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Short Communication

Immunohistochemical Analysis of *P53* Gene Expression and other Protein Indicators in Chinese Hepatocellular Carcinoma Patients

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ABSTRACT

The objective of this study was to analysis P53 gene expression and other protein indicators in patients with hepatocellular carcinoma (HCC) in China. The present study collected a paraffin-embedded tissue sample of 31 patients with HCC from February 2021 to January 2022 from 13 pathology centers in Wuhu. The sample were stained using immunohistochemistry (IHC) method for detection of protein indicators. From the samples examined in this study, it was found that there are positive samples in P53 (n=8, 26.6 percent), RB1 (n=4, 13.3 percent), Cycline-D1 (n=6, 20 percent), c-Fos (n=14, 46.6 percent), and N-ras (n=3, 10 percent). In 85% of the sample, changes were observed in RB1 or Cycline-D1. The present study provides evidence that changes of P53 gene expression and other protein indicators play an important role in creating HCC.

Hepatocellular Carcinoma (HCC) is the fifth most common cancer and the third cause of cancer death in the world (Yin *et al*., 2022). The prevalence of this disease is different in different parts of the world. Its high prevalence in parts of Asia and Africa is due to the weak health system (Chen *et al*., 2006), but in western and developed countries, the reason for its increasing prevalence is due to hepatitis C virus infection, the use of alcohol, and the spread of liver cirrhosis (Davila *et al*., 2007). For the treatment of HCC, in addition to chemotherapy, which has not been very successful, resection and liver transplantation methods are used. Resection is successful when this disease does not have extrahepatic metastasis (Davila *et al*., 2007). Liver transplantation is the most effective treatment for HCC patients without resistance to metastasis (Song *et al*., 2010), although most of the patients are not suitable candidates for this treatment due to the progress of the disease, old age and the like. Also, there are few donors for the liver, so that most of the people waiting to receive the liver die due to the progress of the tumor and the disease at this point in time (Zhou *et al*., 2006). In this cancer, liver transplant surgery is the only treatment method during which the effect of the drug changes and the possibility of the disease returning increases and the probability of the patient's survival is weak and short-term.

Recent advances have led to the identification of indicators that reduce the progress and speed of cancer cells, of which P53 tumor suppressor protein is one of them. Disruption or inactivation of protein P53 leads to cancer (Osada *et al*., 2004). P53 regulates the cell cycle and DNA repair pathways as part of its distinct and important function in maintaining genome stability (Lago *et al*., 2011). Retinoblastoma (RB) tumor suppressor is an important regulator of the cell cycle and a large number of processes associated with tumor growth. Functional inactivation of RB has been sporadically identified in many human tumors, which is involved in the initiation or progression of the disease. Several studies have now shown that the loss of this tumor suppressor creates a selective vulnerability that can be targeted therapeutically and therefore provides a precise approach to exploit the lack of RB (Witkiewicz *et al*., 2018; Robinson *et al*., 2013; Jones *et al*., 2016).

With the progress made in understanding the biology of tumors, studies on carcinogenic molecular biomarkers are growing and increasing day by day due to their potential importance as therapeutic targets. This present study aims to analysis P53 gene expression and other protein indicators in patients with HCC in China.

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Materials and methods

The present study collected a paraffin-embedded tissue sample of 31 patients with HCC from February 2021 to January 2022 from 13 pathology centers in Wuhu. The study was carried out in compliance with guidelines issued by ethical review board committee of Yijishan Hospital, China. The study also obtained written informed consent from all the patients.

For the expression of *P53, RB1, Cycline-D1, c-Fos*, and *N-ras* genes, immunohistochemical technique was used as follows. First, the samples were embedded in paraffin wax, then sections with a thickness of 4-6 µm were cut and then stained with hematoxylin-eosin. The pathologists board committee of Yijishan Hospital made the final confirmation of the disease again.

In the present study, Avidin-Biotin peroxidase method was used to perform the immunohistochemical (IHC) technique as follows: (1) The sections were deparaffinized. (2) Internal peroxidase was inhibited by methanol for 30 min using 0.5% H_2O_2 solution. For this the tissues were dried and then quickly inserted into the peroxidase inhibitor solution and then washed twice in PBS with pH 7.4 for 5 min. (3) The tissues were incubated with 3% horse serum (NHS) for 30 min. (4) The tissues were incubated with an excess of 3% NHS overnight at room temperature with an appropriately diluted primary antibody. (5) The tissues were washed with PBS twice incubated for 30 min at room temperature with the secondary antibody diluted 1:50 with NHS. (6) Washed with PBS twice, and then incubated for 60 min at room temperature with streptavidin conjugated with peroxidase diluted 1:1500 with NHS. (7) After washing with PBS twice, the peroxidase substrate solution was added for about 10 to 20 min. (8) After washing with PBS-pH=7.4, the tissue was stained with hematoxylin.

Analysis of the data related to the expression of genes were evaluated by using SPSS PASW statistic software and the likelihood ratio frequency test was performed to analysis P53 gene expression and other protein indicators in patients with HCC.

Results

Among the 31 eligible patients, one patient was excluded from the study due to the impossibility of follow-up, and the data of the remaining 39 patients were analyzed. The gender distribution was 11 female and 19 male patients aged between 44 and 83 years, with mean age of 63.5±15.74 years and mean body mass index of 24.13±4.98. Ten patients were suffering from severe A disease. They had asymptomatic primary tumors. Thirteen cases were in stage B in terms of disease severity so that they had symptoms. Seven patients were in severity C disease so that they had symptomatic tumors with invasive pattern. Other demographic characteristics of the patients are shown in Table I.

Table I. Frequency distribution of demographic characteristics in the hepatocellular carcinoma patients.

Characteristics	Frequency (n, %)
Age groups	
44-55	10 (33.3)
56-70	16 (53.3)
71-83	4 (13.3)
Gender	
Male	19 (63.3)
Female	11 (36.6)
Education	
Under diploma	18 (60.0)
Above diploma	12 (40.0)
Marital status	
Married	25 (83.3)
Single	5 (16.6)
Severity of disease*	
A	10 (33.3)
B	13 (43.3)
C	7 (23.3)
Income**	
Sufficient	13 (43.3)
Insufficient	17 (56.6)

(*), Severity of disease is based on Barcelona Clinic Liver Cancer (BCLC) criterion, which includes four stages. A, Patients with asymptomatic primary tumors; B, Carcinoma patients with symptoms; C, Symptomatic tumors with invasive pattern; (**), The amount of income of the patients is categorized based on the self-declaration of the patients.

A number of 8 malignant samples (26.6%) were associated with high *P53* gene expression. Twenty two cases of malignant samples lacked *P53* gene expression, while 4 of the patients (13.3%) had positive *RB1* gene expression, 26 had negative. Six samples (20%) were associated with high *Cyclin-D1* gene expression, and the rest lacked it. Fourteen of the patients (46.6%) had positive *c-fos* gene expression but 16 people had negative. Three of the patients (10%) had positive *N-ras* gene expression but 27 people had negative.

Table II shows the correlation between *P53* gene expression and other protein indicators in the patients. In P53 positive samples, *c-Fos* gene was expressed in 5 cases, *RB1* gene in 3 cases, *N-ras* gene 2 cases and *Cyclin-D1* gene was expressed in 4 cases. In the samples

with negative *P53*, *c-Fos* gene expressed in 7 cases, *RB1* gene 2 cases, *N-ras* gene in 3 cases and *Cyclin-D1* gene in 5 cases.

Table II. Correlation between *P53* gene expression and other protein indicators.

Gene expression of proteins	Positive P53	Negative P53
c-Fos		
Positive	5 (62.5%)	7 (31.8%)
Negative	3 (37.5%)	15 (68.2%)
RB1		
Positive	3 (27.3%)	2 (10.5%)
Negative	8 (72.7%)	17 (89.5%)
N-ras		
Positive	2 (25%)	3 (13.6%)
Negative	6 (75%)	19 (86.4%)
Cycline-D1		
Positive	4 (30.8%)	5 (29.4%)
Negative	9 (69.2%)	12 (70.6%)

Discussion

The resent study showed that there is relationship between P53 gene expression and other protein indicators in Chinese HCC patients. Background studies on *P53* gene expression show that this factor plays a role in causing HCC (Guan *et al*., 2007; de Fromentel and Levrero, 2020; Badwei, 2022; Liu *et al*., 2016; Hussain *et al*., 2007). Studies have shown that this gene is mutated in 69-24% of HCC cases (Osada *et al*., 2004). An experimental study in adult female rats showed that there is a significant relationship between *P53* gene expression and HCC so that HCC staging is obtained by detecting the expression of p53, which is a molecular marker (Sakr *et al*., 2022). Yang *et al*. (2022) in a study with sequence of bioinformatics analysis identified novel characteristics in TP53-mutant HCC and provided new insights into a precise individualized therapy for HCC. Studies that investigated *P53* gene expression in HCC with IHC method have provided inconsistent and contradictory information (Qin *et al*., 2002). It is possible to explain and interpret the differences in these results through different methods used, diversity in antibodies and the way of individual analysis. In addition, the absence of detectable P53 protein, such as some mutations, especially frame change and nonsense mutations in which protein stability does not exist, does not necessarily indicate the normal *P53* gene and therefore cannot be detected by IHC method. Therefore, IHC is not a reliable method for assessing P53 states, and DNA mutation analysis requires a more appropriate method to evaluate P53 states.

Our study showed that among 31% of samples with nuclear P53 protein accumulation, 10% of them had N-ras protein accumulation. A study in Guangxi showed among 29 patients with HCC, while 13 cases (44.8%) had *P53* gene expression, 12 cases (41.37%) of the patients had *N-ras* gene mutation (Luo *et al*., 1998). The results of the study showed that apart from *P53* gene, *N-ras* gene may also play a role in causing HCC. Their report also showed that while 62% of the sample with N-ras gene mutation expressed P53 protein, the rest did not, and this indicates that there are other factors in the creation and development of HCC. These findings are consistent with the findings of the present study. In the present study, 29% of the patients with *N-ras* gene mutation expressed P53 protein, but 71% did not. In a study on HCC by Duan *et al*. (2021), it was observed in addition to the fact that Asian HCC patients with RB1 mutation had a shorter overall survival, Caucasian HCC patients with RB1 mutation also had a shorter disease-free survival. Their report showed proportion of infiltrating CD8 T-cells in Asian HCC patients with RB1 mutation was decreasing. They concluded that apart from TP53 mutation, RB1 mutation may also play a role in survival among HCC patients, but these effects are different between Asia and Caucasus. According to another HCC study done by Yuen *et al*. (2001), while *C-fos* gene expression in developed HCC tumor tissues which were in a higher degree of aggressiveness had a high percentage (91%), it was not found among non-tumor tissues. These results also indicated that both *c-fos* and *c-jun* gene expression in HCC patients may play a role in the coordinated tumor cell cycle of progression and proliferation. These findings are consistent with the findings of our study. In our study the expression level of *C-fos* gene in tumor tissues was significantly high.

Conclusion

For the Chinese HCC patients in the study, analysis of *P53* gene expression and other protein indicators based on a immunohistochemical approach appears to change the expression of *P53, RB1, c-Fos* and *Cycline-D1* genes in the creation and development of HCC. Therefore, it can be said that the lack of expression of these genes in HCC accelerates the growth of malignant cells.

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board committee of Yijishan Hospital, First Affiliated Hospital of Wannan Medical College, China. The official letter would be available on fair request to corresponding author.

IRB approval

This study was approved by the Yijishan Hospital, First Affiliated Hospital of Wannan Medical College, Wuhu, Anhui241000, China.

Statement of conflict of interest

The authors have declared no conflict of interest.

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