Wnt-1 Protein as a Prognostic Biomarker for Hepatitis B–Related and Hepatitis C–Related Hepatocellular Carcinoma after Surgery

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Abstract

Background: Up-regulation of Wnt-1 protein has been reported in hepatitis B virus (HBV)–related and hepatitis C virus (HCV)–related hepatocellular carcinoma (HCC) tissues and cell lines. It is known to play a fundamental role in signaling cancer progression, whereas its prognostic role in HCC remains unexplored.

Methods: As a prognostic biomarker, this study analyzed Wnt-1 protein expression in 63 histology-verified HCC patients receiving curative resection. In each paired tumor and nontumor specimen, Wnt-1 levels were semiquantitatively measured by Western blotting and expressed by tumor/nontumor ratio. The data were further correlated with quantitative real-time PCR as well as with β-catenin and E-cadherin expression by immunohistochemistry. Cumulative tumor recurrence-free survival curves were constructed using the Kaplan-Meier method and compared by the log-rank test.

Results: The results showed that 26 (group I) and 37 (group II) HCC patients had an expression ratio of Wnt-1 ≥1.5 and <1.5, respectively. The amount of Wnt-1 estimated by tumor/nontumor ratio correlated with the results by quantitative real-time PCR. High tumor Wnt-1 expression correlated with enhanced nuclear β-catenin accumulation, diminished membranous E-cadherin expression, and increased tumor recurrence after curative tumor resection.

Conclusions: These results suggest that Wnt-1 may be used as a predisposing risk factor for HCC recurrence. The use of tumor Wnt-1 as a prognostic biomarker may identify patients with HBV- and/or HCV-related HCC patients with a high risk of tumor recurrence who may then benefit from further intensive therapy after surgery. (Cancer Epidemiol Biomarkers Prev 2009;18(5):1562–9)

Introduction

Wnt signaling, initially discovered by genetic analysis in the wing development of Drosophila melanogaster, is crucial in regulating many processes during embryonic development, including cell fate, organogenesis, angiogenesis, and stem cell proliferation (1, 2). In humans, 19 WNT genes encoding for 19 WNT proteins have been identified (3, 4). They are similar in size, ranging from 30 to 83% amino acid sequence homology as well as a conserved pattern of 23 to 24 cysteine residues. WNT proteins have also been implicated in bone density maintenance, neurologic conditions during adulthood, and carcinogenesis of many cancers (5-8). The members of the WNT gene family are increasingly being discovered in other species, such as sea anemones (9). The first member of the 19 known human Wnt genes, wingless type mouse mammary tumor virus integration site gene family member 1 (Wnt-1), was discovered because of its oncogenic properties (10). The 19 known human Wnt ligands may interact with at least 10 cognate receptors of the Frizzled or planar cell polarity and/or low-density lipoprotein receptor-related protein 5/6 families to form receptor complexes. However, the signals transduced by different combinations of Wnt ligands and receptors are not yet completely understood (11).

Chronic infections with hepatitis B and C viruses (HBV and HCV) are etiologically linked to the development of hepatocellular carcinoma (HCC; refs. 12-16). Functional proteomics and cDNA microarray studies have shown up-regulation of Wnt-1 in HBV- and HCV-related HCC tissues and cell lines (17-19). Wnt-1 protein processing and secretion have been extensively characterized for the 36-kDa unglycosylated form as well as sequentially glycosylated forms of 38- to 42-kDa forms. The more mature forms, 40 and 42 kDa, are the predominant species in cells that efficiently process and secrete Wnt-1 (20).

Up-regulation of Wnt-1 expression has been shown to correlate with hepatocyte growth stimulated by HCV core protein (17). A link between HBV and Wnt signaling has been reported in hepatoma cell line Huh7, showing
that the X-protein of HBV (HBx) may enhance the stabilization of β-catenin and is essential for the activation of Wnt/β-catenin signaling (21). Moreover, HBx/c-myc transgenic mice have increased development of HCC (22). In a mouse model of breast cancer, Wnt-1 is shown to be fundamental for cancer metastasis progression (23). Enhanced expression of nuclear factor-κB–associated Wnt-1 protein has been identified in a large proportion of HCC specimens with HBV and/or HCV infection (18). The nuclear factor-κB–P50 subunit has been shown to bind to the promoter of the Wnt-1 gene by chromatin immunoprecipitation assay (24). All evidence highly suggests that Wnt-1 plays an important role in HBV- and/or HCV-related hepatocarcinogenesis. This study aims to investigate whether Wnt-1 expression correlates with tumor recurrence in HCC patients receiving curative resection.

Materials and Methods

Study Patients. Among 450 HCC patients with HBV and/or HCV infection from 2000 to 2007, 63 cases with single nodular tumor pattern receiving curative resection at Chi Mei Hospital Liouying and Chi Mei Medical Center (Tainan, Taiwan) were enrolled for follow-up analysis after surgery. Because HCC patients presented with multinodular tumor pattern had a poor prognosis by surgical treatment (12, 16, 25), they were not included in this study. Curative resection was defined as complete excision of the tumor with clear microscopic margin and no residual tumors shown by computer tomograph scan at 1 mo after surgery. Criteria for resectability were that patients with a single tumor nodule, liver functional class of Child-Pugh grade A or B, adequate blood supply to remnant liver after heptectomy, lack of evidence of portal vein tumor thrombosis, and no extrahepatic metastases before surgery. Fresh tissue specimens were separated into tumor and nontumor portions immediately after heptectomy and then transported in liquid nitrogen tank to the Tissue and Serum Bank of Chi Mei Medical Center and stored at −80°C until use. Multiple freezing and thawing was avoided in all the specimens. The study protocol conformed to the ethical guideline of the 1975 Declaration of Helsinki and approved by the Institutional Ethics Committee. Informed consent was obtained from all study subjects.

Postoperative End Point of Follow-up and Alternative Treatments. After surgery, all patients were followed up regularly in the outpatient clinic to monitor any tumor recurrence prospectively by a standard protocol. The protocol included serum α-fetoprotein (AFP) levels assayed monthly and liver ultrasonography or contrast computer tomography scan every 2 to 4 mo. Suspected tumor recurrence was confirmed by hepatic angiography and, if necessary, by percutaneous fine-needle tumor biopsy and/or aspiration cytology. Any confirmed post-operative tumor recurrence was regarded as the end point of follow-up. This study did not differentiate between de novo HCC growth and intrahepatic HCC metastasis from the original tumor, which could not be identified before surgery. After a thorough evaluation, patients with documented postoperative HCC tumors were categorized as tumor recurrent and advised to receive alternative treatments, including transarterial chemoembolization, percutaneous pure ethanol injection, percutaneous acetic acid injection, radiofrequency ablation, local radiotherapy, systemic chemotherapy, or liver transplantation, where feasible and as appropriate to the individual case.

Measurement of Wnt-1 Protein. Western blotting of tumor and nontumor proteins with biotin-conjugated rabbit anti-human WNT1 polyclonal antibody (Zymed Laboratories, Inc.) was conducted by the procedures described previously (18). The amount of Wnt-1 protein was semiquantitatively measured on the immunoblot films with ImageMaster TotalLab version 2.01 (Amersham

**Figure 1.** Differential expression of Wnt-1 proteins in hepatoma cell lines and HCC tumor samples. Western blotting shows differential expression of 36-, 38-, 40-, and 42-kDa Wnt-1 proteins in three hepatoma cell lines Hep3B, HepG2, and PLC/PRF/5. Arrows to the left of PLC/PRF/5 indicated each form of Wnt-1 proteins, respectively. PC12 was used as a positive control in which the 40-kDa glycosylated form Wnt-1 protein was less prominent. Cell lines T1 and T2 were used as negative controls for the blotting. The predominant 42-kDa form Wnt-1 protein was detected in the positive control cell line PC12 as well as the three human hepatoma cell lines and HCC tumor portions from patients 1 and 4. Below each lane, β-actin was used as the protein loading control.
Parmacia Biotech), and all data were expressed as tumor/nontumor ratio. In each assay, β-actin was used as protein loading control. The cell line PC12 (Bioresource Collection and Research Center), a rat pheochromocytoma cell line of neural crest lineage with constitutive Wnt-1 protein production, cross-reactive with that of humans, was used as the positive control for the Wnt-1 assay (26). To show different Wnt-1 expression among different human hepatoma cells that may reflect differential expression in human HCC specimens, the human hepatoma cell lines HepG2 (Bioresource Collection and Research Center), Hep3B (American Type Culture Collection), and Alexander cell line PLC/PRF/5 (Bioresource Collection and Research Center) with hepatitis B surface antigen (HBsAg) production were studied for comparison. In addition, two urothelium cancer cell lines T1 and T2 with no Wnt-1 expression (27) were used as a negative control for the Western blotting.

**Quantitative Real-time PCR Detection of Wnt-1 Expression.** To correlate tumor Wnt-1 expression estimated by Western blotting, quantitative real-time PCR (qPCR) was conducted by the comparative threshold cycle (Ct) method. Before each qPCR detection, RNA integrity was ascertained by electrophoresis of total RNA with 0.8% denaturing agarose gel followed by staining with ethidium bromide. Results showed the 28S and 18S bands and the ratio of 28S/18S is about 2. Total RNA from 1 μg of HCC tumor portion of each paired specimen was reverse transcribed into cDNA (SuperScript II RT, Invitrogen). Equal amounts of cDNA and primers specific for Wnt-1 and β-actin were used for PCR amplification. The β-actin gene was used as a loading control for reverse transcription-PCR detection of Wnt-1 expression. Real-time PCR detection of amplified template was accomplished with SYBR Green I chemistry (Bio-Rad) using an MJ Research DNA Engine Opticon II fluorescence detection system (MJ Research, Inc.). Individual PCR contained 10 μL of cDNA (dilution, 1:50), 1.25 μL of 10 μmol/L forward primers, 1.25 μL of 10 μmol/L reverse primers, and 12.5 μL of SYBR Green I in a final volume of 25 μL. Individual PCR products were analyzed using melting point analysis. Samples were heated from 50°C to 95°C, and the decline in fluorescent signals of each individual sample was assessed. The fluorescence/time-dependent generation of signals was assessed using the manufacturer’s software program. Because an international Wnt-1 RNA standard was not available, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the housekeeping

**Figure 2.** Grouping of HCC patients by tumor/nontumor ratio of 42-kDa Wnt-1 protein expression. Western blotting detection of 42-kDa Wnt-1 expression of the initial 12-paired HCC tumor (T) and nontumor (NT) specimens is illustrated. A. Cases 1 to 7. B. Cases 8 to 12. Both HCC tumor and nontumor portions may express 42-kDa Wnt-1 protein. By tumor/nontumor ratio, group I HCC patients were defined as Wnt-1 ≥ 1.5 and/or undetectable in nontumor portion, and group II < 1.5 and/or undetectable in tumor portion. The densitometry datum 100 shown in nontumor portion of patient 1 on A was arbitrarily selected as the standard of the measurement. Below each lane, β-actin was used as the protein loading control.
The highest $\Delta C_t$ of nontumor portions of the 63 study patients, namely, with the least amount of $Wnt-1$ gene expression, was used for the calibrator in this study.

**Immunohistochemistry.** Representative 4-μm sections were mounted onto positively charged slides, air dried in an incubator at 42°C overnight, and deparaffinized in xylene. After rehydration in graded alcohols followed by PBS, the slides were incubated in 0.1 mol/L EDTA (pH 9.0) and heated in a microwave oven at 600 W for four 5-min cycles. Afterwards, slides were incubated in a blocking solution (PBS with 5% nonfat dry milk and 2% normal rabbit serum) for 30 min at room temperature followed by two 15-min cycles of incubation with avidin-biotin blocking solutions (avidin-biotin blocking kit; Vector Laboratories). The solution was removed and the primary antibody [monoclonal anti-β-catenin, 1:500 (Transduction Laboratories) and monoclonal anti-E-cadherin, 1:5 (Euro-Diagnostica)] was added to the slides for incubation at 4°C overnight. After several rinses with PBS, the bound antibodies were detected using the avidin-biotin complex method and visualized

**Table 1. Demographic features of 63 patients with HCC receiving curative tumor resection**

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>$P$</th>
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<tbody>
<tr>
<td><em><em>Wnt-1 ≥1.5</em> (n = 26)</em>*</td>
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<td></td>
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<tr>
<td>Age (y)</td>
<td>60.3 ± 12.2</td>
<td>53.0 ± 11.1</td>
<td>0.313</td>
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<tr>
<td>Sex (M/F)</td>
<td>48 (34-75)</td>
<td>64 (36-74)</td>
<td></td>
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<tr>
<td>Viral infection</td>
<td>16/10</td>
<td>27/10</td>
<td>0.414</td>
</tr>
<tr>
<td>HbsAg positive</td>
<td>14</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>HbsAg and anti-HCV positive</td>
<td>10</td>
<td>7</td>
<td>0.698</td>
</tr>
<tr>
<td>Wnt-1 (z)</td>
<td>4.17 ± 3.35</td>
<td>2.42 ± 3.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>3.2 ± 3.9</td>
<td>3.0 ± 3.8</td>
<td>0.840</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>23</td>
<td>33</td>
<td>0.862</td>
</tr>
<tr>
<td>AFP (ng/mL)</td>
<td>910.8 ± 2,540</td>
<td>818.9 ± 2,205</td>
<td>0.879</td>
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*Footnotes for table 1.*

- †Tumor/nontumor ratio by densitometry.
- ††Digits represent mean ± SD, median (min-max).
- †Student's $t$ test.
- †† Fisher's exact test.
- †††Relative expression of $Wnt-1$ gene in 1 μg of HCC tumor was given by $2^{\Delta \Delta C_t}$.
- †Mann-Whitney $U$ test.
- †‡$x^2$ test.
- †‡‡$\chi^2$ test.
- †‡‡‡$\chi^2$ test.

*Note: DD represents the difference in threshold cycle (Ct) values between tumor and nontumor portions.*

**Figure 3.** qPCR detection of $Wnt-1$ gene expression by comparative threshold cycle ($C_t$) method. A. Relative $Wnt-1$ gene expression of the two groups is shown by $2^{\Delta \Delta C_t}$ value relative to the calibrator. Group I versus II, $P < 0.001$, Mann-Whitney $U$ test. B. Reverse transcription-PCR showed $Wnt-1$ gene expression in one HCC sample but not in T2 cells. β-Actin gene was used as an internal control.
by 3,3’-diaminobenzidine staining. Slides were lightly counterstained with hematoxylin. In each staining, the nontumor portion of the paired specimens was compared as a control. Two observers (C-J.C. and C-C.T.) independently evaluated the immunostaining results. The concordance ratio was >90%. Differences of opinion were resolved by reaching a consensus with a third evaluator (S-L.T.). When evaluating the $\beta$-catenin protein immunoreaction, $\beta$-catenin accumulation in the nucleus, which may be associated with cytoplasmic and/or membranous reactivity, was interpreted as a positive result. E-cadherin expression was considered decreased when there was a definite reduction and/or loss in E-cadherin immunoreactivity in the tumor cell membrane compared with the adjacent nontumor portion. Grading was done according to the following rules. For $\beta$-catenin, grade 1 indicated <25% of tumor cells with nuclear accumulation; grade 2, 25% to 50%; grade 3, 50% to 75%; and grade 4, >75% of tumor cells. Likewise, grade 1 for tumor E-cadherin expression indicated total loss or <25% tumor cells with membranous immunoreactivity; grade 2, 25% to 50%; grade 3, 50% to 75%; and grade 4, >75% of tumor cells with membranous immunoreactivity. Percentages of immunoreactive cells of both $\beta$-catenin and E-cadherin given are the means from the evaluation of three high-power fields.

**Statistical Analysis.** Statistical analysis was done by using Statistical Package for the Social Sciences software version 12.0 (SPSS, Inc.). $\chi^2$ test, Student’s t test, Fisher’s exact test, and Mann-Whitney U test were used to evaluate differences in patient groups and in tumor characteristics. Tumor recurrence-free survival curves were constructed by the Kaplan-Meier method and compared by log-rank test. Correlation between two ordinal variables was tested with the Kendall’s $\tau$-b test. Due to the lack of normal distribution of the tumor/nontumor ratio of Wnt-1 quantity and HCC tumor size, correlation between them was done by the Spearman correlation test. A $P$ value of <0.05 was considered statistically significant.

**Results**

**Differential Expression of Wnt-1 Proteins in Hepatoma Cell Lines and HCC Samples.** An immunoblot showed differential expression of 36-, 38-, 40-, and 42-kDa Wnt-1 proteins in the three hepatoma cell lines Hep3B, HepG2, and PLC/PRF/5, whereas none expressed in T1 and T2 cell lines and in tumor portion from HCC patients 2 and 3 (Fig. 1). This indicates that Wnt-1 protein expression is variable. Whether these glycosylated forms of Wnt-1 protein involved in its secretion or in its signaling transduction needs further study (1, 2, 4, 8, 20). The levels of 42-kDa Wnt-1 protein expression in tumor portions also varied among different HCC samples from the four HCC specimens with HBV or HCV infection. The predominant 42-kDa form of the
Wnt-1 protein was detected in the positive control cell line PC12 as well as the three human hepatoma cell lines and HCC tumor portions from patients 1 and 4. The 42-kDa form is predominantly expressed and thought to be a fundamental signaling in cancer progression. The significance of the lower 38-kDa or less bands on the Western blots of the cell lines compared with the 42- and 40-kDa isoforms that are in the fresh tumors (Fig. 2) remains to be elucidated.

**Difference in Clinical Features between Two Groups.**

No surgical mortalities occurred in any patient enrolled in this study. As estimated by tumor/nontumor ratio, 26 HCC patients had an expression ratio of Wnt-1 ≥1.5 and/or Wnt-1 undetectable in nontumor tissue, and 37 HCC patients had an expression ratio of Wnt-1 <1.5 and/or Wnt-1 undetectable in tumor tissue, respectively. These 63 patients were arbitrarily placed into groups I and II according to tumor/nontumor ratio and sample size was estimated according to Sample Power 2.0 (SPSS). Figure 2A (initial cases 1-6) and B (initial cases 7-12) illustrated the rationale of the grouping. Except for Wnt-1 expression, there was no significant difference in clinical features between the two groups, including age, sex, viral infections, tumor size measured on surgical specimens, or liver cirrhosis background (Table 1). As estimated by tumor/nontumor ratio, Wnt-1 expression correlated with qPCR assay and had a significantly higher level in group I patients in terms of 2-ΔΔCt value (group I versus II, P < 0.001, Mann-Whitney U test; Fig. 3, Table 1).

**Wnt-1 Expression Correlates with Increased Nuclear β-Catenin Accumulation and with Decreased Membranous E-Cadherin Expression.** By immunohistochemical staining (Fig. 4), high Wnt-1 expression correlated with increased nuclear β-catenin accumulation with or without increased cytoplasmic and membranous immunoreactivity (P < 0.05, Kendall’s τ-b test; Fig. 4A; Table 2A). Conversely, high Wnt-1 expression correlated with decreased membranous E-cadherin immunoreactivity (P < 0.05, Kendall’s τ-b test; Fig. 4B; Table 2B).

**HCC Tumor Wnt-1 Expression Does Not Correlate with Tumor Size.** There was no significant difference in tumor size and serum AFP level between group I and group II. Tumor Wnt-1 expression did not correlate with tumor size (ρ = 0.162, P = 0.209, Spearman correlation analysis; data not shown) or with serum AFP level (ρ = 0.084, P = 0.521, Spearman correlation analysis; data not shown). This indicates that Wnt-1 expression is independent of AFP secretion.

**High Tumor Wnt-1 Expression Correlates with Increased HCC Recurrence.** Cumulative tumor recurrence-free survival curves for group I and II patients were shown in Fig. 5. Group I patients had a tumor recurrence-free survival rate significantly lower than that of group II patients (P < 0.001, log-rank test), indicating that high Wnt-1 expression correlated with increased tumor recurrence.

## Discussion

Recent advances in diagnostic modalities coupled with the use of tumor markers such as AFP and des-γ-carboxyprothrombin in screening early-stage HCC allow early diagnosis (12, 16, 30), and a comprehensive treatment protocol can be initiated without delay. This progress has significantly improved patient survival (31). However, the long-term survival rate of HCC remains very low because of the high recurrence rate after initial treatment (12, 16, 25). For example, in an analysis of 2,820 HCC patients in Taiwan, median survival for HBV-related HCC was only 11.1 months and only 23.9 months for HCV-related HCC (32). This poor prognosis of HCC is because HCC patients cannot achieve a cure or sustained tumor-free survival under current treatment modalities (12, 25, 33), as evidenced by the pooled data of 22 large series reported from year 1990 to 2003 that 5-year survival rate after HCC resection varied from 26% to 76% (33). Most patients with HBV- or HCV-related HCC are cirrhotic; thus, survival may reflect more the underlying liver disease than the progression of HCC. We used tumor recurrence-free survival as the end point to determine the role of Wnt-1 in the progression of this disease. Several factors about the outcome of HCC patients receiving surgical treatment have been reported, but none of these examined Wnt-1 protein as a treatment protocol can be initiated without delay. This progress has significantly improved patient survival (31). However, the long-term survival rate of HCC remains very low because of the high recurrence rate after initial treatment (12, 16, 25). For example, in an analysis of 2,820 HCC patients in Taiwan, median survival for HBV-related HCC was only 11.1 months and only 23.9 months for HCV-related HCC (32). This poor prognosis of HCC is because HCC patients cannot achieve a cure or sustained tumor-free survival under current treatment modalities (12, 25, 33), as evidenced by the pooled data of 22 large series reported from year 1990 to 2003 that 5-year survival rate after HCC resection varied from 26% to 76% (33). Most patients with HBV- or HCV-related HCC are cirrhotic; thus, survival may reflect more the underlying liver disease than the progression of HCC. We used tumor recurrence-free survival as the end point to determine the role of Wnt-1 in the progression of this disease. Several factors about the outcome of HCC patients receiving surgical treatment have been reported, but none of these examined Wnt-1 protein as a...
prognostic biomarker in HCC after tumor resection (33-36).

In the present study, we found that high tumor Wnt-1 expression correlated with increased nuclear β-catenin accumulation accompanied by decreased membranous E-cadherin expression and with increased tumor recurrence after curative resection. This seems to be consistent with the character of cancer cell metastasis. A first step in cancer cell metastasis is the loss of epithelial adhesion by disruption of the E-cadherin/catenin complex. E-cadherin is a cell surface protein involved in homophilic and Ca2+-dependent cell-cell interactions. The cell-cell adhesion is mediated by binding of the cytoplasmic tail of E-cadherin to cytoplasmic catenins, α-, β-, and γ-catenin (37-39). A hallmark of Wnt signaling is the stabilization of cytoplasmic β-catenin followed by its nuclear translocation and association with T-cell factor/lymphoid enhancer factor family proteins, which lead to the transcription of Wnt target genes, such as c-myc, cyclin D1, and c-jun (37-40). β-Catenin, first identified based on its association with cadherin adhesion molecules, is now widely recognized as a central molecule of the Wnt signaling cascade (5, 37, 41, 42).

Many reports have shown that decreased membranous E-cadherin correlated with tumor progression (38, 39, 43, 44). Indirectly, our data could be consistent with reports that enhanced tumor β-catenin nuclear accumulation correlated with tumor progression and postoperative survival in HCC patients (43, 44). The data are also consistent with reports of patients with basal cell carcinoma that Wnt-1 expression is correlated to advanced stages of this cancer (45), and with a mouse model of breast cancer, Wnt-1 was fundamental for metastasis progression (23).

Moreover, genetic alternations of Wnt signaling pathway-associated genes, β-catenin and AXIN1, have been reported in human HCC (15, 46-49). These alternations implicate an important role for the Wnt signaling pathway in hepatocarcinogenesis (50). It has also been shown in children with medulloblastoma and in patients with head and neck squamous cell carcinomas that the Wnt signaling pathway may potentially be used as a therapeutic target for designing new treatment regimens, including immunotherapy (51, 52). Similar approaches may be applied to treat HCC with Wnt-1 expression.

In conclusion, high tumor Wnt-1 expression correlates with increased HCC recurrence. Utilization of Wnt-1 protein as a prognostic biomarker may identify HBV- and/or HCV-related HCC patients with a high risk of tumor recurrence and metastasis progression who may then benefit from further intensive therapy after curative resection.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
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We thank Professor De-Maw Chuang (Section on Molecular Neurobiology, National Institute of Mental Health, NIH, Bethesda, MD) for help in preview and revision of the manuscript and Chin-Li Lu (Department of Medical Research, Chi-Mei Medical Center, Tainan, Taiwan) for statistical analysis of data.

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