**P21\(^{\text{WAF1/Cip1}}\) Gene Expression in Primary Human Hepatocellular Carcinoma and its Relationship with P53 Gene Mutation**

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**Summary:** P21\(^{\text{WAF1/Cip1}}\), an inhibitor of cyclin-dependent kinases, is a critical downstream effector in the P53-specific pathway of growth control. Increased expression of P21\(^{\text{WAF1/Cip1}}\) has been found to reflect the status of the P53 tumor-suppressor pathway. We investigated the expression of P21\(^{\text{WAF1/Cip1}}\) in a relatively small, but well-characterized group consisting of 28 hepatocellular carcinomas. The samples were previously studied for P53 gene mutation. P21\(^{\text{WAF1/Cip1}}\) expression were identified by *in situ* hybridization and immunohistochemistry. Positive ISH for P21\(^{\text{WAF1/Cip1}}\) transcripts was found in 18 of 28 cases (64.3%). All positive cases by ISH showed detectable P21\(^{\text{WAF1/Cip1}}\) protein reactivity by IHC. No relationship was found between P21\(^{\text{WAF1/Cip1}}\) staining and P53 mutational status. No associations were seen with tumor metastasis, size and tumor grade, except for tumor differentiation status which showed higher frequency of P21\(^{\text{WAF1/Cip1}}\) expression in moderate-well differentiated HCCs than poorly differentiated tumors (P<0.05). It is concluded that expression of P21\(^{\text{WAF1/Cip1}}\) is common in HCCs, but does not correlate with P53 mutational status or pathological parameters investigated except for tumor differentiation. Also, there may be other factors beside P53 that regulate P21\(^{\text{WAF1/Cip1}}\) gene expression in HCCs.

**Key words:** hepatocellular carcinoma; P53; P21\(^{\text{WAF1/Cip1}}\)

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P21\(^{\text{WAF1/Cip1}}\) protein (p21) is the product of *waf1* gene, also known as *cip1*, *sdil*\(^{[1,2]}\). P21\(^{\text{WAF1/Cip1}}\) is a universal inhibitor of cyclin-dependent kinase, which are required for G\(_1\) to S transition. P21\(^{\text{WAF1/Cip1}}\) is a critical downstream effector in the P53-specific pathway of growth control in mammalian cells. P53 expression in response to DNA-damaging agents can promote the transcription of P21\(^{\text{WAF1/Cip1}}\) via interaction of P53 with a P53-binding site in the P21\(^{\text{WAF1/Cip1}}\) promoter. It is wild type P53 other than mutant type P53 that can promote P21\(^{\text{WAF1/Cip1}}\) gene transcription. However, P21\(^{\text{WAF1/Cip1}}\) can also be induced in other events, such as growth factor stimulation or cellular differentiation. Early studies indicated that TGF-\(\beta\), FGF, EGF, PDGF etc, could stimulate P21\(^{\text{WAF1/Cip1}}\) gene expression by P53-independent pathway\(^{[3]}\).

Many human tumors have been demonstrated not to express P21\(^{\text{WAF1/Cip1}}\) protein\(^{[4-7]}\). Recently, colorectal carcinoma and breast carcinoma were found to have high frequency of undetectable P21\(^{\text{WAF1/Cip1}}\) expression\(^{[4,5]}\). No relationship between P21\(^{\text{WAF1/Cip1}}\) staining and P53 protein expression or P53 gene mutational status was found in breast carcinoma or lung non-small-cell carcinoma\(^{[5,7]}\). On the other hand, in colonic carcinoma, immunohistochemical expression of P21\(^{\text{WAF1/Cip1}}\) is inversely related to P53 protein overexpression. These data suggest that P21\(^{\text{WAF1/Cip1}}\) expression in human neoplasm may be regulated in a tissue-specific way.

In this paper, we investigated the expression of P21\(^{\text{WAF1/Cip1}}\) at both immunohistochemical and mRNA levels in a series of P21\(^{\text{WAF1/Cip1}}\) primary human HCC, with an attempt to evaluate P21\(^{\text{WAF1/Cip1}}\) expression frequency in HCC and to find its association with P53 gene mutation.

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**1 MATERIALS AND METHODS**

**1.1 Tissue**

A cohort of 28 specimens of HCC and 5
non-cancerous adjacent liver were obtained from livers surgically removed in Tongji Hospital during the first half year of 1995. Twenty-three males and 5 females, aged from 27 to 64 years (average of 50.3 years) were involved. The tumor lesions included 14 poor differentiated, 9 moderately and 5 well differentiated liver carcinomas. Two patients had intrahepatic metastasis and 4 cases had extrhepatic metastasis. According to TNM system (1987), 2 HCCs were in grade I; 13 HCCs were in grade II; 8 tumors were in grade III; the remaining 5 cases were in grade IV. All cases were selected on the basis of availability of frozen materials for study and on the absence of extensive chemotherapy-induced tumor necrosis.

1.2 In Situ Hybridization (ISH) Analysis

The P21WAF1/CIP1 cDNA probe was kindly provided by Dr. SJ Elledge (Houston, USA). The probe was labeled and detected using a Dig DNA labeling and detection kit which was purchased from Boehringer Mannheim Biochemica, Germany. Briefly, 4% paraformaldehyde-fixed paraffin embedded samples were cut at 7 μm and adhered to APES-treated slides. The sections were dewaxed, rehydrated through a graded series of ethanol. Pre-treated the sections by 0.2 mol/L HC1 for 20 rain at room temperature. Digested the sections by proteinase K (10 μg/ml) for 30 rain at 37°C. Then pre-hybridized the samples at 42°C for 2 h. Hybridization solution containing 2 μg/ml probe was subjected to hybridization at 42°C for 36 h. Then added anti-Dig antibody and incubated the samples for another 3 h. Hybridization buffer containing no probe served as negative control.

1.3 Immunohistochemistry

Mouse monoclonal antibody that recognizes the human P21WAF1/CIP1 protein was Santa Cruz product. StreptAvian-Biotin-enzyme Complex (SABC) kit was purchased from Boster Biotechnology Inc. (Wuhan, China). Briefly, 7 μm tissue sections were deparaffinized, rehydrated through a graded series of ethanol, and heated in 0.01 mol/L sodium citrate solution at 94°C—96°C for 10 min. The antibodies were diluted to 1:30. Representative tissue sections were immuno-labeled with PBS as a negative control for the immunohistochemistry.

1.4 PCR-SSCP Analysis

Genomic DNA was extracted from frozen tissues described above by sodium dodecylsulfate, proteinase K and phenol-chloroform treatments and stored at 4°C for future use. The Faculty of Pathology, Beijing Medical University synthesized the primers, by following procedures: Exon 5 5’-TAC TCC CCT GCC CTC AAC AAG A-3’ and 5’-CGC TAT CTG AGC AGC GCT GAT A-3’, the PCR product was 181bp in length; Exon 6 5’-GAT TGC TCT TAG TGC TGG CCC CTC CTC AGC-3’ and 5’-CAG ACC TCA GGC GGC TCA TAG G-3’, 132bp; Exon 7 5’-CTA GGT TGG CTC TGA CTG TAC CAC CAT CAT CC-3’ and 5’-TAG CCT GGA GTC TTC CAG TGT G-3’, 119bp; Exon 8 5’-GTA GTG GTA ATG TAC TGG GAC GGA ACA GA-3’ and 5’-CTC GCT TAG TGC TCC GGG GC-3’ 143bp. The PCR reaction mixture contained 0.1 μg—0.2 μg genomic DNA, 1.5 U of Tag DNA polymerase, 200 μmol/L each of dGTP, dATP, dCTP, dTTP, 5 μmol of each of oligonucleotide primer, MgCl2 1.5—3 μmol/L in a final volume of 50 μl. The first PCR cycle was performed at 95°C for 5 min to denature the DNA, at 55°C for 40 s to anneal the primer and at 72°C for 20 s to synthesize the DNA. The next 33 cycles were performed at 94°C for 60 s, 55°C for 40 s and 72°C for 20 s. The last cycle was at 94°C for 60 s, 55°C for 40 s and 72°C for 5 min. 5 μl of PCR products were examined to ensure that each sample was successfully amplified. After 5 μl of 1 mol/L NaOH was added into the remaining 45 μl products, the mixture was heated at 95°C for 5 min. Then the mixture was chilled in ice water for 5 min. 50 μl of SSCP loading mixture was added to each tube. The treated mixture was applied to the wells of 10% non-denatured polyacrylamide gel. Electrophoresis was performed at 50 V for 14—15 h at room temperature. The gel was fixed in 10% acetic acid for 10—12 h, washed in 30% acetic acid and distilled water for two times. Then the gel was put in silver staining solution (0.1% AgNO₃) for 30 min. Develop-