Reduced $p21^{WAF1/CIP1}$ protein expression is predominantly related to altered p53 in hepatocellular carcinomas

Y-Z Shi, A-M Hui, T Takayama, X Li, X Cui and M Makuuchi

Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Summary To investigate the relationship between the expression of $p21^{WAF1/CIP1}$ protein and p53 status and the possible role of the two proteins in hepatocellular carcinomas (HCCs), we examined the expression of $p21^{WAF1/CIP1}$ and p53 immunohistochemically in 81 tumours from 65 patients with hepatocellular carcinoma. $p21^{WAF1/CIP1}$ protein was absent from 59 of 81 tumours (72.8%), and altered p53 expression was found in 43 (53.1%). $p21^{WAF1/CIP1}$ expression was significantly associated with p53 status ($P=0.0008$); 38 of 59 tumours lacking $p21^{WAF1/CIP1}$ protein were accompanied by altered p53 expression. Further analyses showed that $p21^{WAF1/CIP1}$ expression was inversely correlated with p53 expression in hepatitis C virus (HCV)-related HCCs, but not in HBV-related hepatocellular carcinomas and hepatocellular carcinomas without viral infection. All 11 tumours with intrahepatic metastasis showed altered $p21^{WAF1/CIP1}$ or p53 expression. In contrast, no intrahepatic metastasis was found in any of the 17 tumours without abnormal expression of either of the two proteins. These results suggest that: (1) different modes of $p21^{WAF1/CIP1}$ regulation are involved in HCCs differing in their hepatitis viral infection status, and $p21^{WAF1/CIP1}$ protein can be inducible in p53-null cells; (2) disruption of the p53–$p21^{WAF1/CIP1}$ cell-cycle-regulating pathway may contribute to malignant progression of HCC. © 2000 Cancer Research Campaign

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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. $p21^{WAF1/CIP1}$ protein, a universal CDK inhibitor (Xiong et al, 1993), and p53 protein are two important components of the G1 restriction point.

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

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

We previously investigated $p21^{WAF1/CIP1}$ mRNA expression by reverse-transcriptase polymerase chain reaction (RT-PCR) and p53 mutational status by PCR single-strand conformation polymorphism (SSCP) and direct DNA sequencing in hepatocellular carcinomas (HCCs). We suggested that $p21^{WAF1/CIP1}$ mRNA expression is regulated predominantly by a p53-dependent pathway and that reduced $p21^{WAF1/CIP1}$ mRNA expression may contribute to hepatocarcinogenesis (Hui et al, 1997). However, we did not evaluate the expression of $p21^{WAF1/CIP1}$ and p53 at the protein levels. 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 $p21^{WAF1/CIP1}$ 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; Finlay et al, 1988; Iggo et al, 1990; Hall et al, 1991; Farmer et al, 1992; Yew and Berk, 1992; Steegenga et al, 1996; Somasundaram and El-Deiry, 1997).

We investigated the expression of $p21^{WAF1/CIP1}$ and p53 proteins in 81 tumours from 65 patients with HCC by immunostaining to determine whether $p21^{WAF1/CIP1}$ expression depends on p53 functional status, and the relationship between the two proteins’ expression and clinicopathological features.
MATERIALS AND METHODS

Patients and specimens

Eighty-one primary HCC tissues were obtained from 65 patients who underwent surgical resection at the Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan. 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 years). 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 two were positive for both markers). The remaining nine 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 tumours varied from 0.6 to 15 cm (mean 4.0 ± 3.5 s.d.). The 81 tumours comprised 23 well-, 43 moderately, and 15 poorly differentiated HCCs. Portal vein tumour thrombi were found in 18 (22.2%) HCCs, and intrahepatic metastatic lesions were found in 11 (13.6%).

Immunohistochemical staining for p21WAF1/CIP1 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 non-specific binding activity, slides were incubated with blocking serum at room temperature for 30 min. Sections then were incubated with primary monoclonal antibody against p21WAF1/CIP1 (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 colour reagent, and haematoxylin was used as a counterstain. Normal oesophageal squamous epithelium and HCC with known p53 gene mutation and p53 protein overexpression were used as positive controls for p21WAF1/CIP1 and p53 respectively. Negative controls were obtained by omitting primary antibody. Only nuclear staining was considered to be positive for p21WAF1/CIP1 and p53. Based on

Figure 1  Representative examples of immunohistochemical staining of p21WAF1/CIP1 and p53. Tumour shows positive nuclear staining for p21WAF1/CIP1 (A) while not for p53 (B). Tumour is completely negative for p21WAF1/CIP1 (C), while p53 is diffusely stained (D). (× 350)
the previously published criteria, positive staining of p21WAF1/CIP1 was considered when ≥ 5% of tumour 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

χ² test or Fisher’s exact test was used to examine differences and relationships between groups of patients classified by p21WAF1/CIP1 and p53 staining. Differences at P < 0.05 were judged to be statistically significant.

RESULTS

p21WAF1/CIP1 expression

p21WAF1/CIP1 immunoreactivity was always nuclear, and no cytoplasmic staining of p21WAF1/CIP1 was seen in any specimen. The HCCs showed heterogeneous expression of p21WAF1/CIP1; the percentage of p21WAF1/CIP1-positive cells ranged from 0% to 60% (median 5%). Twenty-two of 81 HCCs (27.2%) showed positive staining for p21WAF1/CIP1 protein (Figure 1A). Most (86.4%) p21WAF1/CIP1-negative tumours showed < 1% positive cells or complete loss of immunoreactivity. p21WAF1/CIP1 expression was not associated with viral infection, histological features of non-tumourous livers, tumour size, tumour differentiation, intrahepatic metastasis, or tumour portal or hepatic vein involvement (P > 0.05; Table 1).

p53 expression

Forty-three of 81 HCCs (53.1%) showed positive staining for p53 protein (Figure 1D). No positive p53 expression was observed in the corresponding non-tumourous liver tissue. p53 overexpression was more frequent in moderately differentiated tumours (23/43 HCCs, 53.5%) than in well-differentiated tumours (8/23 HCCs, 34.8%), and was even more frequent in poorly differentiated tumours (12/15 HCCs, 80%) (poorly vs well-differentiated, P = 0.0064; Table 1). A trend approaching significance was observed between positive p53 expression and tumour portal vein invasion (P = 0.06; Table 1). No association was found between positive p53 expression and any other clinicopathological parameters examined.

Relationship between p21WAF1/CIP1 and p53 expression

The expression of p21WAF1/CIP1 was significantly associated with p53 status (P = 0.0008, Table 2); 38 of the 43 tumours (88.4%) with aberrant p53 expression showed loss of p21WAF1/CIP1 expression and 38 of the 59 tumours (64.4%) without p21WAF1/CIP1 expression were accompanied by altered p53 expression (two tumours showing an inverse staining pattern of p21WAF1/CIP1 and p53 are shown in Figure 1A–D). However, 21 of the 59 (35.6%) tumours lacking p21WAF1/CIP1 protein showed p53-negative staining, and five tumours showed concurrent positive staining of p21WAF1/CIP1 and p53.

The relationship between p21WAF1/CIP1 and p53 expression was further evaluated in HCV-related HCCs, HBV-related HCCs and HCCs with no virus infection (Table 2). The expression of

### Table 1 Relationship between p21WAF1/CIP1 and p53 protein expression and clinicopathological features in HCCs

| Clinicopathology                        | p21WAF1/CIP1 (–) | p-value | p53 (+) | P-value |
|----------------------------------------|------------------|---------|---------|---------|
| Virus infectiona                        |                  |         |         |         |
| HBV                                    | 11/13 (84.6%)    | 0.39    | 5/13 (38.5%) | 0.71 |
| HCV                                    | 37/54 (68.5%)    | 0.85    | 30/54 (55.6%) |       |
| HBV + HCV                              | 3/5 (60.0%)      | 0.46    | 3/5 (60.0%)  |       |
| Negative                                | 8/9 (88.9%)      |         | 5/9 (55.6%)  |       |
| Background liver disease                |                  |         |         |         |
| Chronic hepatitis                       | 15/18 (83.3%)    |         | 9/18 (50.0%)  | 0.85 |
| Liver cirrhosis                         | 44/63 (69.8%)    |         | 34/63 (54.0%) |       |
| Tumour size (cm)                        |                  |         |         |         |
| <2                                     | 21/29 (72.4%)    | 0.52    | 15/29 (51.7%)  | 0.73 |
| 2–5                                    | 23/29 (79.3%)    |         | 17/29 (58.6%)  |       |
| ≥5                                     | 15/23 (65.2%)    |         | 11/23 (47.8%)  |       |
| Tumour differentiation                  |                  |         |         |         |
| Well                                    | 15/23 (65.2%)    | 0.40    | 8/23 (34.8%)  |       |
| Moderate                                | 34/43 (79.1%)    |         | 23/43 (53.5%)  |       |
| Poor                                    | 10/15 (66.7%)    |         | 12/15 (80.0%)  |       |
| Intrahepatic metastasis                 |                  |         |         |         |
| Positive                                | 9/11 (81.8%)     | 0.47    | 7/11 (63.6%)  | 0.45 |
| Negative                                | 50/70 (71.4%)    |         | 36/70 (51.4%)  |       |
| Portal involvement                      |                  |         |         |         |
| Positive                                | 12/18 (66.7%)    | 0.50    | 13/18 (72.2%)  | 0.06 |
| Negative                                | 47/63 (74.6%)    |         | 30/63 (47.6%)  |       |
| Venous involvement                      |                  |         |         |         |
| Positive                                | 4/7 (57.1%)      | 0.33    | 4/7 (57.1%)  | 0.82 |
| Negative                                | 55/74 (74.3%)    |         | 39/74 (52.7%)  |       |

*a HBV, HBsAg-positive; HCV, anti-HCV-antibody-positive; HBV + HCV, both markers positive; Negative, both markers negative. †Well vs poorly differentiated, P = 0.0064; ‡Moderately vs poorly differentiated, P = 0.07.
p21\textsuperscript{WAF1/CIP1} was significantly correlated with p53 expression in HCV-related HCCs, but not in HBV-related HCCs or HCCs without virus infection.

The tumours were divided into two groups based on the expression patterns of the two proteins: I, p21\textsuperscript{WAF1/CIP1}/p53\textsuperscript{−} (17 HCCs; no abnormality in either protein); and II, p21\textsuperscript{WAF1/CIP1}/p53\textsuperscript{+} (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).

**DISCUSSION**

p21\textsuperscript{WAF1/CIP1} plays a key role in p53-mediated arrest of the cell cycle in response to DNA damage (El-Deiry et al, 1993, 1994; Xiong et al, 1993; Dulic et al, 1994). p21\textsuperscript{WAF1/CIP1} 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 (Gu et al, 1993; Harper et al, 1993; Xiong et al, 1993). Strikingly, in normal cells, p21\textsuperscript{WAF1/CIP1} 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\textsuperscript{WAF1/CIP1} expression plays an important role in tumorigenesis. Our results showed that absence of p21\textsuperscript{WAF1/CIP1} expression was very common (59/81; 72.8%) in HCCs, suggesting that the absence of p21\textsuperscript{WAF1/CIP1} expression may be involved in hepatocarcinogenesis.

There are several possible mechanisms that down-regulate p21\textsuperscript{WAF1/CIP1} expression. First, the expression of p21\textsuperscript{WAF1/CIP1} 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\textsuperscript{WAF1/CIP1} mRNA than tumours with mutant p53 (Hui et al, 1997). In the present study, absence of p21\textsuperscript{WAF1/CIP1} 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\textsuperscript{WAF1/CIP1} expression in HCCs.

Second, 21 of the 59 (35.6%) tumours lacking p21\textsuperscript{WAF1/CIP1} protein showed p53-negative staining. Considering that negative immunoreactivity of p53 reflects functional p53, we hypothesize that, in these cases, p21\textsuperscript{WAF1/CIP1} is probably down-regulated by other factor(s). Recently, it has been reported that HCV core protein suppresses the transcriptional activity of the p21\textsuperscript{WAF1/CIP1} promoter (Ray et al, 1998). In the present study, 59 (including five 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 down-regulates p21\textsuperscript{WAF1/CIP1} expression in HCV-related HCCs. In this study group, 13 patients (20%) were HBsAg-positive. No direct evidence that HBV virus represses p21\textsuperscript{WAF1/CIP1} promoter activity has been reported.

Third, proteins can be regulated at the post-transcriptional level. Down-regulation of p21\textsuperscript{WAF1/CIP1} 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 down-regulates p21\textsuperscript{WAF1/CIP1} 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\textsuperscript{WAF1/CIP1} protein absence (72.8%) than the incidence of reduced p21\textsuperscript{WAF1/CIP1} mRNA expression (38.1%) further supports this hypothesis. These observations also suggest that analysis of p21\textsuperscript{WAF1/CIP1} status at the protein level may be more sensitive than analysis at the mRNA level.

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

We found concurrent positive expression of p21\textsuperscript{WAF1/CIP1} and p53 in five HCCs. Because p53 overexpression is a hallmark of non-functional p53, the expression of p21\textsuperscript{WAF1/CIP1} 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\textsuperscript{WAF1/CIP1} and p53 at the protein level in HCCs, although the results were controversial (Qin et al, 1998; Naka et al,
metastasis in HCCs. A tendency for a relatively higher rate of p21WAF1/CIP1 expression was observed in HCCs that altered p53 may be involved in the progression of HCCs. We also found that there was an inverse relationship between p21WAF1/CIP1 and p53 expression in HBV-related HCCs or HCCs without viral infection, suggesting that different modes of p21WAF1/CIP1 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 tumour 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 tumours with portal vein invasion than in tumours without. These observations suggest that altered p53 may be involved in the progression of HCCs. Increased p21WAF1/CIP1 mRNA levels can make metastatic human melanoma cells lose their metastatic potential (Jiang et al., 1995). It is striking that we found no intrahepatic metastasis in p53 knockout mice (El-Deiry et al., 1997). It is evident that p53 plays an important role in suppressing tumour metastasis in HCCs.

We previously showed that other CDK inhibitors, p16INK4 (Hui et al., 1996b) and p27kip1 (Hui et al., 1998), are involved in hepatocarcinogenesis. p16INK4, p21WAF1/CIP1 and p27kip1 appear to be independently inactivated and dysfunction of CDK inhibitors is a very common event in HCCs (Hui et al., 1996a, 1998; Hui and Makuchii, 1999). 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 p21WAF1/CIP1 expression is very common in HCCs; (2) p27kip1 expression is probably regulated via different pathways in HCCs differing in their hepatitis viral infection status, and p21WAF1/CIP1 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 p21WAF1/CIP1 promoter and post-transcriptional regulation are alternate pathways by which p21WAF1/CIP1 expression can be down-regulated; (4) disruption of the p53–p21WAF1/CIP1 cell-cycle-regulating pathway may contribute to malignant progression of HCC.

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