Claudin-10 Expression Level is Associated with Recurrence of Primary Hepatocellular Carcinoma

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ABSTRACT

Purpose: Hepatocellular carcinoma (HCC) patients with the same clinicopathologic features can have remarkably different disease outcomes after curative hepatectomy. To address this issue, we evaluated the cDNA microarray gene expression profiles of HCCs and identified claudin-10 expression level was associated with disease recurrence. The aim of the current study is to validate the microarray data by an alternative research method applicable for routine practice.

Experimental Design: Quantitative reverse transcription–PCR (RT-PCR) was used to validate the microarray data on claudin-10 expression level. The assay was repeated on a separate HCC sample set to consolidate the prognostic significance of claudin-10.

Results: Claudin-10 expression level by quantitative RT-PCR and by microarray measurement showed a high concordance (r = 0.602, P < 0.001). Quantitative RT-PCR was repeated on a separate HCC sample set and the association of claudin-10 expression with recurrence was again confirmed (hazard ratio, 1.2; 95% confidence interval, 1.0–1.4; P = 0.011). By multivariable Cox regression analysis, claudin-10 expression and pathologic tumor-node-metastasis stage were independent factors for prediction of disease recurrence.

Conclusion: Claudin-10 expression of HCC can be used as a molecular marker for disease recurrence after curative hepatectomy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common lethal malignancy and is among the five leading causes of cancer death worldwide (1). The incidence is rising in the United States (2), United Kingdom (3), and Japan (4). In China, liver cancer is the second major cause of cancer death (5). Epidemiologic studies have shown that hepatitis B (HBV) and C virus (HCV) infections, alcohol-induced liver injury, and consumption of aflatoxin are closely associated with liver cancer. Extensive studies have been done to better understand the clinicopathologic features to improve the management of patients with HCC (6–11). However, conventional clinicopathologic parameters have limited predictive power, and patients with the same pathologic tumor-node-metastasis (pTNM) stage of disease can have very different disease outcomes (12, 13). Thus, we systematically analyzed the gene expression profiles of patients with HCC (14) to identify genes that the expression level associated with recurrence after curative hepatectomy. Claudin-10 was examined further to consolidate its clinical significance, as it ranks high in prognosis prediction and is a membrane-bound protein with potential therapeutic value. Because microarray facilities are not commonly available in routine laboratories, quantitative reverse transcription–PCR (RT-PCR) assay method is used as an alternative technique for transcript measurement. Furthermore, the prognostic prediction has to be validated in an independent data set to confirm that it works in general and not only in the group of patients from whom the data were derived (15).

In the current study, the claudin-10 expression level and its prognostic value as a novel molecular marker for HBV-related HCC was presented. The claudin-10 gene was annotated by the Ensembl automatic analysis pipeline (http://www.ensembl.org). The claudin-10 gene locates at chromosome 13q31-q34 spanning 25.51 kb with 5 exons, and the predicted protein contains four potential transmembrane domains. This gene encodes a member of the claudin family in which claudins are integral membrane proteins and components of tight junction strands (refer to ref. 16 for a review). The exact function of claudin-10 is unknown and its role in cancer development and progression is mysterious. Interestingly, the claudin family members have been shown to facilitate cell invasion and migration (16). In this report, the claudin-10 (NM_006984, encodes 228 amino acids) was characterized for its clinical significance as the predominant isoform observed in various tissue organs (National Center for Biotechnology Information GenBank) and in liver,α and was reported to be overexpressed in lung cancer cell lines (17) and papillary thyroid carcinoma (18).
PATIENTS AND METHODS

Patients and Samples. We have recently reported the global gene expression profiles of HCC and the adjacent non-tumor liver tissues examined by the cDNA microarray approach (14). In the present study, gene expression profiles from 48 patients undergoing curative partial hepatectomy for HCC during the period March 1999 to April 2000 at Queen Mary Hospital, Hong Kong, were included for patient outcome analysis. Patients were excluded from the present disease outcome analysis if the pathologic examination of the resected specimen showed positive resection margin or mixture of other tumor cell types (e.g., cholangiocarcinoma); if they had received chemotherapy before or after resection; if they had undergone liver transplantation instead of partial hepatectomy; if the resection was for recurrence or palliative intent; or if the resection was followed by hospital death. Another 53 HCCs operated on during the period April 2000 to March 2002 in the same institute with the same exclusion criteria were recruited for validation study. Informed consent had been obtained for specimen collection. The study protocol was approved by the Ethics Committee of the University of Hong Kong.

Diagnosis of HCC recurrence was based on typical imaging findings in a contrast-enhanced computed tomography scan and an increased serum α-fetoprotein (AFP) level. In case of uncertainty, hepatic arteriography and a post-Lipiodol computed tomography scan were done, and if necessary, fine-needle aspiration cytology was used for confirmation. Up to the date of analysis, 59 of the total 101 patients developed recurrence and the median disease-free period was 5.7 months (range, 0.9-32.7 months). For the remaining 42 patients who were disease free, the median follow-up period was 34.0 months (range, 14.9-48.8 months). The age of the patients ranged from 13 to 79, with a median age of 52 years. There were 81 men and 20 women. Serum hepatitis B surface antigen was positive in 92 patients (91.1%). Tumors were staged according to the Union Internationale Contre Cancer pTNM tumor classification 1997 version (19), because the 2002 version did not clearly stratify the patients into different stages in terms of survival rate (20). The clinicopathologic features were prospectively collected into the HCC clinical database.

Microarray Expression Study. The cDNA microarray slides were printed with about 23,000 cDNA clones including 17,400 genes. Samples, RNA preparations, and hybridization protocols had been established and described in detail previously (14, 21). Data were deposited into the Stanford Microarray Database (http://genome-www5.stanford.edu/MicroArray/SMD/; ref. 22). A total of 1,404 cDNA clones with expression levels different by at least 4-fold from the mean in at least two samples were selected for further Cox regression analyses.

Quantitative RT-PCR. Quantitative RT-PCR was done as described (23). Human 18S rRNA primer and probe reagents (Predeveloped TaqMan Assay Reagents, Applied Biosystems, Foster City, CA) were used as the normalization control for subsequent multiplexed reactions. The relative amount of claudin-10, which had been normalized with control 18S for RNA amount variation and calibrator for plate-to-plate variation, was presented as the relative fold change in log 2 base. Transcript quantification was done in at least triplicates for every sample. Quantification was done using the ABI Prism 7700 sequence detection system (Applied Biosystems). Primers and probe for claudin-10 were CLDN10-F, 5′-CTGTG GAAG CGTGC GTTA-3′; CLDN10-R, 5′-CAAAG AAGCC CAGGC TGACA-3′; and CLDN10-P, 5′-6FAM CCTCC ATGCT GGCGC MGBNFQ-3′.

Statistical Methods. Cox regression analyses with gene expression data as continuous variables were computed to examine gene expression that was associated with disease recurrence after curative resection. The technical concern of microarray data reproducibility was addressed by using quantitative RT-PCR for validation. Expression data by microarray and quantitative RT-PCR data were continuous variables assessed by Pearson’s correlation coefficient (r). The association of claudin-10 expression and disease-free survival was validated in another independent sample set, and we used quantitative RT-PCR as a different assay technique for the transcript quantitation in the independent sample set.

The claudin-10 expression data were modeled as categorical variables only in the Kaplan-Meier analyses. The Youden index (sensitivity + specificity – 1; ref. 24) was used to determine the optimal cutoff point of claudin-10 expression for the prediction of 3-year disease-free survival. Other cutoff values including the mean, median, and 75th percentile had also been considered and examined, and they were all able to segregate the patients with clinical implications. The Youden index was used to maximize the sensitivity and specificity of the prediction simultaneously.

The association of gene expression and clinicopathologic parameters with patient outcome was examined by a multivariable Cox proportional hazards regression with the forward stepwise selection procedure. The claudin-10 expression data were modeled as continuous variable, and all the clinicopathologic parameters were modeled as categorical variables in the Cox regression analyses. The associations of claudin-10 expression level with clinicopathologic features were assessed by Spearman correlation and Mann-Whitney U test where appropriate. Differences were considered significant when P < 0.05. The statistical analyses were aided by SPSS version 11.0 software package (SPSS Inc., Chicago, IL).

Additional Microarray Information. The microarray study was carried out following the minimum information about microarray experiment guidelines issued by the Microarray Gene Expression Data Group (25). The original data are available in the Stanford Microarray Database (http://genome-www5.stanford.edu). Information is also available from the authors on request.

RESULTS

Claudin-10 Expression and Recurrence. Cox regression analyses with gene expression modeled as a continuous variable were computed to identify gene expression that predicts disease recurrence after curative resection (HCCs, n = 48; Supplementary Table 1). Claudin-10 ranks high in prognosis prediction and is a membrane-bound protein with potential therapeutic value. Claudin-10 encodes a member of the claudin family in which claudins are integral membrane proteins and components of tight junction strands. The claudin-10 level by cDNA microarray was significantly associated with recurrence [hazard ratio (HR), 1.7;
At the analyses.

grades 3 and 4).
liver remnant), tumor encapsulation (absence versus presence of tumor capsule), and Edmondson-Steiner histologic grade (grades 1 and 2 versus HBV association (absence versus presence of serum hepatitis B surface antigen), chronic liver disease (normal and hepatitis versus cirrhosis of the liver remnant), tumor encapsulation (absence versus presence of tumor capsule), and Edmondson-Steiner histologic grade (grades 1 and 2 versus

Tumor size (cm)
pTNM stage
n
Venous infiltration
Absence
Presence
Tumor nodule
Single
Multiple
Microsatellite nodules
Absence
Presence
Serum AFP level (ng/mL)
Claudin-10†

95% confidence interval (CI), 1.1-2.6; P = 0.014). To verify the technical concern on cDNA microarray reproducibility, quantitative RT-PCR was done on the same HCC sample set. Results derived from the two research methods showed a high concordance (Pearson correlation coefficient, r = 0.602, P < 0.001).

To provide an independent test of the association between claudin-10 expression and disease recurrence, a second set of primary HCCs was used (n = 53). Quantitative RT-PCR was used to measure the abundance of the claudin-10 transcript. The claudin-10 level was treated as a continuous variable, and Cox regression analysis was used to examine the relationship of the transcript level with disease recurrence of the patients after curative HCC surgery. Results indicated that the transcript level of claudin-10 was significantly associated with recurrence (HR, 1.2; 95% CI, 1.1-1.3, P = 0.002), gender (male versus female), age (<60 versus >60 years old), HBV association (absence versus presence of serum hepatitis B surface antigen), chronic liver disease (normal and hepatitis versus cirrhosis of the liver remnant), tumor encapsulation (absence versus presence of tumor capsule), and Edmondson-Steiner histologic grade (grades 1 and 2 versus 3 and 4).

The claudin-10 expression level (relative fold change in log 2 base) examined by quantitative RT-PCR was modeled as continuous variable in the analyses.

### Table 1

<table>
<thead>
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<th>Variables*</th>
<th>n</th>
<th>HR (95% CI)</th>
<th>P</th>
<th>Adjusted HR (95% CI)</th>
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<td>pTNM stage</td>
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<td>Stages I and II</td>
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<td>1</td>
<td></td>
<td></td>
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<td>Stages III and IVa</td>
<td>58</td>
<td>3.0 (1.7-5.4)</td>
<td>&lt;0.001</td>
<td>2.6 (1.4-4.7)</td>
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<tr>
<td>≤5</td>
<td>39</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td>62</td>
<td>2.2 (1.2-3.8)</td>
<td>0.006</td>
<td>2.7 (1.5-4.9)</td>
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<tr>
<td>Presence</td>
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<td>Microsatellite nodules</td>
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<td>Absence</td>
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<tr>
<td>Presence</td>
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<td>1.7 (1.0-2.9)</td>
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<td>0.002</td>
<td>1.2 (1.1-1.3)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Insignificant variables with P > 0.05 were not listed in the table, including gender (male versus female), age (<60 versus >60 years old), HBV association (absence versus presence of serum hepatitis B surface antigen), chronic liver disease (normal and hepatitis versus cirrhosis of the liver remnant), tumor encapsulation (absence versus presence of tumor capsule), and Edmondson-Steiner histologic grade (grades 1 and 2 versus grades 3 and 4).

†The claudin-10 expression level (relative fold change in log 2 base) examined by quantitative RT-PCR was modeled as continuous variable in the analyses.

By univariable Cox regression analysis, claudin-10 expression (HR, 1.2; 95% CI, 1.1-1.3, P = 0.002), late pTNM stage (HR, 2.6; 95% CI, 1.4-4.7, P = 0.002), large tumor size (HR, 2.7; 95% CI, 1.5-4.9; P = 0.001), and high serum AFP level (HR, 2.2; 95% CI, 1.2-4.0; P = 0.010) were independent prognostic factors for disease recurrence. The other clinicopathologic features did not add independent prognostic information.

The Kaplan-Meier plot was used to further examine the prediction power by using the claudin-10 expression level alone or together with the pTNM stage system because these two factors were independent prognostic indicators by Cox regression analysis. By Youden index, the optimal cutoff value of claudin-10 expression was 1.23 (relative fold change in log 2 base) to segregate patients into low or high claudin-10 expression group. Using this cutoff value, there were 60 patients in the low claudin-10 expression group (range, 0-1.15) and 41 patients in the high claudin-10 expression group (range, 1.30-11.21). By using the claudin-10 factor alone to segregate the patients, the cumulative 3-year disease-free survivals for patients with low and high claudin-10 levels were 53.3% (32/60) and 24.4% (10/41), respectively (log-rank test, P < 0.001; Fig. 1). The analysis was repeated based on the claudin-10 level and pTNM stages of the patients. The cumulative 3-year disease-free survival was 75% (21/28) for early-stage (stages I and II) patients with low claudin-10 level, 40.0% (6/15) for early-stage patients with high claudin-10, 34.4% (11/32) for late-stage (stages III and IVa) patients with low claudin-10, and 15.4% (4/26) for late-stage patients with high claudin-10 (log-rank test, P < 0.001).
Decreased Claudin-10 Expression Was Associated with Older Patients, Presence of Tumor Capsule, and Non-cirrhotic Liver. To better understand the significance of claudin-10 expression, we analyzed the association of claudin-10 expression level with the clinicopathologic parameters of the patients with HCC. The down-regulation of claudin-10 expression in tumor was significantly associated with older patients ($r = -0.223$, $P = 0.025$), presence of tumor capsule ($P = 0.011$), and noncirrhotic liver remnant ($r = 0.257$, $P = 0.009$). The claudin-10 expression level in tumor was not significantly associated with the pTNM stages, venous infiltration, tumor size, multiple tumor nodules, microsatellite nodules, gender, HBV association, serum AFP level, or Edmondson-Steiner histologic grade.

**DISCUSSION**

Stratifying patients with different risk of disease recurrence will become more important for patient benefit. In the present study, we validated the microarray data in another independent sample set and used quantitative RT-PCR for transcript quantitation in the independent sample set. Both data sets examined by different assay techniques showed that down-regulation of claudin-10 expression was associated with prolonged disease-free period after curative surgery. Our results indicated that prognosis for patients with HCC can be derived from the gene expression of primary tumors. The use of quantitative RT-PCR to assess the claudin-10 level is particularly feasible for the clinical setting, as the test is sensitive and the assay facilities are commonly available in routine laboratories for practical application. Cox regression multivariate analysis indicated that claudin-10 expression was independent of pTNM stage in predicting prognosis, and gene expression data used together with pTNM stage can have added power to provide more accurate prediction for disease outcome (Fig. 1).

This is the first report on claudin-10 expression associated with disease-free survival in patients with HCC after hepatectomy. We and others have reported the expression profiles of HCCs with the microarray approach (14, 21, 26–31), although there have been few reports on the association of gene expression with HCC patient outcomes. Notably, a recent report by Iizuka et al. showed a correlation of gene expression, a predictive system consisting of 12 genes, with early post-hepatectomy intrahepatic recurrence within 1 year (32). Claudin-10 did not reveal prognostic significance in the Iizuka et al. report, and the prognostic genes reported in the two cohorts of patients did not overlap. The discrepancy may be due to several reasons. First, in the study by Iizuka et al., the patients were mostly HCV related (22/33, 66.7%), whereas the majority of our patients were HBV related (92/101, 91.1%). Different HCC etiologies may actually involve different genes and thus recurrence-associated genes in HBV- and HCV-related HCC may be different. Second, the fundamental difference in clinical end point consideration (only intrahepatic recurrence within the first year after surgery in the report of Iizuka et al.; both intra- and/or extrahepatic recurrence within 3 years in our report) may account for the differences, because different genes may be responsible for early recurrence (within the first year) or late recurrence (after the first year). Furthermore, we considered both intra- and extrahepatic recurrence within 3 years as clinical end-point assessments because recurrence outside the liver was also important for disease management and the longer follow-up period would have included the majority of recurrence after curative surgery. It would thus be important to evaluate if...
claudin-10 expression level can predict 3-year disease recurrence in HCV-related HCCs.

The functional annotation of genes provides an insight into the underlying biological mechanism leading to cancer recurrence. The biological function of claudin-10 is unknown. Particularly, claudin family members have been shown to associate with cell invasion and migration (16). Overexpression of claudin-2 transforms a “tight” tight junction into a “leaky” tight junction in epithelial cells (33). Overexpression of claudin-11 induces proliferation and enhances migration in an oligodendrocyte cell line (34). Nonetheless, the role of claudins in human cancer is diverse. Overexpression of claudin-4/claudin-3 has been reported in pancreatic (35, 36), colorectal (37), and ovarian (38) cancer. Notably, claudin-4 expression decreases cell invasion and metastatic potential of pancreatic cancer (39). On the other hand, down-regulation of claudin-7/claudin-1 has been reported in head and neck squamous cell carcinomas (40) and breast cancer (41, 42). Claudin-10 has not been well characterized (16). Notably, claudin-10 is reported to be highly expressed in lung cancer cell lines (17) and papillary thyroid carcinoma (18). In HCC, low claudin-10 expression was associated with the more favorable features including older age of patients, presence of tumor capsule, and noncirrhotic liver remnant. More advanced stages of the HCCs were observed in young patients (9, 43). Absence of tumor capsule was an aggressive HCC feature and associated with early recurrence (7, 10). Operative mortality was higher in patients with cirrhotic liver, which was related to hepatic function reserve (11, 44). The biological role of the decreased claudin-10 level in contribution to favorable HCC prognosis is not clear. Preliminary immunohistochemistry analysis on the cell origin of claudin-10 indicated that in the HCCs with high level of claudin-10 transcript, strong membranous signal and granular cytoplasmic staining were observed in the neoplastic hepatocytes (Supplementary Fig. 1). Nonetheless, further investigation is required to define the role of the prognostic gene claudin-10 in carcinogenesis to delineate the exact molecular pathways leading to disease recurrence.

Our results indicate that claudin-10 expression can predict disease recurrence after curative surgery. We have commenced to examine the functional role of claudin-10 in the contribution to disease recurrence, targeting to provide a more complete picture on cancer progression for better disease management.

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