Promoter Hypermethylation of the p16 Gene Is Associated with Poor Prognosis in Recurrent Early-Stage Hepatocellular Carcinoma

Eunkyung Ko,1,2 Yujin Kim,1,2 Sung-Joo Kim,2 Jae-Won Joh,2 SangYong Song,3 Cheol-Keun Park,3 Joobae Park,1,4 and Duk-Hwan Kim1,4

1Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon, Korea; 2Departments of Surgery and Pathology, Samsung Medical Center; and 3Center for Genome Research, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Seoul, Korea

Abstract

Despite significant advances in the detection and treatment of hepatocellular carcinoma, the prognosis of patients with hepatocellular carcinoma remains very poor, in part due to the high incidence of recurrence. This study was aimed at identifying a prognostic indicator of recurrence in patients with hepatocellular carcinoma. We retrospectively analyzed CpG island hypermethylation of the p14, p15, p16, GSTP1, integrin α4, SYK, and CDH1 genes in fresh-frozen tissues from 265 patients with hepatocellular carcinoma using the methylation-specific PCR. The expression levels of p16 and p53 were evaluated by immunohistochemistry. CpG island hypermethylation was detected in 6% for p14, 21% for p15, 67% for p16, 75% for GSTP1, 23% for integrin α4, 12% for SYK, and 57% for CDH1. Recurrence was observed in 102 (38%) of the 265 patients. There was no association between the risk for recurrence and hypermethylation of any gene studied. However, p16 methylation was associated with a poor survival after surgery for recurrent stage I to II hepatocellular carcinomas (hazard ratio, 4.05; 95% confidence interval, 1.15-14.20; P = 0.03). In addition, the hazard of failure after recurrence was about 3.80 (95% confidence interval, 1.03-14.20; P = 0.04) times higher in patients with p16 methylation than in those without. Negative expression of p16 at a protein level was also associated with poor survival in recurrent stage I to II hepatocellular carcinomas, but p53 expression did not have a synergistic effect on the poor prognosis. In conclusion, the present study suggests that p16 methylation may be associated with a poor prognosis in recurrent early-stage hepatocellular carcinomas. (Cancer Epidemiol Biomarkers Prev 2008;17(9):2260–7)

Introduction

Hepatocellular carcinoma is the sixth most common cancer worldwide, and it is the third most common cause of cancer-related death (1). About 600,000 people are diagnosed with hepatocellular carcinoma every year, with the highest incidences occurring in eastern and southeastern Asia, sub-Saharan Africa, and Melanesia. Although the incidence of hepatocellular carcinoma is low in developed areas, Latin America, and south-central Asia, the incidence of hepatocellular carcinoma is rising in Western countries, and it is therefore becoming an important issue in these countries. Despite significant improvements in diagnostic methods, treatments, and perioperative care for hepatocellular carcinoma, the prognosis is unsatisfactory in part due to the high incidence of recurrence. Even in patients who undergo curative surgical resection of hepatocellular carcinoma, the recurrence rates are as high as 50% at 2 years and approach 75% to 100% at 5 years (2-4). Prolonged survival after hepatocellular carcinoma resection depends largely on the aggressive management of recurrence. Aggressive management of postoperative recurrence with therapies such as transarterial chemoembolization could prolong survival. Thus, the identification of prognostic factors for recurrence would not only provide guidance in the choice of efficient treatments, but it may also aid in selecting patients for adjuvant therapy. In addition to the identification of numerous clinicopathologic factors that might serve as prognostic tools for the prediction of patient outcome after curative resection of hepatocellular carcinoma, many attempts have recently been made to predict the prognosis for patients with hepatocellular carcinoma using epigenetic alteration of promoter regions in genes associated with hepatocellular carcinoma. The aberrant methylation of normally unmethylated CpG islands has become widely recognized as a means of inducing the transcriptional silencing of tumor suppressor genes in a variety of human cancers. Hypermethylation of CpG islands in the promoter region of >20 tumor suppressor genes has been reported in hepatocellular carcinoma.

It is necessary for transformed residual cancer cells to acquire the ability to replicate limitlessly and...
Materials and Methods

Study Population. Fresh samples of hepatocellular carcinoma tissues were obtained from 265 patients who underwent curative surgical resection for hepatocellular carcinoma between February 1999 and November 2004 at the Samsung Medical Center in Seoul, Korea. Written informed consent for the use of the surgically removed tumor tissues and paraffin blocks, which were composed and administered according to the protocols previously approved by the institutional review board at the Samsung Medical Center, was provided by all of the patients with hepatocellular carcinoma before the operation. Information about the patients’ demographics and lifestyle variables was obtained using an interviewer-administered questionnaire. None of the patients had any preoperative chemotherapy. All patients had no serum antibody against hepatitis C virus. The histopathologic features of hepatocellular carcinomas examined were tumor size, tumor necrosis, pathologic stage, intrahepatic metastasis, tumor capsule formation, and cirrhosis. The pathologic stage was determined according to the American Joint Committee on Cancer cancer staging criteria (15). Intrahepatic metastasis was matched to the criteria of the Liver Cancer Study group of Japan (16). The 265 patients consisted of 218 men (82%) and 47 women (18%), ranging in age from 28 to 78 years. The mean age at the time of the surgical resection for the hepatocellular carcinoma was 52.5 years. Recurrence or death was evaluated from information obtained from our hospital records and those from other hospitals as of October 31, 2007. We defined the recurrence as evidence of an overt new growing mass in the remaining liver or as new multiple lung nodules in three-phase helical Computed Tomography images of the liver, including the chest.

Methylation-Specific PCR. Genomic DNA was extracted from fresh-frozen tissues of hepatocellular carcinoma as previously described (17). Methylation-specific PCR was done to analyze the methylation status of seven genes, as previously described by Herman et al. (Fig. 1; ref. 18). Primer sequences and annealing temperatures for methylation-specific PCR have been previously described by several groups (5-12). DNA from the peripheral blood lymphocytes of healthy subjects was used as a negative control for the methylation-specific assays. Lymphocyte DNA from healthy volunteers was treated with SsI methyltransferase (New England BioLabs), followed by treatment with sodium bisulfite, and then used as a positive control for the methylated alleles. Bisulfite-modified DNA from normal lymphocytes served as a positive control for the unmethylated alleles. The unconverted DNA from normal lymphocytes was used as a negative control for methylated alleles.

Tissue Microarrays and Immunohistochemistry of p16 and p53. For the preparation of tissue microarrays, one normal region and three tumor regions from a donor block were carefully selected by H&E staining. Cores (1.5 mm in diameter) were punched from each area and transferred to a recipient paraffin block using a tissue microarrayer (Beecher Instruments). Five-micrometer-thick sections of the resulting tissue microarray blocks were immunostained with the p16 mouse monoclonal

![Figure 1. Methylation-specific PCR for the p14, p15, p16, GSTP1, integrin α4, SYK, and CDH1 genes. Patient identification numbers are given. Pos, positive controls for the methylated (M) and unmethylated (U) allele. Negative control samples without DNA were included for each PCR.](image-url)
antibody (p16INK4a Ab-6, Neomarker; Fig. 2A and B) and p53 mouse monoclonal antibody (DO-7, BD Biosciences; Fig. 2C and D), as previously described (19, 20). Negative controls (isotype-matched irrelevant antibody as primary antibody) were made to run simultaneously. For assessment of the positivity of immunostaining for each section, only nuclear staining was regarded as positive. We regarded p16 as positive when ≥10% of tumor cell nuclei showed positive immunostaining and p53 as positive when ≥5% of tumor cell nuclei showed positive immunostaining (20). We counted tumor cells with clearly brown reaction products in nuclei and the mean value of three tumor regions was used. All available blocks and slides were reviewed by pathologists (SY Song and C-K Park).

**Statistical Analysis.** The clinicopathologic differences and the methylation status of the seven genes studied in patients with and without recurrence were analyzed using a *t* test (or Wilcoxon rank sum test) and $\chi^2$ test (or Fisher’s exact test) for continuous and categorical variables, respectively. Multivariate logistic regression analysis was carried out to examine the relationship between the development of recurrence and the covariates determined to be statistically significant by univariate analysis and to calculate odds ratios after controlling for potential confounding factors. The effect of promoter methylation on the time to death or recurrence was estimated by the Kaplan-Meier method, and the significance of differences in survival or recurrence between the two groups was evaluated by the log-rank test. Cox proportional hazards model was used to evaluate the significance of the independent variables that were identified as important for survival in Kaplan-Meier analysis after controlling for potential confounding factors. All statistical analyses were two-sided with a type I error rate of 5%.

**Results**

**Clinicopathologic Characteristics and Recurrence.** At a median follow-up of 31 months, 102 (38%) of the 265 patients had developed disease recurrence. The associations between disease recurrence and their clinicopathologic features are listed in Table 1. The mean ages of the patients with and without recurrence were similar ($P = 0.19$). The tumor size was larger in patients with recurrence than in those without (4.1 cm versus 3.1 cm; $P = 0.04$). The percentage of necrosis was significantly higher in patients with recurrence than in those without (10.5% versus 6.5%, respectively; $P = 0.002$). Recurrence was more prevalent in male patients (39%) than in female patients (33%), but the difference was not statistically significant ($P = 0.49$). A significant relationship was also found between recurrence and pathologic stage ($P = 0.0004$). Recurrence occurred in 26% of the stage I cancers, in 27% of the stage II cancers, in 54% of the stage III cancers, and in 25% of the stage IV cancers. Patients with intrahepatic metastasis had a higher risk for recurrence than those without (60% versus 34%; $P = 0.001$). Recurrence was more prevalent in patients without capsule formation than in those with capsule formation ($P = 0.01$). Recurrence was unrelated to

**Figure 2.** Immunohistochemical staining of p16 and p53 in hepatocellular carcinoma. Immunohistochemical stainings of p16 (A) and p53 (C) show rare nuclear immunoreactivity in hepatocellular carcinoma. These tumors show moderate or intense nuclear staining for p16 (B) and p53 (D). Original magnification, ×200.
The activity of hepatitis was scored by the histologic activity index. The mean histologic activity index score was 4.2 ± 2.1 for the patients who were positive for hepatitis B surface antigen. Recurrence was more prevalent in Hepatitis B e antigen-positive cancers than in HBeAg-negative cancers, but the difference was not statistically significant (P = 0.14). No significant relationship was found between recurrence and liver cirrhosis (P = 0.53).

DNA Methylation and the Risk for Recurrence. The relationship between recurrence and the CpG island hypermethylation of the seven genes studied was investigated to identify biomarkers that would be useful for predicting recurrence after surgery in patients with hepatocellular carcinoma (Table 2). Of the 265 hepatocellular carcinoma tissue samples, hypermethylation was detected in 6% for p14, 21% for p15, 67% for p16, 75% for GSTP1, 23% for integrin α4, 12% for SYK, and 57% for CDH1. Because tumor recurrence was significantly associated with pathologic stage at the time of curative resection (P = 0.0004; Table 1), we stratified the data according to pathologic stage and reanalyzed the relationship between CpG island hypermethylation and recurrence. The number of patients in stage I and IV tumors was very small (31 and 4, respectively), and therefore, the patients were grouped into two tumor stage groups: stages I to II and stages III to IV. Recurrence was found in 39 (27%) of 147 stage I to II hepatocellular carcinomas and in 63 (53%) of 118 stage III to IV hepatocellular carcinomas. The prevalence of recurrence in stage I to II and in stage III to IV hepatocellular carcinomas was not significantly different between those with and without the hypermethylation of any gene (Table 2). Using recursive partitioning analysis, we also analyzed the relationship between the co-hypermethylation of any of the genes and recurrence, but found no relationship between them in tumors at any stage of malignancy (data not shown). Stratified multivariate logistic regression analysis was done to control for the potential confounding effects of variables such as age, tumor size, tumor necrosis, intrahepatic metastasis, capsule formation, and HBeAg status and to calculate the odds ratios. However, there was no relationship between the hypermethylation of any gene studied and the risk for recurrence in the stage I to II or stage III to IV hepatocellular carcinomas after adjusting for possible confounding factors (data not shown).

Association of Survival with DNA Methylation. Recurrence-free survival, overall survival, and survival after recurrence were compared between patients with and without the hypermethylation of any of the seven genes studied. The recurrence-free 5-year survival rates

### Table 1. Relationship between the recurrence and clinicopathologic features of hepatocellular carcinomas

<table>
<thead>
<tr>
<th></th>
<th>Recurrence</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Age*</td>
<td>53 ± 9</td>
<td>51 ± 11</td>
</tr>
<tr>
<td>Sex</td>
<td>3.1 ± 2.5</td>
<td>4.1 ± 3.5</td>
</tr>
<tr>
<td>Necrosis (%)*</td>
<td>6.5 ± 14.4</td>
<td>10.5 ± 16.8</td>
</tr>
<tr>
<td>Pathologic stage</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>Intrahepatic metastasis</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Capsule formation</td>
<td>18</td>
<td>27</td>
</tr>
<tr>
<td>HBsAg</td>
<td>125</td>
<td>64</td>
</tr>
<tr>
<td>HBeAg</td>
<td>136</td>
<td>85</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>145</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>110</td>
<td>65</td>
</tr>
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</table>

Abbreviation: HBsAg, hepatitis B surface antigen.

### Table 2. Association between the methylation and recurrence of hepatocellular carcinomas

<table>
<thead>
<tr>
<th>Met</th>
<th>Recurrence (stages I-II)</th>
<th>P</th>
<th>Recurrence (stages III-IV)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>p14</td>
<td>98</td>
<td>37</td>
<td>0.52*</td>
<td>53</td>
</tr>
<tr>
<td>p15</td>
<td>10</td>
<td>2</td>
<td>0.16*</td>
<td>42</td>
</tr>
<tr>
<td>p16</td>
<td>84</td>
<td>35</td>
<td>0.77</td>
<td>34</td>
</tr>
<tr>
<td>CDH1</td>
<td>51</td>
<td>20</td>
<td>0.66</td>
<td>33</td>
</tr>
<tr>
<td>GSTP1</td>
<td>31</td>
<td>10</td>
<td>0.71</td>
<td>11</td>
</tr>
<tr>
<td>Integrin α4</td>
<td>78</td>
<td>32</td>
<td>0.23</td>
<td>47</td>
</tr>
<tr>
<td>SYK</td>
<td>93</td>
<td>35</td>
<td>0.78*</td>
<td>46</td>
</tr>
</tbody>
</table>

Abbreviation: Met, methylation.

*Fisher's exact test.
were 76% for stage I cancers, 72% for stage II cancers, and 47% for stage III cancers, and these differences were statistically significant \((P < 0.001)\). The overall 5-year survival rates for stage I, II, and III cancers were 82%, 79%, and 61% \((P < 0.001)\), respectively. Recurrence-free survival and overall survival were not significantly different according to pathologic stage in patients with hypermethylation of genes and those without (data not shown). Although p16 methylation was not associated with the overall survival of all patients in stage I to II

Figure 3. Kaplan-Meier survival plots in 265 patients and 39 recurrent stage I to II hepatocellular carcinomas. A and B. Overall survival of all patients in stage I to II \((P = 0.11)\) and stage III to IV hepatocellular carcinomas \((P = 0.71)\) is not significantly associated with p16 methylation. C. In recurrent stage I to II hepatocellular carcinomas, patients with p16 methylation have poorer overall survival than those without p16 methylation \((P = 0.02)\). D. In addition, the survival after recurrence is also poorer in patients with p16 methylation than those without \((P = 0.03)\).

Figure 4. Kaplan-Meier survival plots in 39 recurrent stage I to II hepatocellular carcinomas. The overall survival (A) and the survival after recurrence (B) are also poorer in patients with negative expression of p16 than in those with positive expression of p16, regardless of p53 expression. The p53 expression does not have a significant synergistic effect on the poor survival resulting from the negative expression of p16. The \(P\) values were calculated for all four groups using the log-rank test. Negative sign, the lack of expression at the level of protein.
Table 3. Cox proportional hazards analysis in recurrent stage I to II hepatocellular carcinomas

<table>
<thead>
<tr>
<th></th>
<th>p16 Methylation</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall survival</td>
<td>No</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>4.05 (1.15-14.20)</td>
<td>0.03</td>
</tr>
<tr>
<td>Survival after recurrence</td>
<td>No</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.80 (1.03-14.02)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

NOTE: Adjusted for resection margin, Edmondson-Steiner grade, α-fetoprotein, cirrhosis, and serum albumin levels.

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

The 3-year overall survival rates for the 39 patients that experienced recurrence of stage I to II hepatocellular carcinomas were 41% and 83% in patients with p16 methylation and those without, respectively, and this difference was statistically significant (P = 0.02). The 2-year survival rates after recurrence were 37% and 81% in patients with p16 methylation and those without, respectively, and this difference was also statistically significant (P = 0.03). The median survival times after surgery and recurrence were 27 and 19 months, respectively, in the recurrent stage I to II hepatocellular carcinomas with p16 methylation. For the stage III to IV hepatocellular carcinomas, p16 methylation was not associated with survival after recurrence or overall survival in recurrent cancers (data not shown).

Association of Survival with p16 or p53 Protein Expression. The relationship between p16 and p53 expression was analyzed to understand the possible mechanism underlying the poor prognosis in the recurrent patients with hepatocellular carcinoma with p16 methylation. The p16 expression was negative in 172 (96%) of 180 hepatocellular carcinoma tissues with p16 methylation, suggesting that p16 methylation was functional in this study (P < 0.001; Fisher’s exact test). The expression of p53 was positive in 52 (20%) of the 265 patients. Survival in recurrent stage I to II hepatocellular carcinomas was finally examined with respect to p16 and/or p53 expression. Of the 39 patients that did experience recurrence in stage I to II hepatocellular carcinomas, both overall survival (Fig. 4A) and survival after recurrence (Fig. 4B) were found to be extremely poor in patients with negative expression of p16. However, p53 expression did not have a synergistic effect on the survival of patients with negative expression of p16. The 3-year overall survival rates were 37% in patients with negative expression of p16 and positive expression of p53, and 41% in those with negative expression of p16 and p53; however, this difference was not statistically significant (P = 0.73).

Cox Proportional Hazards Analysis. A stratified Cox proportional hazards analysis was conducted according to pathologic stage to determine whether p16 methylation in recurrent cases is an independent prognostic factor of survival after controlling for potential confounding factors. A number of variables are known to be associated with patient prognosis after surgery or recurrence (21-23) and were considered to estimate hazard ratios in univariate Cox proportional hazards analyses for overall and recurrent-free survival and survival after recurrence. Resection margin, Edmondson-Steiner grade, α-fetoprotein, cirrhosis, and serum albumin levels for recurrent hepatocellular carcinomas were found to be statistically significant in the univariate analysis and were adjusted in the multivariate Cox proportional hazards analysis to estimate a prognostic effect of p16 methylation in recurrent cases. Overall survival in the recurrent stage I to II hepatocellular carcinomas was poorer in patients with p16 methylation than in those without p16 methylation (hazard ratio, 4.05; 95% confidence interval, 1.15-14.20; P = 0.03). The hazard of failure after recurrence in the stage I to II hepatocellular carcinomas was about 3.80 (95% confidence interval, 1.03-14.02; P = 0.04) times higher in patients with p16 methylation than in those without p16 methylation (Table 3). However, there was no relationship between hazard of failure and p16 methylation in the recurrent stage III to IV hepatocellular carcinomas after adjusting for confounding factors (data not shown).

Discussion

The long-term survival of patients with hepatocellular carcinoma is unsatisfactory, in part because of the high rate of recurrence after curative surgical resection of hepatocellular carcinoma. Therefore, it is critically important to identify groups at a high risk for recurrence and to achieve effective treatment after surgery. Although there have been many reports on the prognostic significance of epigenetic alterations at the promoter region of genes associated with hepatocellular carcinoma, these studies have produced conflicting results in patients with hepatocellular carcinoma. Lee et al. (24) reported that patients with hepatocellular carcinoma with E-cadherin or GSTP1 methylation showed poorer survival than those without, whereas Anzola et al. (25) did not find an association between GSTP1 methylation and prognosis. Some groups reported that the hypermethylation of p15 or p16 genes was not associated with patient prognosis (6, 7, 24). However, Wong et al. (26) found that 75% of 12 patients with concurrent methylation of p15 and p16 genes were more likely to develop recurrent disease following resection. Matsuda et al. (27) also reported that the association between the loss of p16 and poor prognosis was significant when p27 expression was high (P < 0.01). These conflicting observations may be due to (a) the difference in the etiologic and underlying antecedent factors of hepatocellular carcinoma, (b) the very small number of patients with hepatocellular carcinoma, ~50 to 120 patients, or (c) a lack of control for possible confounding factors.

In a study population in which hepatitis B virus is a major cause of hepatocellular carcinoma, anti–hepatitis C virus antibody–positive cases may act as an effect modifier when studying the relationship between DNA methylation and a patient’s prognosis because of the different pathogenetic effects of hepatitis C virus and hepatitis B virus on DNA methylation in hepatocarcinogenesis. Thus, anti–hepatitis C virus antibody–positive cases were not included in this study because their incidence is very low (7%) in Korea. The 34%
of patients with hepatocellular carcinoma in this study did not have coexistent cirrhosis. It is known that cumulative incidence of hepatocellular carcinoma is higher in patients with cirrhosis due to hepatitis C rather than due to hepatitis B or alcohol. In Korea, the main cause of cirrhosis is alcohol consumption and hepatitis B viral infection, which may result in high incidence of noncirrhotic liver. In the present study, 38% (102 of 265) of the patients experienced recurrence after curative resection, and the prognosis of patients with recurrence was poorer than that of those without recurrence ($P < 0.001$). In addition, p16 methylation was significantly associated with poor prognosis in recurrent stage I to II cases.

The p16 gene is one of the major targets for human hepatocellular carcinogenesis. Promoter methylation of the gene is known to be the primary mechanism underlying the transcriptional silencing of the p16 gene in hepatocellular carcinoma. A number of studies have reported that p16 methylation is found not only in cancerous and precancerous tissues of HCC patients but also in noncancerous liver tissues of patients with hepatocellular carcinoma. Fine-needle aspiration biopsy of tissues from patients with chronic hepatitis B showed that the expression of the hepatitis B virus X protein was higher in patients with p16 methylation than in those without (25). p16 methylation is also found in 20% to 30% of patients with chronic hepatitis, in 30% to 50% of patients with cirrhotic liver tissue, and in 70% to 90% of cancerous tissues (29-31). In addition, hepatocellular carcinomas within dysplastic nodules shows high prevalence [15 (83.3%) of 18] of p16 hypermethylation, and the prevalence increased from cirrhotic nodules [15 (62.5%) of 24] to dysplastic nodules [26 (70.3%) to 37; ref. 32]. These observations suggest that p16 methylation is required for the malignant transformation of hepatocytes. A recurrent carcinoma develops from residual cancer cells that are left in or near the "macroscopically normal" surgical margins. Therefore, a risk factor of recurrence may play an important role in the proliferation, but not malignant transformation, of remnant cancer cells after surgery until detection in a clinical setting. In the present study, p16 methylation was not associated with the risk for recurrence. Based on these observations, it is therefore likely that methylated p16 in remnant cancer cells after surgery would play a minor role in the proliferation of remnant cancer cells until the detection of recurrence.

In the present study, p16 methylation was associated with poor prognosis in recurrent early-stage hepatocellular carcinomas. It remains unclear how p16 methylation affects prognosis in these cases. One possibility is that p16 methylation may be involved in the invasion and metastasis of residual cancer cells after hepatic resection, although p16 methylation is not involved in the proliferation of remnant cells. To gain insight into the potential mechanism underlying the poor prognosis in recurrent early-stage cases with p16 methylation, we analyzed the expression of p16 and p53 using immunohistochemistry. Lewis et al. (33) reported that the absence of p53 had no effect on tumor initiation but greatly influenced tumor behavior in hepatocellular carcinoma. They found that liver tumors induced after the somatic introduction of mouse polyoma virus middle T antigen to albumin-to-a transgenic mice with germ line deletion of Trp53 metastasizes to the lung in ~40% of cases. Chen et al. (34) recently reported that tumor cell lines generated from tumors lacking both Trp53 and Ink4a/Arf showed strong migration and invasion activity in vitro in comparison to those lacking Trp53 alone. These observations suggest that the inactivation of p14 and/or p16 may affect patient prognosis by accelerating invasion and metastasis in recurrent cases with p53 loss.

Negative expression of p16 protein by immunohistochemistry in this study was associated with poor survival in recurrent hepatocellular carcinomas, but p53 expression did not affect patient prognosis. This finding is not consistent with the previous results (33, 34). This may result from a marked heterogeneity in the dominant negative effect over wild-type p53 (antimorphic) and gain of function activity (neomorphic) of various p53 mutants in human hepatocellular carcinomas. Using a library of 2,314 distinct p53 missense mutants, Kato et al. (35) studied a correlation of the p53 transactivation function with p53 structure and different mutations and found a marked heterogeneity in the loss of function of the various mutants. Mouse models also showed heterogeneity of phenotype in p53 knockout mice and knock-in mice expressing various p53 mutants (36). For p53 mutants found in human cancer, some mutants show complete loss of their mutant activities, but other mutants still retain the activities, transactivating target genes. In addition to the heterogeneity, tissue specificity of p53 mutants and genetic backgrounds of hepatocellular carcinomas studied may influence the inconsistent result. The lack of an association between p16 methylation and patient prognosis in advanced stages may have resulted from the fact that tumor invasion has already occurred in stage III to IV tumors.

Before stratification of data by stage, we determined whether the strength of association between recurrence after surgery and methylation of seven genes was identical across stages. We applied the Breslow-Day test for homogeneity of the odds ratio and found evidences of heterogeneity in some genes (data not shown). The variable stage was treated as an effect modifier in this study; we divided patients into two groups (stages I-II and III-IV) and computed a different odds ratio for each group rather than a single summary value for the overall relative odds. This study was severely limited by a small number of recurrent cases and by the lack of data regarding the genetic alteration of preneoplastic cells in a given field. Thus, we could not discriminate between a recurrent carcinoma and a second field tumor that developed from preneoplastic precursor cells clonally related to the cells of the index tumor or a true second primary tumor defined as an independently evolved carcinoma. These may have led to an incorrect conclusion regarding the association between p16 methylation and patient prognosis. In addition, the small number of recurrent cases may bias the association between p16 methylation and poor survival in recurrent stage I to II cases. Further studies with larger samples are needed to precisely determine these relationships. It also needs to validate the effect of p16 methylation status on an independent set of resected patients with hepatocellular carcinoma and appropriately done in vitro or in vivo studies to show a plausible mechanism for the effect of p16 on early-stage hepatocellular carcinoma.

In conclusion, this study suggests that recurrent early-stage hepatocellular carcinomas with p16 methylation
may have a poor prognosis after surgical resection. We strongly recommend that aggressive treatment should be considered in early-stage hepatocellular carcinomas with p16 methylation and high risk factors of recurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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