Loss of p16 Contributes to p27 Sequestration by Cyclin D1-Cyclin-dependent Kinase 4 Complexes and Poor Prognosis in Hepatocellular Carcinoma

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ABSTRACT
Purpose: p27Kip1 (p27) might act as an adverse prognostic marker for various types of cancers. However, its clinical usefulness remains uncertain, because it is sometimes overexpressed in aggressive types.

Experimental Design: To precisely evaluate the practical significance of p27 in hepatocellular carcinoma (HCC), we immunohistochemically compared the level of p27 expression with Ki-67 labeling in 74 HCCs and focused on tumors in which cell proliferation increased despite a high level of p27 expression. We then analyzed the status of p27 and related cell-cycle regulators using kinase and immunoprecipitation assays, Western blotting, and methylation-specific PCR to understand the rationale for the functional inactivation of p27 in HCC. We also evaluated relationships between the key biological characteristics of HCC and survival.

Results: Immunohistochemical studies showed that 40 (54%) of 74 HCCs expressed high levels of p27 (>50% of the tumor cells). Of these, the Ki-67 labeling index was low (<20%) in 26 (65%) and high (>20%) in 14 (35%). Increased proliferative activities were closely correlated with elevated kinase activities, sequestration of p27 protein, and p16 gene methylation. The association between a loss of p16 and poor prognosis was significant when p27 expression was high (P < 0.01).

Conclusions: The loss of p16 appears to be closely related to the functional inactivation of p27, and assessment of p16 status may be useful for a precise prognostic prediction of individuals with HCC expressing high levels of p27.

INTRODUCTION

p27Kip1 (p27) is a tumor suppressor of the KIP family of CDK inhibitors. Null mice or those encoding a heterozygous p27 gene are predisposed to cancer (1) and expression of this gene product is reduced in various types of human cancers. One remarkable finding is that decreased p27 expression in the nuclei of cancer cells is often associated with a poor prognosis, indicating that p27 is a promising adverse prognostic factor (2). Because p27 exerts a major role in inhibiting S-phase entry in the cell cycle via inactivating CDK2 (3, 4), tumor aggressiveness might be attributable to decreased p27 expression.

However, recent studies have generated much conflicting evidence. In some aggressive types of cancers, p27 increases abnormally in the nucleus and/or cytoplasm (5–10). Increased levels of p27 expression are associated with a poorer prognosis in B-cell lymphocytic leukemia (11). Taken together, the biological behavior of p27 may be somewhat complex and might differ among types of cancer.

HCC is a highly aggressive disease with a poor prognosis that requires a reliable prognostic marker. Several studies have suggested that a decrease in the level of p27 expression could indicate a poor prognosis for patients with HCC (12–15). However, anomalous p27 expression in this type of tumor has not been investigated. To precisely evaluate its practical significance, the functional status of p27 expressed in cancer cells should be thoroughly investigated. We, therefore, examined levels of p27 expression and cell-proliferation status in HCCs by immunohistochemical analysis and focused on tumors with increased proliferative activities despite high levels of p27 expression. We investigated the compositional state of p27 in these tumors to understand the regulatory mechanism involved in the functional inactivation of p27 in HCC. We also examined the expression of a series of related cell-cycle regulators to determine differences in the biological characteristics between tumors with and without increased cell proliferation among high p27 expressers.

MATERIALS AND METHODS

Tissue Samples. HCC tissues (mean size, 6.5 ± 3.8 cm) were obtained from 74 patients, some of whom had been subjects in our previous study (16). All underwent curative surgery without postoperative systemic chemotherapy (66
Follow-up data of the postoperative outcomes were retrospectively obtained from all of the patients, and the follow-up period ranged from 5 to 120 months (mean, 81 months). Twelve patients were positive for HBV surface antigen, 54 were positive for HCV antibody, and 8 were positive for both. Histological grades were classified according to the General Rule for Clinical and Pathological Study of Primary Liver Cancer adopting the Tumor-Node-Metastasis classification (Liver Cancer Study Group of Japan, 4th edition, 2000) as well (n = 12), moderately (n = 51), and poorly differentiated (n = 11). Tissue samples were immediately processed after surgical removal. For histological examination, all tumorous and surrounding nontumorous tissue portions were fixed in formalin and embedded in paraffin. Protein was analyzed in 38 snap-frozen tumorous and adjacent nontumorous tissue samples that were stored at −80°C.

Control nontumorous tissue samples were obtained from four normal, three HBV-related cirrhotic, and five HCV-related cirrhotic livers that were obtained at surgery for other conditions. All individuals provided written, informed consent to participate in the study, which was approved by the institutional guidelines of Niigata University Graduate School of Medicine and Dental Science.

**Immunohistochemical Analysis of p27 and Ki-67.** Deyara-fined tissue sections were microwave-heated to retrieve antigen and reacted with a monoclonal antibody against full-length p27 (K25020; Transduction Laboratories, Lexington, KY) at a dilution of 1:500 for 2 h at room temperature. Color was developed using the Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine. Staining specificity was evaluated by previous absorption of the antibody with recombinant p27 protein (Santa Cruz Biotechnology, CA), and negative control slides were reacted with normal mouse immunoglobulin under similar conditions. Counting 1000 cells assessed the L.I. of p27-positive cells. Samples below or above the cutoff value of 50% of the L.I. were categorized as “low” or “high” p27 expressers, respectively (13–15). We assessed the proliferation index in the tumor area using a monoclonal antibody against Ki-67 nuclear antigen (Clone Ki-S5; DAKO, Copenhagen, Denmark; diluted 1:25) as described by the manufacturer.

**Western Blotting for p27.** To confirm the results of p27 immunostaining, aliquots of tissue lysates (10 μg of protein)
from 38 snap-frozen HCC samples were resolved by SDS-PAGE, transferred onto Immobilon-P membranes (Millipore, Bedford, MA), and incubated with polyclonal antibody against p27 (C-19; Santa Cruz Biotechnology). Protein bands were visualized using the Amersham ECL detection system (Amersham Life Science, Arlington Heights, IL) and quantified with a Bio-Image analyzer (LAS 2000; Fuji Photo Film Co. Ltd., Tokyo, Japan).

Kinase Assay of High p27 Expressers. Tissue lysates (300 µg of protein) obtained from high p27 expressers were clarified over 50% protein A-Sepharose (Pharmacia, Piscataway, NJ; see Ref. 19). The beads were washed in reaction buffer consisting of 50 mM HEPES (pH 8.0) containing 2.5 mM ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 10 mM MgCl₂, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 10 mM β-glycerophosphate, 0.1 mM sodium vanadate, 1 mM sodium fluoride, 10 µM ATP, and 20 µM [γ-³²P]ATP (5 µCi; Pharmacia Biotech, Piscataway, NJ). Reacted products were resolved by SDS-PAGE and visualized by autoradiography, and phosphorylation was assessed using a Bio-Image analyzer. To evaluate heat-stable CDK-inhibitory activity, tissue lysates (300 µg of protein) were heated at 95°C for 5 min and clarified by centrifugation (18). The supernatant was incubated with 100 µg of untreated lysate of the same tissue sample for 20 min at 30°C, and kinase was assayed as described above.

Immunoprecipitation Assay of High p27 Expressers. Tissue lysates (250 µg of protein) obtained from high p27 expressers were incubated with antibodies against cyclin D₁ (M-20), CDK2 (M2), and CDK4 (C-22), as well as Protein G Plus/Protein A agarose beads (Calbiochem; Oncogene Research Products, La Jolla, CA; see Ref. 19). The beads were washed in lysis buffer, resolved by SDS-PAGE, and reacted with an antibody against p27 (C-19; Santa Cruz Biotechnology). Products, La Jolla, CA; see Ref. 19). The beads were washed in lysis buffer, resolved by SDS-PAGE, and reacted with an antibody against p27 (C-19; Santa Cruz Biotechnology). Products, La Jolla, CA; see Ref. 19).

Analysis of Cell-Cycle Regulator Expression in High p27 Expressers. Expression of a series of related cell-cycle regulators in high p27 expressers was examined by Western blotting under conditions similar to those described above. Polyclonal antibodies were directed against p15 (K-18), p16INK4a (p16; C-20), CDK4 (C-22), cyclin D₁ (M-20), cyclin D₃ (C-16), and cyclin E (M-20; Santa Cruz Biotechnology).

Immunohistochemical analysis for p16 proceeded using the Cox proportional hazard model to assess the relationship between overall survival and potential categories of prognostic varieties. The association between p27 and p16 and clinicopathological varieties of the patients in a group of high p27 expressers was assessed by the χ² test and by Fisher’s exact test. Overall survival was measured from the date of sample collection to the date of the last follow up or death, and survival curves plotted according to the Kaplan-Meier method (21) were used to assess possible correlations between each of the subgroups and overall survival period. Differences in survival curves were estimated by the log-rank test using Stat View 5.0 software (SAS Institute, Cary, CA). All reported P values were two sided, and data were considered statistically significant at P < 0.05.

RESULTS

Expression of p27 in HCCs. Immunohistochemical staining for p27 showed that 34 of 74 HCCs (46%) expressed <50% of the L.I. (low p27 expresser; Fig. 1A), whereas 40 (54%) expressed >50% of the L.I. (high p27 expresser; Fig. 1B). Within a group of high p27 expressers, cytoplasmic staining was concomitant in >50% of the cells in sections from seven patients (Fig. 1C). Western blotting of tissue lysates obtained from 38 HCC samples closely correlated with the results of the immunohistochemical analysis. Among 18 snap-
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The antibody. Western blot for p27 of each sample is shown in the corresponding nontumorous tissue samples (Fig. 1). The remainder was relatively lower than that of each of their corresponding tissue samples (Fig. 1). Among 20 high p27 expressers, band intensity was equivalent to an 8-fold increase over that of surrounding nonneoplastic tissues (Fig. 1E, 3 and 4; others not shown).

Proliferative Activities Differ among High p27 Expressers. We examined the proliferative activities of HCC in a group of high p27 expressers by immunostaining for Ki-67. The L.I.s of Ki-67 varied from low to high, and the tumors could be categorized into two groups according to a threshold of 20% of the index (Fig. 2). Twenty-six of 40 (65%) high p27 expressers were within 20% (2–13%) of the average of Ki-67-positive cells, whereas 14 (35%), including all 7 with cytoplasmic p27 immunostaining, were >20% of the index, ranging from 22% to 42%.

CDK2 Activities of High p27 Expressers Correlate with the Proliferative Activities. We obtained 20 samples from high p27 expressers for protein analysis. Of these, 12 were <20% of the Ki-67 L.I. in the tumor area, and 8 were >20%. We detected CDK2 activities in all high p27 expressers examined, and their corresponding nontumorous tissues expressed faint activities (Fig. 3A). The correlation was relatively close between the level of kinase activities and Ki-67 L.I.s in high p27 expressers. Kinase activities in 8 of 12 tumors with <20% Ki-67 labeling were low, with a densitometric intensity that was 1–2-fold higher, whereas the intensity in the remaining 4 was 5–8-fold higher than their corresponding adjacent nontumorous tissues (Fig. 3A, 1; others not shown). In contrast, kinase activities in all eight samples from a subgroup with an increased Ki-67 index (>20%) were significantly higher, from 20 to 50-fold when compared with their matched nontumorous liver samples (Fig. 3A, 4 and 5; others not shown). Heating tissue extracts from the same sources effectively inactivated CDK2 activities (Fig. 3A; bottom).

Composition of Complexes Containing p27 in High p27 Expressers. To understand the diversity of kinase activities among high p27 expressers, we immunoprecipitated the 20 tissue lysate samples described above. In 12 high p27 expressers in which kinase activities were <8-fold that in adjacent nontumorous tissues, p27 was closely associated with CDK2 in 8 samples (Fig. 3B, 1–3; others not shown) and faintly with cyclin D1–CDK4 in 4 samples (Fig. 3B, 1 and 3; others not shown). In contrast, we detected a high level of cyclin D1–CDK4-bound p27 in all eight HCC samples, with increased kinase activities ranging from 20- to 50-fold higher than in corresponding nontumorous tissues (Fig. 3B, 4 and 5; others not shown).

Expression Profiles of Cell-Cycle Regulators in High p27 Expressers. We examined the expression of a series of cell-cycle regulatory factors among high p27 expressers using the tissue lysates that were processed for immunoprecipitation assays (Fig. 3C). Western blotting showed variable

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### Table 1 Relationship between Ki-67 L.I. and p16 expression in the cases of high p27 expressers (P < 0.0001)

<table>
<thead>
<tr>
<th>Ki-67 L.I.</th>
<th>P16 expression</th>
<th>Total</th>
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<tbody>
<tr>
<td>≤20%</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>&gt;20%</td>
<td>12</td>
<td>2</td>
</tr>
</tbody>
</table>

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frozen tissues from low p27 expressers, a protein band corresponding to p27 was undetectable in eight, and the intensity in the remainder was relatively lower than that of each of their corresponding nontumorous tissue samples (Fig. 1E, 1 and 2; others not shown). Among 20 high p27 expressers, band intensity was equivalent to an 8-fold increase over that of surrounding nonneoplastic tissues (Fig. 1E, 3 and 4; others not shown).
levels of CDK4 and cyclin D1, D3, and E expression and no remarkable correlation with the status of complexes containing p27. The expression of p15 was not altered as compared with nontumorous tissues. The correlation between the level of p16 expression and the status of complexes containing p27 was close. In 12 high p27 expressers in which p27 was dominantly associated with CDK2, band intensity corresponding to p16 was faint but detectable in 3 (Fig. 3C, 1; others not shown) and intense in 9 (Fig. 3C, 2 and 3; others not shown). In contrast, p16 was undetectable in all eight samples in which p27 was closely associated with cyclin D1-CDK4 (Fig. 3C, 4 and 5; others not shown).

**Fig. 4** Comparison between p27 and p16 expression and Ki-67 L.I. in high p27 expressers. A. Immunohistochemical staining for p27, Ki-67, and p16. Left, tissue sections of the same high p27 expresser with low Ki-67 L.I. (2%) and high p16 expression. Right, tissue sections of high p27 expresser with high Ki-67 L.I. (22%) and loss of p16 expression. Original magnification, ×50. B. MSP of p16 in high p27 expressers. Primer sets used for amplification were unmethylated p16 (U) and methylated p16 (M). N1, normal liver; N2, nontumorous cirrhotic liver; 1 and 2, high p27 expressers showing positive p16 staining by immunohistochemical analysis; 3 and 4, high p27 expressers with loss of p16 staining. Left lane shows φX174/HaeIII digest.

**p16 Expression Inversely Correlates with Ki-67 Labeling in High p27 Expressers.** To precisely investigate the relationship between p16 expression and Ki-67 labeling in high p27 expressers, we examined the immunohistochemical expression of p16. Among 40 high p27 expressers, 28 showed positive nuclear heterogeneous to homogenous p16 staining in the tumor cells, and 12 were negative for p16. All of seven negative p16 expressers showed cytoplasmic p27 staining. The relationship between p16 expression and Ki-67 L.I.s was close and inverse in high p27 expressers (Table 1). p16 staining was positive in all of the subsets in a group of high p27 expressers with a Ki-67 index <20% (n = 26; Fig. 4A,
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Loss of p16 Is Correlated with DNA Methylation in High p27 Expressers. To address whether DNA methylation is associated with a loss of p16 expression in subsets of high p27 expressers, we performed MSP on tissue sections. A methylated p16 gene promoter was undetectable in all of 26 samples (100%) that were immunohistochemically positive for p16 (Fig. 4B, 1 and 2), but detectable in 10 of 12 tumors (83%) with negative p16 immunostaining (Fig. 4B, 3 and 4).

Survival Analysis. Within the follow-up period, 12 of 34 (35%) low p27 expressers and 7 of 40 (18%) high p27 expressers died of cancer recurrence. Kaplan-Meier survival curves showed that the 5-year survival rate was 62% and 93% for the low and high p27 expressers, respectively. Log-rank tests showed that low p27 expression was associated with a relative risk of dying from HCC when compared with high p27 expressers (P = 0.002; Fig. 5A). The HCC patients in a group of high p27 expressers (n = 40) were further divided according to the status of p16 expression determined by immunohistochemical analysis into groups A (p16-negative; n = 12) and B (p16-positive; n = 28). The status of p16 expression was not significantly associated with clinical variables such as age, sex, size, and histological type of the tumors (data not shown). Five of 12 (42%) patients in group A died of HCC, whereas two of 28 (7%) died in group B within the follow-up period. Kaplan-Meier curves and the log-rank test showed that the 5-year overall survival rates were 70% and 100% for groups A and B, respectively, and that the loss of p16 expression was closely correlated with a poorer clinical outcome in the group of high p27 expressers (P = 0.0004; Fig. 5B). Multivariate analysis by the Cox proportional hazard model showed that the loss of p16 expression was an independent poor prognostic factor in the high p27 expressers when compared with standard prognostic variables (P = 0.03; Table 2).

DISCUSSION

Deregulated cell-cycle progression is one of the most significant alterations in cancer cells. The step during G1 to S phase is thought to be a key target of tumorigenesis, which is, in part, negatively regulated by two types of CDK inhibitors. The INK4 family of CDK inhibitors (p15, p16, p18, and p19) specifically inhibit D-type cyclin-CDK4/CDK6 complexes and the KIP/CIP family of CDK inhibitors (p21, p27, and p57) associates with different types of cyclin-CDK complexes. Of these, p27 is presently regarded as a potent adverse prognostic factor because it is abundantly expressed in quiescent cells and is down-regulated in many aggressive cancers (2–4). The function of p27 is regulated at the levels of transcriptional induction, mRNA stabilization, ubiquitin proteasome-mediated proteolytic degradation, and noncovalent sequestration (2). Because our immunohistochemical studies showed that p27 was reduced in 34 of 74 HCCcs (46%), one of the main mechanisms for abrogating p27 in HCC is its decreased expression, as observed in other types of cancers (2–4, 12–15).

Table 2 Univariate and multivariate analyses of prognostic factors in 40 HCC patients in a group of high p27 expressers

<table>
<thead>
<tr>
<th>Factor</th>
<th>Hazard ratio</th>
<th>95% Confidence Interval</th>
<th>P *</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>0.946</td>
<td>0.838–1.069</td>
<td>0.375</td>
</tr>
<tr>
<td>Tumor size</td>
<td>0.966</td>
<td>0.784–1.191</td>
<td>0.748</td>
</tr>
<tr>
<td>Histology (well-mod./poor)</td>
<td>0.420</td>
<td>0.076–2.128</td>
<td>0.283</td>
</tr>
<tr>
<td>Clinical stage (I–II/III–IV)</td>
<td>0.491</td>
<td>0.109–2.206</td>
<td>0.353</td>
</tr>
<tr>
<td>Ki-67 (high/low L.I.)</td>
<td>7.525</td>
<td>1.438–39.384</td>
<td>0.0168</td>
</tr>
<tr>
<td>p16 (−/+</td>
<td>0.089</td>
<td>0.017–0.471</td>
<td>0.004</td>
</tr>
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* Analyzed by Cox’s proportional hazards general linear model.

The hazards ratio is given as well or moderate (mod.) vs. poor in the differentiation.

The hazards ratio is given as stage I or II versus III or IV defined by the General Rules for the Clinical and Pathological Study of Primary Liver Cancer adopting the tumor-node-metastasis classification.
in 14 of 40 HCCs (35%), together with intense p27 expression. CDK2 activities were high in all eight samples obtained from this subset. These results suggest that another mechanism of p27 inactivation is involved in the increased proliferative status in HCCs. Because p27 is heat-stable, we examined whether CDK2 inhibition in these tumors would also be heat-stable. Heating lysates from tumor samples effectively reduced CDK2 activities, indicating that p27 per se was functional and not inactivated by either DNA mutation or by genetic loss. Immunoprecipitation assays showed that p27 was exclusively sequestered to cyclin D1-CDK4 in these tissues. Therefore, a compositional change in the complex containing p27 may be an alternative reason for inactivating p27 in HCC. This environment might be crucial for inhibiting CDK activities because it results in a blockade of the p27 function and the stabilization of active cyclin D1-CDK4/CDK6 complexes (22, 23).

The reason why subsets of HCCs lost p27 function via sequestration is a concern. Several cell-cycle regulatory factors influence the compositional status of complexes containing p27. Cyclin D1 and CDK4 mobilize p27 from cyclin E-CDK2 to D-type cyclin-CDK4 (4), whereas p15 and p16 shift p27 to associate with cyclin E-CDK2 (4, 24, 25). Our results showed that p16 was obvious in 9 of 12 samples with dominant association between p27 and CDK2, but negligible in all 8 HCCs in which cyclin D1-CDK4 was closely associated with p27. These data suggest that the status of p16 affects cell proliferation in HCC via maintaining p27-containing complexes. Immunohistochemical analysis may support this hypothesis because p16 was lost in 12 of 14 high p27 expressers with an increased level of Ki-67. We are now investigating the presence of prominent biological features in the remaining two samples.

As far as we know, the first to show that p16 is closely associated with the compositional status of p27 in tumorous tissues, which substantiates the results of experiments (24, 25). Although many investigators have examined the expression of p16 and p27 in human tumors, only Sanchez-Beato et al. (26) identified a close relationship between p16 loss and anomalous p27 expression. Further studies are needed to address whether the status of p16 influences any clinicopathological characteristics in human tumors expressing high levels of p27. We also found that the loss of p16 in these subsets was associated with DNA methylation, as found in other types of cancer (27). Epigenetic alterations in the p16 gene might be the main cause of p27 inactivation in HCCs in which a considerable amount of p27 is expressed.

All HCCs with cytoplasmic p27 immunostaining displayed increased cell-proliferative activities and a poor prognosis in the present study, and investigating this mechanistic nature should be of interest. Several aggressive cancers express cytoplasmic p27, which is mostly associated with cyclin D3 (3, 4), suggesting that an association between subcellular p27 and cytoplasmic D-type cyclins allows active D-type cyclin-CDK complexes to freely move in the nucleus (10, 19). Akt phosphorylates p27 at Thr198 and promotes binding to 14-3-3 protein, allowing cytoplasmic translocation (28). Phosphorylated p27 at Ser10 also contributes to the cytoplasmic displacement of p27 by recruiting binding to the nuclear export protein chromosome region maintenance 1 (29, 30). We are presently investigating the mechanism of cytoplasmic p27 expression by using more HCC samples.

The most notable finding of the present study was that all of the tumors expressing cytoplasmic p27 lost p16. Although it remains unclear which of these two independent events occurred first, they might have synergistically acted to promote tumor aggressiveness.

Finally, the present study shows that the level of p27 expression does not fully explain its functional value in HCCs. Thus, p16 expression and cell-proliferative activity should be examined to resolve this inconsistency. We surmise that a coordinate examination of p16 and p27 status could become a more accurate tool with which to predict the prognosis of HCC.

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