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TOPIC HIGHLIGHT

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Molecular mechanism underlying the functional loss of cyclindependent kinase inhibitors p16 and p27 in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common human cancers, and its incidence is still increasing in many countries. The prognosis of HCC patients remains poor, and identification of useful molecular prognostic markers is required. Many recent studies have shown that functional alterations of cell-cycle regulators can be observed in HCC. Among the various types of cell-cycle regulators, p16 and p27 are frequently inactivated in HCC and are considered to be potent tumor suppressors. p16, a G1-specific cell-cycle inhibitor that prevents the association of cyclindependent kinase (CDK) 4 and CDK6 with cyclin D1, is frequently inactivated in HCC *via* CpG methylation of its promoter region. p16 may be involved in the early steps of hepatocarcinogenesis, since p16 gene methylation has been detected in subsets of pre-neoplastic liver cirrhosis patients. p27, a negative regulator of the G1-S phase transition through inhibition of the kinase activities of Cdk2/cyclin A and Cdk2/cyclin E complexes, is now considered to be an adverse prognostic factor in HCC. In some cases of HCC with increased cell proliferation, p27 is overexpressed but inactivated by sequestration into cyclin D1-CDK4-containing complexes. Since loss of p16 is closely related to functional inactivation of p27 in HCC, investigating both p16 and p27 may be useful for precise prognostic predictions in individuals with HCC.

Key words: Hepatocellular carcinoma; Cell-cycle regulator; Cyclin-dependent kinase inhibitor; DNA methylation; DNA methyltransferase; p16; p27; FoxM1b

INTRODUCTION

The liver is a remarkable organ, since it can regenerate after significant tissue loss. When liver tissue is partially lost or injured, residual hepatocytes immediately enter the cell cycle from a quiescent (G0) state through pre-replicative (G1), DNA synthesis (S) and mitosis (M) states. Significantly, once the original volume of the liver is achieved, the hepatocytes revert to their quiescent state and restore the tissue volume to a physiological condition. This exquisite regenerative potential of the liver has been regarded as potentially being governed by sequential activation of a series of positive and negative cell-cycle regulators in hepatocytes. Briefly, cell-cycle entry is mainly promoted by cyclins A, D and E with cyclin-dependent kinase (CDK) 2 and 4 and the activities of these CDKs are negatively regulated by the cyclin-dependent kinase inhibitors (CDKIs) p16 and p27 to avert excessive hepatocellular replication. Therefore, it is understandable that many oncologists have sought to elucidate the molecular mechanism underlying cell-cycle regulators during hepatocarcinogenesis[1,2].

To date, many studies have reported genetic and epigenetic alterations of cell-cycle regulators in hepatocellular carcinoma (HCC). In particular, both p16 and p27 are frequently altered in HCC, suggesting that they are potent tumor suppressors. Of note, epigenetic alterations of p16 in liver cirrhosis have recently been reported[3-5], supporting the idea that p16 may be involved in the early stage of hepatocarcinogenesis. Moreover, we and others have found a close relationship between p16 and p27 during the progression of HCC[6-8], indicating that comprehensive analyses of these CDKIs may

be valuable for further understanding of the clinical significance of cell-cycle regulators in HCC. In this review, an overview of the molecular mechanism underlying p16 and p27 inactivation in HCC is presented, and the clinical significance of the relationship between these CDKIs in individuals with a high risk of HCC is described.

FREQUENT INACTIVATION OF p16 IN HCC

p16 is a specific inhibitor of CDK4 and CDK6 and potentially blocks G1 cell-cycle progression *via* dephosphorylation of Rb through its inhibitory effect on CDK4/cyclin D1 complex activity[9,10]. The *CDKN2/MTS1* gene encoding p16 is frequently deleted or inactivated by 5'-CpG island hypermethylation in various types of cancer cells, and p16 is considered to be a potent tumor suppressor[10,11]. Many studies have focused on searching for the genetic/epigenetic status of p16 in HCC with a view to investigating the molecular mechanism of hepatocarcinogenesis. Biden *et al* reported that p16 was frequently mutated or deleted in human HCC in an Australian population[12], while Chaubert *et al* reported germline mutations of p16 in a subset of familial HCC in Switzerland[13] and Piao *et al* reported the presence of a homozygous deletion of p16 in 61% of HCC cases in a Korean population[14]. On the contrary, we examined the genetic status of p16 in 60 HCC cases in Japan but did not find any homozygous loss of *p16*[15]. Only 4 of 60 (6.6%) HCC cases showed intragenic mutations in exon 2 of *p16*, and no hotspots for amino acid changes were detected in our study. Significantly, however, we found that the p16 gene promoter was methylated in 27 of 60 (45%) HCC cases. A close relationship between loss of p16 immunostaining and hypermethylation of the p16 gene was found, indicating that epigenetic alterations induced loss of p16 protein expression in HCC[15].

Interestingly, 24 of 60 (40%) HCC cases showed high levels of methylation in the p16 gene promoter region, ranging from 60%-85% of the CpG islands as assessed by methylation-sensitive single nucleotide primer extension[16]. The reason why the patterns of p16 inactivation differ among the studies is unclear. It should be noted that Li *et al* reported that HCCs with *p16* methylation were only found in individuals with hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and not in virus-negative individuals[4]. Since many other studies have found rare homozygous deletions and infrequent mutations of *p16* in HCCs[17-19], epigenetic changes may be the main reason for p16 inactivation in hepatitis virus-associated HCC, while genetic mutations of p16 may occur in specific geographical conditions and pedigrees.

MOLECULAR MECHANISM OF p16 DNA METHYLATION IN HCC

The molecular basis of the aberrant hypermethylation of CpG islands of p16 observed in many cases of HCC is unknown. One possible mechanism is upregulation of DNA (cytosine-5)-methyltransferase (DNMT), which is widely assumed to be responsible for most of the methylation of the human genome. Unfortunately, however, the relationship between DNA methyltransferase and gene methylation in cancer cells is controversial. For example, Eads *et al* reported that deregulation of DNA methyltransferase gene expression in human colorectal cancer cells did not play a role in establishing tumor-specific abnormal DNA methylation patterns[20]. Furthermore, Rhee *et al* reported that the p16 gene remained fully methylated and silenced in colorectal carcinoma cells lacking DNMT1, while genetic disruption of both DNMT1 and DNMT3b nearly eliminated methyltransferase activity and reduced genomic DNA methylation by more than 95%, indicating that cooperation of these two enzymes may be essential for maintaining DNA methylation in cancer cells[21,22]. In contrast, Robert *et al* reported that specific depletion of DNMT1, but not DNMT3a or DNMT3b, markedly potentiated the ability of the demethylating agent 5-aza-2'- deoxycytidine to reactivate silenced tumor-suppressor genes[23], suggesting that DNMT1 may be sufficient for maintaining CpG island methylation in human cancer cells.

Histone deacetylation is another possible mechanism of p16 methylation, since DNA methylation and repressive chromatin characterized by histone deacetylation appear to act as synergistic layers for the silencing of global genes. However, the molecular relationship between p16 and histone deacetylation is not clear. Cameron *et al* reported that hypermethylated genes, including p16, cannot be transcriptionally reactivated with trichostatin A (a specific inhibitor of histone deacetylase)[24]. Zhu *et al* reported that p16 repressed in human lung cancer cells was induced by synergistic cooperation of depsipeptide (an inhibitor of histone deacetylase) and 5-aza-2'-deoxycytidine[25]. Interestingly, however, they also reported that cells treated with higher concentrations of 5-aza-2'-deoxycytidine and depsipeptide showed decreased p16 expression together with significant suppression of cell growth[25]. These lines of evidence indicate that the molecular mechanism of p16 DNA methylation in cancer cells may vary among different types of cancer cells.

It is noteworthy that Guan *et al* reported a close relationship between K-ras mutations and p16 methylation in colon cancer, by showing that a K-ras-transformed colon cancer cell line exhibited increased DNA methyltransferase activity and p16 gene methylation[26]. In HCC, Weihrauch *et al* reported that frequent K-ras mutations and p16 methylation were found in vinyl chloride-induced HCC[27], suggesting that K-ras mutations may be important events in p16 inactivation in chemically-induced HCC. Although there have been no reports of the relationship between hepatitis virus infection and K-ras mutation, these reports may shed new light on why p16 is inactivated in many cases of HCC. A thorough examination of the relationship between p16 methylation and K-ras mutations in HCC is awaited.

p16 GENE IS METHYLATED DURING THE EARLY STEPS OF HEPATOCARCINOGENESIS

Several recent studies have revealed that *p16* is methylated in some sets of individuals with non-HCC liver tissues

associated with chronic hepatitis virus infection. Kaneto *et al* reported that *p16* methylation was detected in 5 of 17 (29.4%) cirrhosis patients and 4 of 17 (23.5%) chronic hepatitis patients, all of whom were associated with HBV or HCV infection, but not in normal liver and other non-viral liver diseases[3]. Similarly, Li *et al* reported that the *p16* gene was methylated in 6 of 38 (16%) HBV-infected or HCV-infected chronic hepatitis and cirrhosis patients bearing HCC[4]. We found that p16 was methylated in 4 of 112 (4%) liver cirrhosis patients[5], all of whom were infected with HCV (personal communication). Although the ratio of *p16* DNA methylation in non-HCC liver tissues observed in our study was low compared with previous studies, these lines of evidence suggest that hepatitis virus infections may play roles in the induction of *p16* promoter methylation. Very recently, Jung *et al* reported that HBV X protein induced DNA hypermethylation of the p16 promoter to repress its expression and led to transcriptional activation of DNMT1 *via* the cyclin D1-CDK4/6-pRb-E2F1 pathway[28], suggesting a close relationship between hepatitis virus infections and aberrant methylation of the p16 gene. Therefore, examinations of the degree of *p16* DNA methylation in non-HCC liver tissues with hepatitis virus infections, by bisulfate sequencing for example, may be of use for further elucidating the roles of hepatitis viruses in p16 inactivation in the liver.

CLINICAL SIGNIFICANCE OF p16 IN HCC

To investigate the clinical significance of p16 inactivation in HCC, we evaluated the labeling index (LI) of p16 in HCCs by immunohistochemical staining and found that the proportion of tumors with negative staining increased as the histopathologic grading of the tumors tended to become more poorly differentiated. Similarly, Ito *et al* reported that the p16 LI was significantly decreased in cases with advanced-stage HCC[29]. However, regarding the prognosis of individuals with HCC, p16 does not seem to be an independent risk factor. Tannapfel *et al* reported that they were unable to establish alterations of the p16 locus as an independent prognostic factor for HCC[30]. Anzola *et al* found no associations between p16 inactivation and clinicopathological characteristics or prognosis[31], and suggested that this may arise because p16 potentially plays an important role in hepatocarcinogenesis and is frequently altered from the early stages of HCC. As described later, we recently found that the status of p16 affects the prognosis of HCC with p27 overexpression[6]; indicating p16 may become a supportive prognostic factor in HCC when combined with other prognostic factors. In other words, p16 may become an important therapeutic target for HCC. Although there have been little studies of the relationship between the level of CDK4/6 activities and p16 inactivation in HCC patients, the fact that p16 is widely inactivated in HCC does give us the idea that a clinical trial of CDK4/6-selective inhibitor in patients with HCC may be hopeful.

REGULATORY MECHANISM OF p27

P27 is a member of the KIP family of CDKIs[32,33], which negatively regulates the G1-S phase transition by inhibiting the kinase activities of CDK2/cyclin A and CDK2/cyclin E complexes. During the cell cycle, the p27 protein level is highest at G0/G1 phase and lowest at S phase, and is mainly regulated by protein degradation *via* a Skp1-Cullin-F-box protein (SCF)-type ubiquitin ligase complex that contains Skp2 as the substrate-recognizing subunit[34,35]. It has also been found that Skp2 acts as the main rate-limiting regulator for p27 degradation[36-38] and that cyclin kinase subunit 1 (Cks1) is essential for efficient Skp2-dependent destruction of p27[39,40].

p27 IS AN ADVERSE PROGNOSTIC FACTOR IN HCC

It is noteworthy that p27 is widely regarded as an adverse prognostic indicator in many types of cancers, since decreased or absent expression of p27 is frequently observed in cell nuclei in various types of human cancers with poor prognoses[41]. In HCC, many studies have reported that decreased p27 expression is closely associated with clinical invasiveness of the tumors. Ito *et al* reported that the p27 LI was significantly decreased in cases with portal invasion, poor differentiation, larger size and intrahepatic metastasis among 104 HCC cases examined[29], and suggested that p27 can act as an independent predictor of HCC recurrence among several types of G1-S cell-cycle regulators (e.g., pRb, p21, p16, p53, cyclin D1 and cyclin E). Fiorentino *et al* examined 54 HCC cases and reported that high expression of p27 was a favorable independent prognostic parameter[42]. Tannapfel *et al* reported that p27 was decreased in advanced cases in a series of curatively resected HCCs[43]. Qin *et al* reported that longer disease-free survival rates were observed in patients whose tumors had higher p27 (KIP1) expression [44]. Armengol *et al* reported that p27 constituted an independent predictor of recurrence after surgical resection in 46 cirrhotic patients with small HCCs[45]. Zhou *et al* reported that the LI of p27 was associated with differentiation, invasiveness and metastasis of tumors among 45 HCC cases examined[46]. Similar to these previous reports, we found that HCCs expressing low levels of p27 (low-p27 expressers; p27 LI of $< 50\%$ in the tumor cells), as evaluated by immunohistochemical staining, showed significantly favorable prognoses compared with individuals with HCCs showing high p27 expression (high p27 expressers; p27 LI of $> 50\%$). Kaplan-Meier survival curves revealed that the 5-year survival rates of the low-p27 and high-p27 expressers were 62% and 93%, respectively, and log-rank tests revealed that low p27 expression was associated with a higher relative risk of dying from HCC than high p27 expression[6]. Taken together, p27 can be regarded as a powerful clinical indicator for prognosis prediction in individuals with HCC.

p27 IS OVEREXPRESSED IN SOME SETS OF AGGRESSIVE HCCS

As described above, many studies have suggested that decreased p27 expression can be regarded as a risk factor in individuals with HCC. Recently, however, extensive

studies have unraveled a novel aspect of the role of p27. Sganmbato *et al* reported that the levels of p27 were significantly increased in some breast cancer cell lines that exhibited exponential growth and high levels of cyclin D1 and cyclin E[47]. Fredersdorf *et al* reported that p27 expression was increased in some cases of highly proliferative breast cancer cells overexpressing cyclin D1[48]. Interestingly, Ciaparrone *et al* reported that a subset (35%) of colorectal carcinomas displayed diffuse cytoplasmic staining for p27 by immunohistochemical staining[49], and Singh *et al* reported that cytoplasmic localization of p27 was associated with decreased survival in Barrett's associated adenocarcinoma[50].

To comprehensively investigate the clinical significance of p27, we thoroughly examined the p27 status in HCC by immunohistochemical staining and found that the Ki-67 LI, a proliferation marker, varied from low to high among the high-p27 expressers (p27 LI of > 50%). Our results indicated that the tumors could be categorized into two groups according to a Ki-67 LI threshold of 20%, revealing that 26 of 40 (65%) high-p27 expressers had a Ki-67 LI of < 20% (2%-13%), while the remaining 14 (35%), including all 7 cases with cytoplasmic p27 immunostaining, had a Ki-67 LI of > 20% (22%-42%)[6]. To address the reason for the different cell proliferation status among high-p27 expressers, the kinase activity of CDK2 was analyzed in HCC samples from the high-p27 expressers. The results revealed that the CDK2 activities were low in 8 of 12 (67%) tumors with a Ki-67 LI of < 20%, but 5-8-fold higher in the remaining 4 cases. In contrast, the kinase activities in all 8 HCC cases in a subgroup with a Ki-67 LI of > 20% were significantly higher (20-fold-50-fold higher than their matched non-tumorous liver samples). Heating the tumor sample lysates effectively reduced the CDK2 activities, indicating that p27 *per se* was functional and not inactivated by either DNA mutations or genetic loss, since p27 is heat-stable. These lines of evidence indicate that p27 does not simply act as a tumor suppressor in some types of cancer cells, and that a thorough examination of the biological status of p27 should be performed.

FUNCTIONAL INACTIVATION OF p27 IS CLOSELY ASSOCIATED WITH SEQUESTRATION TO CYCLIN D1-CDK4-COMPLEXES

To understand the molecular mechanism of the different levels of kinase activities among the high-p27 expressers, examination of the components of p27-containing complexes may be useful, since it has been reported that sequestration of p27 from CDK2 to cyclin D1-containing complexes results in a blockade of p27 functions as well as stabilization of active cyclin D1-CDK4/CDK6 complexes[51,52]. Therefore, we performed immunoprecipitation assays using 20 tissue sample lysates from high-p27 expressers and found that, in 12 tumors with low levels of kinase activities (< 8-fold relative to the levels in adjacent non-tumorous tissues), p27 was closely associated with CDK2 in 8 samples and faintly associated with cyclin D1-CDK4 complexes in 4 samples. In contrast, in all 8 HCC samples with high levels of kinase activities (20-fold-50-fold higher relative to the levels in adjacent non-tumorous tissues), high levels of cyclin D1-CDK4-bound p27 were detected. These pieces of evidence strongly indicate that compositional changes in the complexes containing p27 may be an alternative reason for the increased cell proliferation in p27-overexpressing HCC.

THE COMPOSITIONS OF COMPLEXES CONTAINING p27 VARY AMONG HIGH p27 EXPRESSERS

Several cell-cycle regulatory factors are known to influence the compositional status of p27-containing complexes. For example, cyclin D1 and CDK4 mobilize p27 from cyclin E-CDK2 complexes to cyclin D-CDK4 complexes, while p15 and p16 shift p27 to associate with cyclin E-CDK2 complexes[53,54]. We examined the expressions of these cell-cycle regulatory factors in high-p27 expressers, and found that p16 was closely correlated with the status of complexes containing p27, while CDK4 and cyclins D1, D3 and E were not. Our results revealed that p16 was expressed in 9 of 12 high-p27 expressers in which p27 was predominantly associated with CDK2, but was undetectable in all 8 HCCs in which p27 was closely associated with cyclin D1-CDK4 complexes[6]. Although there have been no other reports of the molecular relationships between p16 and p27-containing complexes, it is noteworthy that Sanchez-Beato *et al* reported a close relationship between p16 loss and anomalous p27 expression in aggressive B-cell lymphomas[55]. Further studies are needed to address whether the status of p16 influences any clinicopathological characteristics in human tumors expressing high levels of p27.

p16 DETERMINES THE PROGNOSIS OF HCCS WITH p27 OVEREXPRESSION

To further investigate the functional role of p16 in the cell proliferation status of highp27 expressers, we examined the LIs of p16 and Ki-67 in HCCs and found that the p16 LI was inversely correlated with the Ki-67 LI in high-p27 expressers. Immunohistochemical analyses revealed that p16 staining was positive in all 26 (100%) high-p27 expressers with a Ki-67 LI of < 20%, but negative in 12 of 14 (85%) high-p27 expressers with a Ki-67 LI of > 20%. Interestingly, methylation of the *p16* gene promoter was not detected in all 26 (100%) samples with positive p16 immunostaining, but was detected in 10 of 12 (83%) samples with negative p16 immunostaining. Therefore, we surmise that epigenetic changes of the *p16* gene may be the main cause of the p27 inactivation in HCCs that express considerable amounts of p27. Since we also found that loss of p16 expression was an independent prognostic factor for a poor outcome in high-p27 expressers compared with standard prognostic variables[6],

assessment of the *p16* status may be useful for precise prognostic prediction in individuals with HCCs expressing high levels of p27.

FORKHEAD TRANSCRIPTION FACTORS AND p27

Recently, Forkhead box M1B (Foxm1b), a ubiquitously expressed member of the Forkhead Box (Fox) transcription factor family, has been focused upon as another regulatory factor of p27 expression. Foxm1b expression is restricted to proliferating cells and mediates both hepatocyte entry into DNA synthesis and mitosis during liver regeneration[56,57]. Wang *et al* showed that regenerating livers in aged mice exhibited diminished Foxm1b expression with significant reductions in hepatocyte proliferation and increased levels of p27 expression[58], thereby providing new evidence that Foxm1a regulates p27 at the protein level. Kalinichenko *et al* reported that Foxm1b was essential for the development of a rodent HCC model, and its mechanism was associated with reduced expression of p27 and increased expression of the CDK1-activator Cdc25B phosphatase[59]. More significantly, they also showed that Foxm1b was a novel inhibitory target of the p19 (ARF) tumor suppressor, and that a peptide containing p16 may represent a potential therapeutic agent toward Foxm1boverexpressing cancer cells. Since FoxM1 is essential for transcription of Skp2 and Cks1[60], which are specificity subunits of the SCF ubiquitin ligase complex that targets p27, examining the relationship between Foxm1b and p27 in human HCC may help toward understanding of the molecular mechanism of hepatocarcinogenesis.

CONCLUSION

HCC is one of the most common human cancers, and its incidence is still increasing in civilized countries. HCC represents the fifth most-common malignant disease in the world and is the third most-common cause of cancer-related death worldwide[61,62]. The etiology of HCC is unique in that most HCC cases are associated with liver cirrhosis or chronic hepatitis attributable to HBV or HCV infection, chronic alcohol abuse and, more recently, non-alcoholic steatohepatitis[63]. Since the prognosis of HCC patients remains poor, identification of useful molecular prognostic markers for HCC is required. As described in the present review, coordinated examination of the p16 and p27 statuses may become a more accurate tool for predicting the prognosis of HCC. Further studies of cell-cycle regulators will provide better insights into the mechanism of hepatocarcinogenesis.

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