

日本財団補助金による  
2000年度日中医学学术交流促進事業

①. 調査・共同研究助成

(3) 肝癌発生の分子機構に関する研究

研究テーマ：肝癌発生の分子機構に関する研究

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## 要旨

日本では肝癌の発生の70%はC型慢性肝炎・肝硬変がベースになっている。これに対し、中国では7割の肝癌はB型肝炎ウイルスに起因するものである。今度B型肝炎ウイルス(HBV)とC型肝炎ウイルス(HCV)による肝発癌の分子機構の対比研究を行った。

p21はp53依存性またはp53非依存性的に調節され、細胞周期調節因子として働いている。本研究は肝癌におけるp21とp53蛋白の発現を免疫組織化学の手法により検索した。肝癌65例の81腫瘍の中、p53異常は43(53%)例、p21蛋白発現低下は59(73%)例において認められた。p21とp53の相関性を検討すると、HCVに起因する肝癌ではp21蛋白発現低下はp53異常との相関は強く認められ、p21発現の調節はp53依存的であり、HBVに起因する肝癌及び肝炎ウイルスの感染の伴わなかった肝癌ではその相関性は認められなく、p21発現異常の調節はp53非依存的であることが示唆された。

p16は細胞周期G1期を制御する重要な遺伝子であり、その蛋白欠失は多くのヒト癌で報告されている。p16不活性のメカニズムとして遺伝子の欠失、変異およびDNA過剰メチル化による転写ブロックが考えられたが、肝細胞癌ではp16遺伝子の欠失及び変異が検出されなかった。本研究は肝細胞癌よりDNAを抽出し、肝癌におけるp16遺伝子promoterのメチル化状況をMethylation-specific PCR法を用いて調べた。p16遺伝子promoterの過剰メチル化は50例中の29例(58%)において認められた。それらの肝癌におけるp16蛋白の発現を免疫染色法を用いて検索するとp16遺伝子promoterの過剰メチル化と蛋白欠失の相関が認められた。さらに、肝炎ウイルス感染との関係を見ると、HBVに起因する肝癌と比べて、HCVに起因する肝癌ではp16遺伝子の過剰メチル化する症例が比較的が多かった。p16遺伝子の過剰メチル化は肝発癌、特にHCVによる発癌に関与することが示唆された。

**Keywords:** p21<sup>WAF1/CIP1</sup>; p53; p16; hepatocellular carcinoma

## 研究報告

### 背景と目的

Cell cycle progression is governed by several checkpoints, which are regulated by a family of protein kinases, the cyclin-dependent kinases (CDKs) and the cyclins (Hunter and Pines, 1994). The restriction point is one of the most important checkpoints in the late G1 phase. Disruption of the checkpoints is one mechanism of oncogenesis. p21<sup>WAF1/CIP1</sup> protein, a universal CDK inhibitor (Xiong et al, 1993), and p53 protein are two important components of the G1 restriction point.

Recently, the p21<sup>WAF1/CIP1</sup> gene was cloned and mapped to the 6p21.2 chromosome region (El-Deiry et al, 1993; Noda et al, 1994). p21<sup>WAF1/CIP1</sup> inhibits a wide variety of cyclin-CDK complex activities by binding to the complexes (Xiong et al, 1993; Harper et al, 1993). p21<sup>WAF1/CIP1</sup> has also recently been shown to bind to and inactivate proliferation cell nuclear antigen, the processivity subunit of DNA polymerase (Waga et al, 1994). These observations suggest that p21<sup>WAF1/CIP1</sup> may play a dual role in blocking entry into S phase (Noda et al, 1994). In addition, p21<sup>WAF1/CIP1</sup> has also been suggested to play a role in inducing differentiation and apoptosis (Michieli et al, 1994; Sheikh et al, 1995), and introduction of p21<sup>WAF1/CIP1</sup> cDNA suppresses the growth of human tumor cells in culture (El-Deiry et al, 1993).

Cells lacking functional p53 express a very low level of p21<sup>WAF1/CIP1</sup> and the p21<sup>WAF1/CIP1</sup> promoter contains a p53-binding site, suggesting that expression of p21<sup>WAF1/CIP1</sup> depends on p53 function (Xiong et al, 1993; El-Deiry et al, 1993, 1994). However, p21<sup>WAF1/CIP1</sup> can be inducible in p53-null cells, showing that the expression of p21<sup>WAF1/CIP1</sup> can also be induced by p53-independent pathways (Michieli et al, 1994; Zhang et al, 1995).

We previously investigated p21<sup>WAF1/CIP1</sup> mRNA expression by reverse-transcriptase polymerase chain reaction and p53 mutational status by polymerase chain reaction single-strand conformation polymorphism and direct DNA sequencing in hepatocellular carcinomas (HCCs). We suggested that p21<sup>WAF1/CIP1</sup> mRNA expression is regulated predominantly by a p53-dependent pathway and that reduced p21<sup>WAF1/CIP1</sup> mRNA

expression may contribute to hepatocarcinogenesis (Hui et al, 1997). However, we did not evaluate the expression of p21<sup>WAF1/CIP1</sup> and p53 at the protein level. Because post-transcriptional regulation is also an important mechanism in gene expression (Hui et al, 1996b; Loda et al, 1997; Maki and Howley, 1997), and loss of function of p53 is caused not only by gene mutation but also when p53 protein interacts with viral or cellular oncoproteins (Sarnow et al, 1982; Farmer et al, 1992; Yew and Berk 1992; Steegenga et al, 1996; Somasundaram and El-Deiry, 1997), it is necessary to investigate p21<sup>WAF1/CIP1</sup> and p53 at the protein level. The wild-type p53 protein has a short half-life of about 20 min, and in general it cannot be detected by immunohistochemistry. However, a p53 gene mutation or p53 interacting with oncoproteins may stabilize p53 protein and result in altered expression of p53 that can be detected by immunohistochemistry (Sarnow et al, 1982; Farmer et al, 1992; Yew and Berk, 1992; Steegenga et al, 1996; Somasundaram and El-Deiry, 1997; Iggo et al, 1990; Finlay et al, 1988; Hall et al, 1991).

We investigated the expression of p21<sup>WAF1/CIP1</sup> and p53 proteins in 81 tumors from 65 patients with HCC by immunostaining to determine whether p21<sup>WAF1/CIP1</sup> expression depends on p53 functional status, and the relationship between the two proteins' expression and clinicopathological features.

## 方法

### Patients and specimens

Eighty-one primary HCC tissues were obtained from 65 patients. Fifty patients were found to have underlying cirrhosis, and 15 had chronic hepatitis. The study included 13 (20%) women and 52 (80%) men with a mean age of 61 years (range 13–74 yr). Fifty-six patients had serological evidence of viral infection (11 were positive for hepatitis B surface antigen [HBsAg], 43 were positive for hepatitis C virus [HCV] antibody and 2 were positive for both markers). The remaining 9 patients were negative for both hepatitis markers. Tissue samples were obtained at the time of operation, fixed in 10% formalin, and histopathologically examined. The size of the tumors varied from 0.6 to 15 cm (mean, 4.0 ± 3.5 [SD]). The 81 tumors comprised 23 well, 43 moderately, and 15 poorly differentiated HCCs. Portal vein tumor thrombi were found in 18 (22.2%) HCCs, and intrahepatic metastatic lesions were found in 11 (13.6%).

### **Immunohistochemical staining for p21<sup>WAF1/CIP1</sup> and p53**

Immunohistochemistry was done using the avidin–biotin–peroxidase complex method. Paraffin-embedded sections (4 μm thick) were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and then treated with 3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After brief washing with distilled water, tissue sections were processed in 10 mM citrate buffer (pH 6.0) and heated to 120°C in an autoclave for 10 min for antigen retrieval (Hui et al, 1999a; 1999b; 2000; Li et al, 2000). Slides were allowed to cool at room temperature for 20 min and then rinsed with phosphate-buffered saline (PBS). To inhibit nonspecific binding activity, slides were incubated with blocking serum at room temperature for 30 min. Sections then were incubated with primary monoclonal antibody against p21<sup>WAF1/CIP1</sup> (clone EA10, Oncogene Science, Cambridge, MA, USA) diluted at 1:200, or with monoclonal antibody against p53 (clone DO-7; DAKO A/S, Denmark) at 1:100, at 4°C in a moist chamber overnight. The sections were then incubated with biotinylated anti-mouse immunoglobulins (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min, and avidin–biotin complex (Vector Laboratories) for 30 min at room temperature, with washing in PBS before each incubation. 3,3'-Diaminobenzidine tetrahydrochloride was used as the color reagent, and haematoxylin was used as a counterstain. Normal esophageal squamous epithelium and HCC with known p53 gene mutation and p53 protein overexpression were used as positive controls for p21<sup>WAF1/CIP1</sup> and p53, respectively. Negative controls were obtained by omitting primary antibody. Only nuclear staining was considered to be positive for p21<sup>WAF1/CIP1</sup> and p53. Based on the previously published criteria, positive staining of p21<sup>WAF1/CIP1</sup> was considered when ( 5% of tumor cells were stained (Ogawa et al, 1997). Positive scoring for p53 was considered when ( 10% of the nuclei were stained, according to the criteria used previously (Esrig et al, 1994; Cote et al, 1998).

### **Statistical analysis**

χ<sup>2</sup> test or Fisher's exact test was used to examine differences and relationships between groups of patients classified by p21<sup>WAF1/CIP1</sup> and p53 staining. Differences at  $P < 0.05$  were judged to be statistically significant.

## 結果

### p21<sup>WAF1/CIP1</sup> expression

p21<sup>WAF1/CIP1</sup> immunoreactivity was always nuclear, and no cytoplasmic staining of p21<sup>WAF1/CIP1</sup> was seen in any specimen. The HCCs showed heterogeneous expression of p21<sup>WAF1/CIP1</sup>; the percentage of p21<sup>WAF1/CIP1</sup>-positive cells ranged from 0% to 60% (median 5%). Twenty-two of 81 HCCs (27.2%) showed positive staining for p21<sup>WAF1/CIP1</sup> protein. Most (86.4%) p21<sup>WAF1/CIP1</sup>-negative tumors showed < 1% positive cells or complete loss of immunoreactivity. p21<sup>WAF1/CIP1</sup> expression was not associated with viral infection, histological features of non-tumorous livers, tumor size, tumor differentiation, intrahepatic metastasis, or tumor portal or hepatic vein involvement ( $P > 0.05$ ).

### p53 expression

Forty-three of 81 HCCs (53.1%) showed positive staining for p53 protein. No positive p53 expression was observed in the corresponding non-tumorous liver tissue. p53 overexpression was more frequent in moderately differentiated tumors (23/43 HCCs, 53.5%) than in well differentiated tumors (8/23 HCCs, 34.8%), and was even more frequent in poorly differentiated tumors (12/15 HCCs, 80%) (poorly *vs.* well differentiated,  $P = 0.0064$ ). A trend approaching significance was observed between positive p53 expression and tumor portal vein invasion ( $P = 0.06$ ). No association was found between positive p53 expression and any other clinicopathological parameters examined.

### Relationship between p21<sup>WAF1/CIP1</sup> and p53 expression

The expression of p21<sup>WAF1/CIP1</sup> was significantly associated with p53 status ( $P = 0.0008$ ); 38 of the 43 tumors (88.4%) with aberrant p53 expression showed loss of p21<sup>WAF1/CIP1</sup> expression and 38 of the 59 tumors (64.4%) without p21<sup>WAF1/CIP1</sup> expression were accompanied by altered p53 expression (two tumors showing an inverse staining pattern of p21<sup>WAF1/CIP1</sup> and p53 are shown in Figures 1A–D). However, 21 of the 59 (35.6%) tumors lacking p21<sup>WAF1/CIP1</sup> protein showed p53-negative staining, and 5 tumors showed concurrent positive staining of p21<sup>WAF1/CIP1</sup> and p53.

The relationship between p21<sup>WAF1/CIP1</sup> and p53 expression was further evaluated in HCV-related HCCs, HBV-related HCCs and HCCs with no virus infection. The expression of p21<sup>WAF1/CIP1</sup> was significantly correlated with p53 expression in HCV-related HCCs, but not in HBV-related HCCs or HCCs without virus infection.

The tumors were divided into two groups based on the expression patterns of the two proteins: I, p21<sup>WAF1/CIP1</sup>+ / p53- (17 HCCs; no

abnormality in either protein); and II, p21<sup>WAF1/CIP1</sup>+/p53+, p21<sup>WAF1/CIP1</sup>-/p53- or p21<sup>WAF1/CIP1</sup>-/p53+ (64 HCCs; at least one altered expression involved in the two proteins). When this was done, a trend approaching significance was observed between the two groups ( $P = 0.066$ ); intrahepatic metastasis was observed more frequently in group II (11/64, 17.2%) than in group I (0/17, 0%). There was no significant relationship between either group and any of the clinicopathological features examined ( $P > 0.05$ ).

### 考察

p21<sup>WAF1/CIP1</sup> plays a key role in p53-mediated arrest of the cell cycle in response to DNA damage (Xiong et al, 1993; El-Deiry et al, 1993, 1994; Dulic et al, 1994). p21<sup>WAF1/CIP1</sup> inhibits transition from G1 to the S phase by inhibiting a wide variety of cyclin-CDK complexes, including cyclin D-CDK4, cyclin D-CDK2, and cyclin E-CDK2 (Xiong et al, 1993; Harper et al, 1993; Gu et al, 1993). Strikingly, in normal cells, p21<sup>WAF1/CIP1</sup> is associated with quaternary complexes of most cyclins, CDKs, and proliferation cell nuclear antigens, but is absent from these complexes in most transformed cells (Xiong et al, 1993). These observations suggest that reduction or loss of p21<sup>WAF1/CIP1</sup> expression plays an important role in tumorigenesis. Our results showed that absence of p21<sup>WAF1/CIP1</sup> expression was very common (59/81; 72.8%) in HCCs, suggesting that the absence of p21<sup>WAF1/CIP1</sup> expression may be involved in hepatocarcinogenesis.

There are several possible mechanisms that downregulate p21<sup>WAF1/CIP1</sup> expression. First, the expression of p21<sup>WAF1/CIP1</sup> is transcriptionally induced by wild-type but not mutant p53 (El-Deiry et al, 1993). We have shown previously that HCCs with wild-type p53 express significantly greater p21<sup>WAF1/CIP1</sup> mRNA than tumors with mutant p53 (Hui et al, 1997). In the present study, absence of p21<sup>WAF1/CIP1</sup> protein was significantly associated with altered p53 expression ( $P = 0.0008$ ). Drawing together the observations of our present and previous (Hui et al, 1997) studies, we suggest that the p53-dependent transcriptional pathway is the main mechanism that regulates p21<sup>WAF1/CIP1</sup> expression in HCCs.

Second, 21 of the 59 (35.6%) tumors lacking p21<sup>WAF1/CIP1</sup> protein showed p53-negative staining. Considering that negative immunoreactivity of p53 reflects functional p53, we hypothesize that, in these cases, p21<sup>WAF1/CIP1</sup> is probably downregulated by other factor(s). Recently, it has been reported that HCV core protein suppresses the transcriptional activity of the p21<sup>WAF1/CIP1</sup> promoter (Ray et al, 1998). In the present study, 59

(including 5 HCCs positive for both HCV and HBV markers) of the 81 HCCs were associated with HCV infection. Therefore, we consider it is highly probable that HCV core protein downregulates p21<sup>WAF1/CIP1</sup> expression in HCV-related HCCs. In this study group, 13 patients (20%) were HBsAg-positive. No direct evidence that HBV virus represses p21<sup>WAF1/CIP1</sup> promoter activity has been reported.

Third, proteins can be regulated at the post-transcriptional level. Downregulation of p21<sup>WAF1/CIP1</sup> expression through degradation by a ubiquitin-dependent proteolytic pathway has recently been reported (Maki and Howley, 1997). Therefore, we suggest that post-transcriptional regulation may be another mechanism that downregulates p21<sup>WAF1/CIP1</sup> expression in HCCs. Drawing together the results of our present and previous (Hui et al, 1997) studies, we consider that the higher incidence of p21<sup>WAF1/CIP1</sup> protein absence (72.8%) than the incidence of reduced p21<sup>WAF1/CIP1</sup> mRNA expression (38.1%) further supports this hypothesis. These observations also suggest that analysis of p21<sup>WAF1/CIP1</sup> status at the protein level may be more sensitive than analysis at the mRNA level.

Fourth, a mutation rate of 5% in p21<sup>WAF1/CIP1</sup> has been reported in HCCs (Furutani et al, 1997), so mutation of p21<sup>WAF1/CIP1</sup> may be a possible mechanism leading to altered p21<sup>WAF1/CIP1</sup> expression in a small proportion of HCCs.

We found concurrent positive expression of p21<sup>WAF1/CIP1</sup> and p53 in five HCCs. Because p53 overexpression is a hallmark of nonfunctional p53, the expression of p21<sup>WAF1/CIP1</sup> in these cases may be induced by p53-independent pathways (Michieli et al, 1994; Zhang et al, 1995).

Two previous studies have evaluated the relationship between the expression of p21<sup>WAF1/CIP1</sup> and p53 at the protein level in HCCs, although the results were controversial (Qin et al, 1998; Naka et al, 1998). Naka et al demonstrated a significant inverse correlation between expression of the two proteins, whereas Qin et al did not. This discrepancy may be due to the difference in hepatitis viral infection status between the two groups of patients, since the molecular basis underlying HCV-related and HBV-related HCCs appears to differ, at least partially. In Qin's group (Chinese patients), the majority (81/97, 84%) of the HCCs were HBV-related, and a small subset (16/97, 16%) were HBsAg-negative. No information about HCV infection was given for this group. On the other hand, in Naka's group (Japanese patients), 38% of HCCs were HBV-related, 44% were HCV-related, and 18% had no hepatitis virus infection. In both of these studies, the relationship between p21<sup>WAF1/CIP1</sup> and p53 expression in HBV-related HCCs or HCV-related HCCs or HCCs without viral infection was

not studied separately. In contrast to the situation in China, the majority (more than 65%) of HCCs in Japan are associated with HCV, and only a small portion (10-15%) are caused by HBV infection (Okuda, 1997). In our study group, 54 of the 81 HCCs (67%) were HCV-related. We found that there was an inverse relationship between p21<sup>WAF1/CIP1</sup> and p53 expression in HCV-related HCCs, but failed to establish such a relationship in HBV-related HCCs or HCCs without viral infection, suggesting that different modes of p21<sup>WAF1/CIP1</sup> regulation are involved in HCCs differing in their hepatitis viral infection status. Our data are generally consistent with the results of Qin et al and Naka et al, and offer a credible explanation for the discrepancy between the conclusions of the above two studies.

We found that p53 overexpression was significantly associated with poor tumor differentiation. This is consistent with results of a previous study of p53 in HCCs (Ng et al, 1995). We also found that p53 overexpression was more frequent in tumors with portal vein invasion than in tumors without. These observations suggest that altered p53 may be involved in the progression of HCCs.

Increased p21<sup>WAF1/CIP1</sup> mRNA levels can make metastatic human melanoma cells lose their metastatic potential (Jiang et al, 1995). We found a tendency for a relatively higher rate of p21<sup>WAF1/CIP1</sup> expression in the group without intrahepatic metastasis than in the group with intrahepatic metastasis, indicating a potential role of p21<sup>WAF1/CIP1</sup> dysfunction in the process of HCC metastasis. A correlation between loss of p21<sup>WAF1/CIP1</sup> expression and tumor metastasis has been previously reported in gastric carcinoma (Ogawa et al, 1997). It is striking that we found no intrahepatic metastasis in 17 HCCs with no abnormality in either the p21<sup>WAF1/CIP1</sup> or the p53 protein, and that all HCCs with intrahepatic metastasis had altered expression of p21<sup>WAF1/CIP1</sup>, p53, or both. These observations strongly suggest that the p53-p21<sup>WAF1/CIP1</sup> cell-cycle-regulating pathway may play an important role in suppressing tumor metastasis in HCCs.

We previously showed that other CDK inhibitors, p16<sup>INK4</sup> (Hui et al, 1996b) and p27<sup>Kip1</sup> (Hui et al, 1998), are involved in hepatocarcinogenesis. p16<sup>INK4</sup>, p21<sup>WAF1/CIP1</sup>, and p27<sup>Kip1</sup> appear to be independently inactivated and dysfunction of CDK inhibitors is a very common event in HCCs (Hui et al, 1996a, 1998; Hui and Makuuchi, 1999c). Understanding the mechanisms that inactivate these CDK inhibitors may be important for seeking new biological therapies for HCCs.

In conclusion, our present study has shown that: (1) absence of p21<sup>WAF1/CIP1</sup> expression is very common in HCCs; (2) p21<sup>WAF1/CIP1</sup> is probably regulated via different pathways in HCCs differing in their

hepatitis viral infection status, and p21<sup>WAF1/CIP1</sup> expression appears to be predominantly related to altered p53 in HCV-related HCCs; (3) in addition to p53-dependent transcriptional regulation, HCV core protein suppression of the transcriptional activity of the p21<sup>WAF1/CIP1</sup> promoter and post-transcriptional regulation are alternate pathways by which p21<sup>WAF1/CIP1</sup> expression can be downregulated; (4) disruption of the p53-p21<sup>WAF1/CIP1</sup> cell-cycle-regulating pathway may contribute to malignant progression of HCC.

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