCC cells not expressing HBV antigens escape from the immune surveillance[31,32], and mutation in HLA class I-restricted T cell epitope on HBsAg contributes to HCC cells escaping from immune surveillance, thus resulting in persistent infection[33]. As the tumor increases in size, an enrichment of cells not expressing HBV antigens is observed. A common feature of HBV infection is the integration of HBV-DNA into host chromatin. With regard to virus sequences, integration commonly involves S and X genes whereas the deletion usually occurs in other fragments[34]. Therefore, HBcAg is seldom observed in liver tissues where integration of HBV is involved. Expression deletion of HBcAg leads to the uncontrolled proliferation of liver cells[35]. Our study suggested that in HCC tissues there were less HBsAg and HBcAg. Loss of attack targets may prevent tumor cells from being destroyed by the host anti-tumor immune and accelerate the hepatocarcinogenesis.

Tissue microarray is a high-throughput analysis tool, which allows rapid validation of molecular targets in as many as thousands of tissue specimens at a time, either at DNA and RNA level or protein level[36,37]. In this method, minute tissues are removed from hundreds or thousands of different primary tissue blocks and placed into one arrayed block with high density. Tissue microarray is suitable for all analyses *in situ* such as antibody staining, immunohistochemistry (ISH), fluorescence *in situ* hybridization, Western blot. In fact, any molecular detection scheme developed for whole tissue sections is adaptable to tissue microarray sections[38-41].

Tissue microarray technology is likely to find applications in many other fields of HCC research including gene detection of hepatocarcinogenesis, exploration and screening of anti-tumor drugs, molecular diagnosis, evaluation of therapy scheme and prognosis, *etc*.[42,43]. It is also possible to use tissue microarray to study molecular pathology and therapy of many other diseases.

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