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Imaging, Diagnosis, Prognosis

Tumor Cell-Microenvironment Interaction Models Coupled with Clinical Validation Reveal CCL2 and SNCG as Two Predictors of Colorectal Cancer Hepatic Metastasis

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Abstract

Purpose: This study aimed to identify novel biological markers for the prediction of colorectal cancer liver metastasis.

Experimental Design: We established two models that mimicked the interactions between colorectal tumor cells and the liver microenvironment. From these models we established subcell lines that had an enhanced ability to metastasize to the liver. Genes that related to hepatic metastasis were screened by microarray. The candidate markers were tested by immunohistochemistry, and their predictive accuracy was assessed by the cross-validation method and an independent test set.

Results: Highly metastatic colon cancer cell sublines SW1116p21 and SW1116v3 were established from the tumor cell-microenvironment interaction models. Seven of the up-regulated genes in the sublines were selected as candidate markers for predicting metastatic potential. A total of 245 colorectal cancer samples were divided into a training set containing 117 cases and a test set containing 128 cases. In the training set, immunohistochemical analysis showed CCL2 and SNCG expression was higher in the hepatic metastasis group than in the nonmetastasis group, and was correlated with poor survival. Logistic regression analysis revealed that CCL2 and SNCG levels in primary tumors, serum carcinoembryonic antigen level, and lymph node metastasis status were the only significant ($P < 0.05$) parameters for detecting liver metastasis. In leave-one-out-cross-validation, the two markers, when combined with clinicopathologic features, resulted in 90.5% sensitivity and 90.7% specificity for hepatic metastasis detection. In an independent test set, the combination achieved 87.5% sensitivity and 82% specificity for predicting the future hepatic metastasis of colorectal cancer.

Conclusion: Our results suggest that these models are able to mimic the interactions between colorectal cancer cells and the liver microenvironment, and may represent a promising strategy to identify metastasis-related genes. CCL2 and SNCG, combined with clinicopathologic features, may be used as accurate predictors of liver metastasis in colorectal cancer. (Clin Cancer Res 2009;15(17):5485–93)

Colorectal carcinoma is one of the major causes of cancer death worldwide (1). Liver is the most common target for metastasis in patients with this disease. It is estimated that approximately 50% of colorectal cancer patients develop liver metastases, with 15% to 25% of synchronous and 20% of heterochronous cases (2). Liver metastasis is the most critical prognostic factor for colorectal cancer. The 5-year overall survival rate of patients with hepatic metastasis is only 25% to 40%

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Translational Relevance

Liver metastasis is the most important prognostic factor of colorectal cancer. Prediction of colorectal cancer liver metastasis may provide useful information for doctors to design treatment strategies to improve patient survival. In the present study, we established subcell lines of colon cancer cells with high hepatic metastasis potential and identified genes that were related to hepatic metastasis. Among these genes, the expression of *CCL2* and *SNCG* was significantly related to hepatic metastasis and poor survival. Statistical analysis revealed that concomitant use of the two immunohistochemical markers and other clinicopathologic features had a significant predictive value with high sensitivity and specificity, and could even correctly predict 87.5% of the future hepatic metastasis in an independent test. These data suggest a promising clinical use for these two biological markers in the prediction of colorectal cancer liver metastasis and may be helpful for the treatment design of this disease.

(3, 4); that of patients without hepatic metastasis is 69.5% to 95.7% (5, 6). Thus, early detection of liver metastasis is important for improving patient survival.

The microenvironment of the target tissue is an important determinant of whether or not metastatic cells are able to invade and survive (7). Specifically, interactions between circulating cancer cells and the endothelia of the host organs play a key role in metastasis (8–10). These interactions are accompanied by changes in gene expressions in tumor cells (9, 11).

Traditional clinicopathologic indices for colorectal cancer hepatic metastasis prediction, including the depth of invasion (12), the presence of venous invasion (13), and lymph node metastasis (14), have only limited prognostic values. Several immunohistochemical markers, such as CD10 (15), CD44 (16), vascular endothelial growth factor (17), transforming growth factor-α (17), matrix metalloproteinase 2 (17), and insulin-like growth factor II (17), have also been shown to be correlated with the probability of liver metastasis. Here we describe the development of two tumor-environment interacting models designed to identify markers for predicting liver metastasis. From the *in vitro* model, we selected a subfraction of SW1116 cells that had a strong ability to interact with human liver sinusoidal endothelial cells (HLSEC) and had a high propensity to metastasize to liver. From the *in vivo* model, we inoculated SW1116 cells into the spleen of nude mice, and got a subcell line with a high potential for liver metastasis from SW1116 cells. We then analyzed the gene expression profiles of the subcell lines to screen candidate markers, and validated their ability to predict liver metastasis of colorectal cancer.

Materials and Methods

Colorectal cancer samples and tissue microarray construction. Fresh surgical resection specimens from 245 colorectal cancer patients were collected from Cancer Institute/Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS&PUMC), Beijing. Primary tumor regions and the matched histologic normal mucosa from the same patients were separated by experienced pathologists and immediately stored at -80°C until use. All the patients received no treatment before surgery. Approval of the present studies was obtained from the Institutional Review Board of Cancer Institute/Hospital, CAMS&PUMC, and the informed consents were signed by all study participants who were treated with the utmost respect and safety. These patients included 143 males and 102 females, with a mean age of 56 y (range, 25-85 y). The detailed information of the patients and tissue clinicopathologic features are described in Supplementary Table S1. Colorectal cancer cases with no history of liver metastasis (104 cases; mean clinical follow-up, 68.7 ± 6.7 mo) were named as CRC-M_0. Colorectal cancer cases with history of liver metastasis were designated as CRC-M_1 (141 cases; mean clinical follow-up, 17.6 ± 14.1 mo). Metastasis was considered as synchronous (93 cases) when it had been detected by computed tomography (CT) scan, ultrasonography, or by surgery at the time of initial diagnosis; and cases were considered as heterochronous (48 cases; mean colon cancer liver metastasis time, 24.8 ± 15.1 mo) when liver metastases, confirmed by CT scan or ultrasonography, occurred after resection of the primary tumor. Immunohistochemical scores of each section were independently scored by two pathologists who were blinded to the patients' outcomes.

Tissue microarrays were prepared from archival formalin-fixed, paraffin-embedded tissue blocks. For each tumor, a representative tumor area was carefully selected from a H&E-stain section. A total of 245 specimens were divided into two groups including a training set (117 cases) and a test set (128 cases) that were respectively placed on two different tissue microarrays. Of these cases, 54 of CRC-M_0 and 63 synchronous cases of CRC-M_1 were randomly selected for the training set. The remaining 50 cases of CRC-M_0 and 30 synchronous cases of CRC-M_1 were then selected for the test set. All heterochronous cases were used for the test set, as the number of heterochronous cases was limited.

Adhesive assay and selection of SW1116 subpopulation by coculture with HLSECs. For adhesion assay with HLSECs or human lung endothelial cells, a 96-well plate, coated by 2% gelatin, was seeded with 1×10^4 endothelial cells per well and cultured until confluence. Tumor cells (5×10^5) labeled by calcein AM (Invitrogen) were then added to each well and cultured for 1 h. Nonadherent cells were removed by 5 times of wash with gentle shaking in serum-free medium. Adherent cells were photographed under fluorescent microscopy in three randomly chosen ×100 fields and counted using Image-Pro Plus Version 5.1 software (Media Cybernetics). Assays were repeated three times. The subfraction of colon tumor cells by coculturing with HLSECs was similar to that described above, except that adherent tumor cells were allowed to remain in culture flask for 72 h.

Immunohistochemistry. The avidin-biotin-complex method was used for immunohistochemical analysis. Briefly, after deparaffinization in xylene and graded alcohols, heated antigen retrieval was done in citrate buffer (10 mmol/L pH 6.0) by water-bath kettle heating for 30 min. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide for 10 min. Nonspecific binding was blocked by incubation in 10% normal animal serum for 10 min. Sections were incubated at 4°C for 24 h with a primary antibody for CCL2 (14-7099, eBioscience; 1:50), CCND3 (sc-6283, Santa Cruz; 1:200), CYR61 (sc-8561, Santa Cruz; 1:100), LGALS3 (sc-32790, Santa Cruz; 1:800), IQGAP1 (H00008826-M01, Abonova; 1:50), PAI-1 (sc-8979, Santa Cruz; 1:300), or SNCG (sc-10699, Santa Cruz; 1:200). Biotinylated secondary antibody and horseradish peroxidase–labeled avidin were subsequently used, and color was developed using the diaminobenzidine method. Expression levels of proteins were scored according to the stain pattern of malignant/epithelial cells. The stains of proteins, including Galectin3, PAI-1, CYR61, and SNCG, were heterogeneous and were determined by malignant/epithelial cells staining intensity and the percentage of immunoreactive cells according to ref. (18). Tissues with no staining were rated as 0, with faint staining or moderate to strong staining in 25% of cells as 1, with moderate staining or strong staining in 25% to 50% of cells as 2, and with strong staining in >50% of cells as 3. Colorectal cancer samples that registered levels 0

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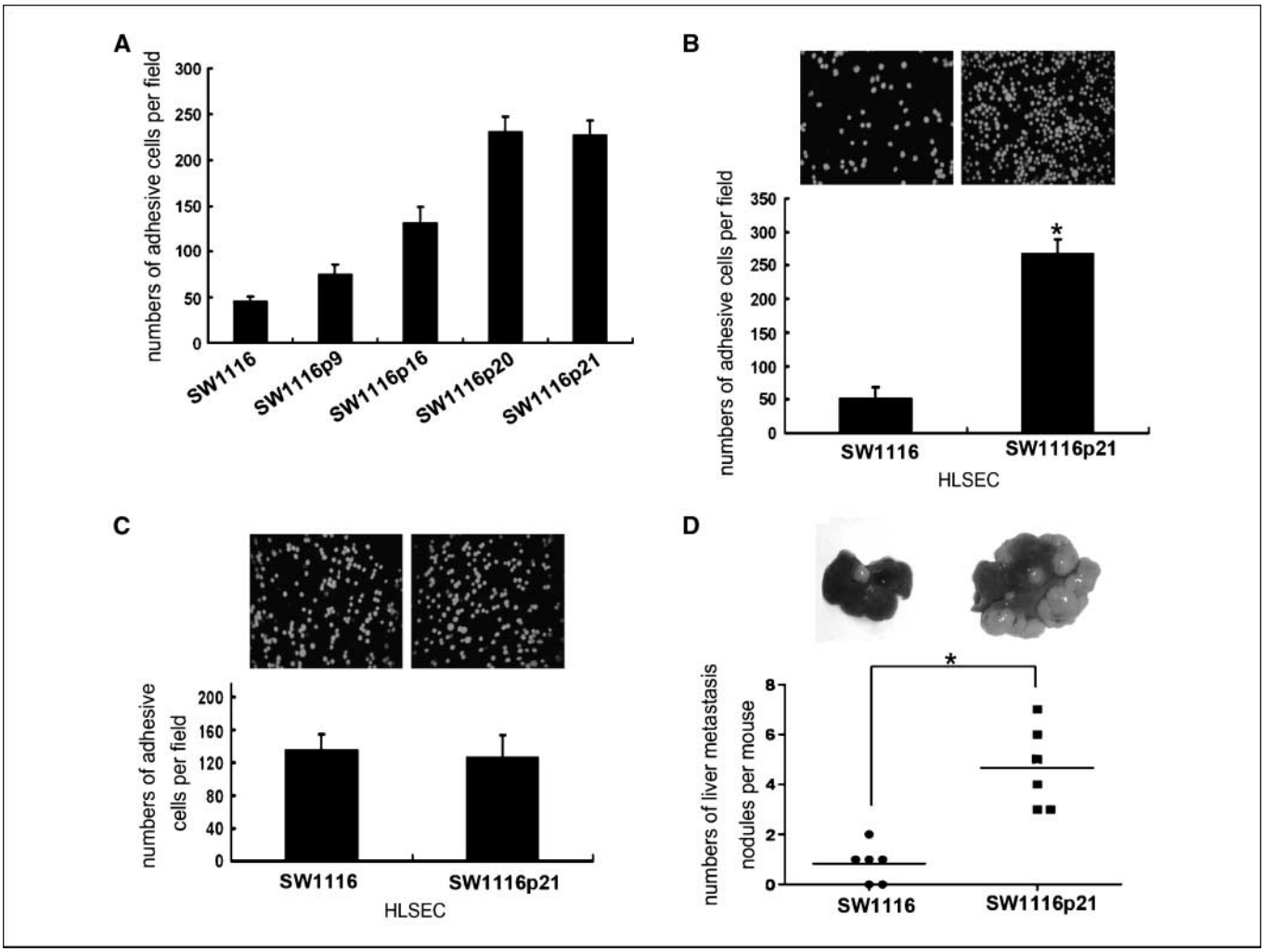

Fig. 1. Establishment of SW1116 subpopulations with high potential for liver metastasis. *A,* the adhesive ability of the indicated round of SW1116 subpopulations with HLSECs. *B,* SW1116p21 showed greater adhesive ability with HLSECs than SW1116 with HLSECs. Top, representative photos of SW1116 and SW1116p21 adhering to HLSECs; bottom, statistical results of the adhesion. *C,* SW1116 and SW1116p21 showed similar adhesive ability with human lung endothelial cells (HLEC). Top, representative photos of SW1116 and SW1116p21 adhering to HLECs; bottom, statistical results of the adhesion. *D,* representative photos of liver metastasis of SW1116 and SW1116p21 cells after injection into the spleen and statistical plots of liver metastasis foci of SW1116p21 and SW1116 cells. *, statistical significance ($P < 0.05$).

and 1 were defined as negative for expression, whereas samples at levels 2 or 3 were defined as positive. The nuclear staining intensity of SNCG is determined by the percentage of immunoreactive cells among total malignant/epithelial cells. Tissues with no staining to 10% of cells were rated as 0, staining in >10% of cells as 1. Colorectal cancer samples of levels 0 were defined as negative for expression, whereas samples of levels 1 were defined as positive. Three other proteins, namely, CCL2, CCND3, and IQGAP1, are normally expressed in a homogenous manner. According to previous studies (19), expression levels were graded across four categories depending on the percentage of positive cell as follows: irrespective of the intensity of the immunoreactive signal, no positive cells, 0; positive in <5% of constituent carcinoma cells, 1; positive in 5% to 50% of constituent carcinoma cells, 2; and positive in >50% of constituent carcinoma cells, 3. Colorectal cancer samples that registered level 0 were considered to be negative for expression, and samples containing levels 1 to 3 were defined as positive.

Statistical analysis. The SPSS 15 software package (SPSS, Inc.) was used for statistical analysis. The method for analysis the mouse liver metastases was two-sided *t*-test. The seven candidate markers expression was first analyzed as continuous numeric data, and the mean staining intensity between the primary tumors of CRC-M_0 and CRC-M_1 was compared with a two-sided *t*-test. According to an optimal cut point described in immunohistochemical analysis, CCL2 and SNCG level was analyzed as a dichotomous variable for further evaluation. The association between the immunoreactive markers and clinicopathologic features was analyzed using χ^2-test or two-sided *t*-test as appropriate. To estimate the variables of immunoreactive markers or clinicopathologic features that may contribute to the prediction of liver metastasis, those of significant difference between CRC-M_0 and CRC-M_1 were then evaluated by logistic regression analysis. The factors of significance in logistic regression analysis were used for training set. All possible combinations of these factors were used for the training set, to build up classifiers that were able to distinguish metastatic cases from nonmetastatic cases. The classifiers were examined using leave-one-out-cross-validation within cases of the training set. Then a discriminant equation and a cut point for the prediction was built according to each type combination of these factors. The discriminant equation and the cut point of the most satisfactory combination were then used to predict the probability of metastasis in an independent test. Receiver operating characteristics curves were generated to compare the predictive sensitivity and specificity, and the area under the curve. The survival rates were assessed by the Kaplan-Meier

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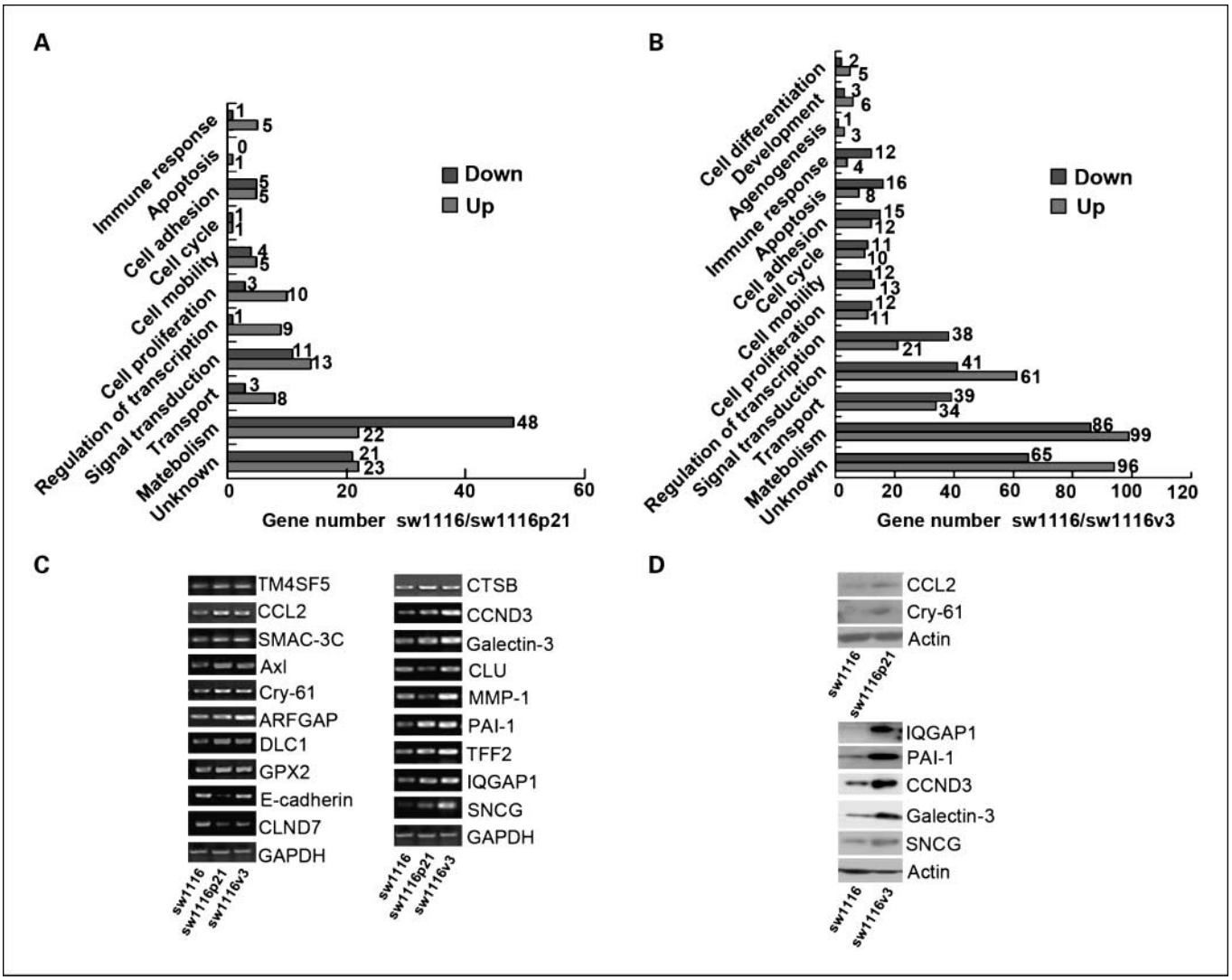

Fig. 2. Gene Ontology (GO) categories and expression validation of the dysregulated genes in the SW1116p21 and SW1116v3 cells. *A,* functional categories of dysregulated genes, SW1116p21 versus SW1116. Gene function is labeled using GO terms. Numbers of genes per category are indicated. *B,* functional categories of dysregulated genes, SW1116v3 versus SW1116. Gene function is labeled using GO terms. Numbers of genes per category are indicated. *C,* reverse transcription-PCR analysis of the selected genes. *D,* Western blot analysis of the seven candidate genes.

method and compared by the log-rank test. Statistical significance was set at $P < 0.05$ (two-tailed).

Results

Establishment of SW1116 subpopulations with high potential for liver metastasis by coculturing with HLSECs. In the present study, we found SW1116 had the lowest ability to adhere to HLSECs among the nine tested cell lines (Supplementary Fig. S1). As we sought to isolate a subpopulation of cells that would show a dramatic increase in adhesive and metastatic potential, we used SW1116 for the coculture selection. SW1116 cells were plated on HLSECs. After 1 hour the nonadherent cells were removed and the adherent cells were allowed to remain in the culture flask for 72 hours. During this time, the cancer cells began to penetrate the monolayer of HLSECs, and ultimately grew up to cancer cell nests. We then removed HLSECs by taking advantage of the fact that they were much more easily trypsinized than the colon cancer cells. The surviving tumor cells were then subjected to another round of selection. We found that the adhesive ability of the 9th, 16th, and 20th selected cells sequentially increased. SW1116p21 cells (the 21th cycle coculture with HLSEC) are 6-fold more adhesive than the parental cell line. However, SW1116p21 cells did not show significantly greater adhesion than SW1116p20 cells, suggesting that they had reached a plateau (Fig. 1A). We also found that SW1116p21 cells showed no greater adhesion to human lung endothelial cells than did the parental cells, which suggested that the SW1116p21 cells interacted specifically with HLSECs (Fig. 1B and C). The SW1116p21 or SW1116 cells were then injected into the spleen of six nude mice for each group. Eight weeks later, the mice were sacrificed and were examined for the presence of metastases. SW1116p21 cells caused significantly more visible liver metastatic foci than did SW1116 cells ($P < 0.05$; Fig. 1D). Lung metastasis was not observed in any of the animals.

Establishment of highly metastatic colon tumor cells by an in vivo selection model. We inoculated SW1116 cells into the spleens of seven nude mice for every selection round, and

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isolated cells from the liver that had most aggressive metastases for the next round of inoculation. This process was repeated three times, yielding the subpopulations SW1116v1, SW1116v2, and SW1116v3. The metastatic abilities of these cell lines were evaluated by macroscopic observation and by assessment of liver weight. Mice injected with either SW1116v2 or SW1116v3 cells developed significantly more visually observable liver nodules than did the mice injected with either SW1116 or SW1116v1 cells (data illustrated in Supplementary Table S3). Liver weight was 1.32 ± 0.12 g for SW1116, 2.28 ± 1.20 g for SW1116v1, 4.46 ± 2.03 g for SW1116v2, and 4.88 ± 1.98 g for SW1116v3, indicating that SW1116v3 cells possessed the highest potential to metastasize to the liver.

Metastasis-related gene expression analysis. The expression profiles of SW1116p21 versus SW1116 and SW1116v3 versus SW1116 cells were analyzed by microarrays. We found 200

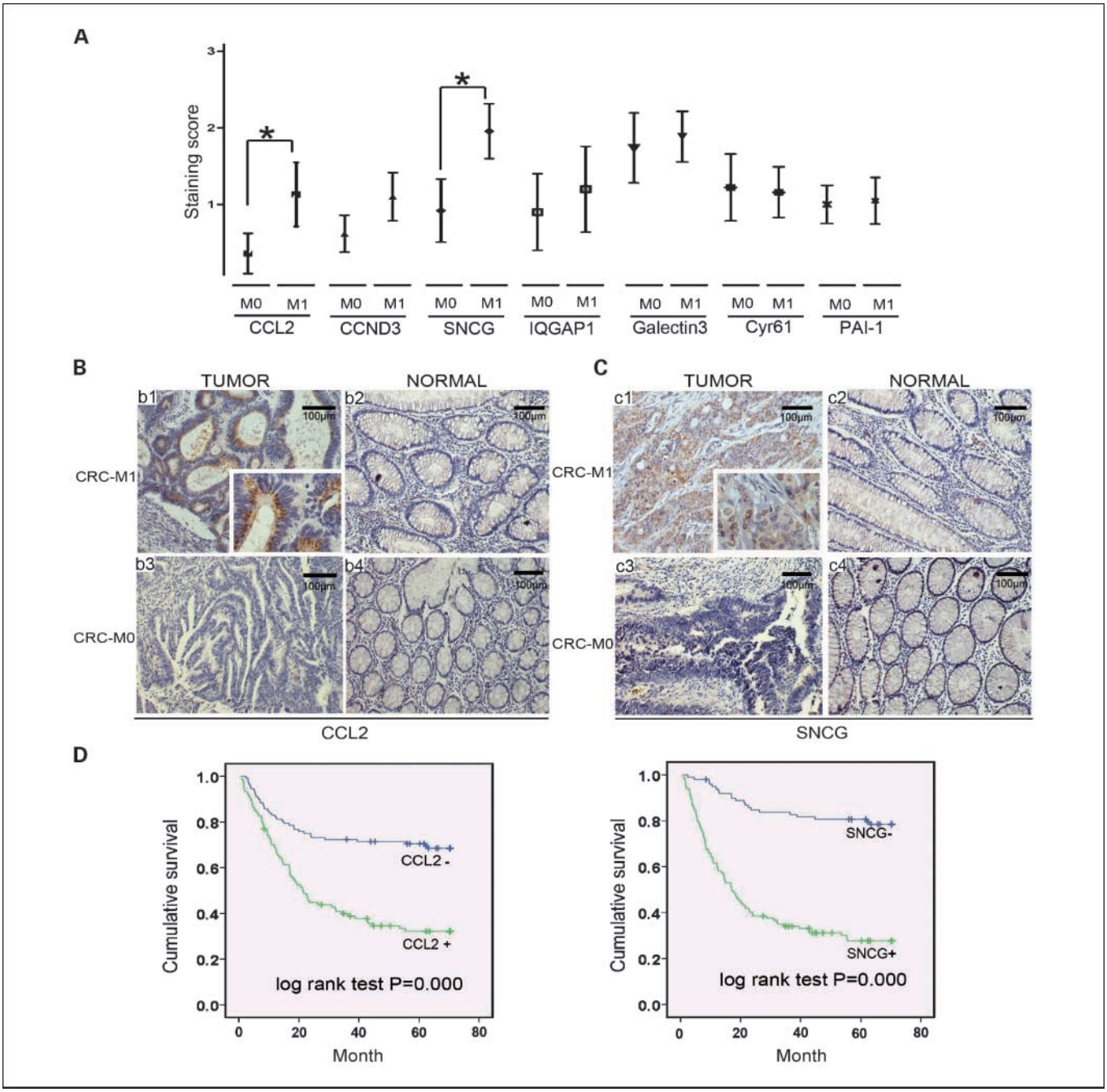

Fig. 3. Immunoscreen of candidate markers. *A,* statistical plots of the immunoreactive intensity of seven markers in 40 colorectal carcinoma cases. *, statistical significance ($P < 0.05$). *B,* representative immunohistochemical staining of CCL2 in colorectal cancer samples and matched normal tissues. The expression of CCL2 is significantly greater in the metastasis group (CRC-M_1) than in the control group (CRC-M_0). *C,* representative immunohistochemical staining of SNCG in colorectal cancer samples and matched normal tissues. The expression of SNCG is significantly greater in the metastasis group (CRC-M_1) than in the control group (CRC-M_0). Scale bar, 100 μm. *D,* Kaplan-Meier survival curve of colorectal cancer patients with CCL2 and SNCG expression. Patients with expression of CCL2 (*left*) and SNCG (*right*) showed significantly lower survival rates. ($P = 0.000$).

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Table 1. Correlation between CCL2 or SNCG expression and clinicopathologic characteristics in 117 cases

	SNCG			CCL2		
	Negative	**Positive**	***P***	**Negative**	**Positive**	***P***
Gender (male: female)	28:27	37:25	0.341	32:33	24:28	0.741
Age (y)	54.9 ± 11.7	55.7 ± 10.8	0.48	54.7 ± 11.8	55.9 ± 10.6	0.55
Serum CEA level			0.004*			0.000*
0	40	32		45	27	
1	7	6		5	8	
2	3	7		1	9	
3	3	19		4	18	
Tumor size (cm)	4.9 ± 2.5	6.15 ± 2.68	0.01*	5.1 ± 2.52	5.98 ± 2.69	0.19
Differentiation						
Well	4	7	0.682	5	6	0.705
Moderate	46	49		44	51	
Poor	4	6		6	4	
Depth of invasion			0.19			0.009*
T_1+T_2	11	6		14	3	
T_3	14	11		10	15	
T_4	30	45		32	43	
Lymph node involvement			0.111			0.011*
N_0	24	19		28	15	
N_1	17	20		13	24	
N_2	12	25		14	23	
Stage						
I	8	6	0.000*	12	2	0.000*
II	16	4		13	7	
III	18	2		16	4	
IV	13	50		15	48	
M			0.000*			0.000*
M_0	42	12		41	13	
M_1	13	50		15	48	

NOTE: CEA: 0, ≤15 ng/mL; 1, >15-≤40 ng/mL; 2, >40-≤100 ng/mL; 3, >100 ng/mL.
*$P < 0.05$.

genes differentially expressed between SW1116p21 and SW1116 cells, including 102 genes that were up-regulated and 98 that were down-regulated with respect to the parental SW1116 cells (cutoff of >2-fold difference, in supplement). When comparing the expression profile of the SW1116v3 with the parental cells, 383 genes were found to be up-regulated, and 356 down-regulated (in supplement). Most of the differentially expressed genes seemed to participate in metabolism and cancer progression, such as signal transduction, invasion, adhesion, and proliferation (supplementary data). Figure 2A and B show the biological terms of the differential expressions. The two dysregulated gene profiles shared 28 common up-regulated genes (in supplement). Most of these common differentially expressed genes seemed to play roles in metabolism, adhesion, transportation, etc.

To verify the results of microarray, we selected 19 genes, which had been reported to play roles in cancer metastasis, from the dysregulated profiles. Two down-regulated and 17 up-regulated genes were examined by reverse transcription-PCR for three times, which showed that the expression changes of 17 selected genes were in consistence with the microarray data, except *ARFGAP* and *CTSB* (Fig. 2C). Then we chose seven genes for Western blot analysis, including *Cyr61* and *CCL2* that overexpressed in SW1116p21 cells, and *CCND3*, *SERPINE1*, *IQGAP1*, *Galectin3*, and *SNCG* overexpressed in SW1116v3 cells. The relative expression levels of the seven proteins in the selected and parental cells were comparable to those measured at the RNA level (Fig. 2D).

Immunohistochemical analysis reveals that CCL2 and SNCG expression are correlated with liver metastasis of colorectal cancer. We initially evaluated the significance of the seven proteins in 40 colorectal cancer cases, including 20 CRC-M_0 and 20 CRC-M_1, which were randomly selected from the 117 cases of the training set. *T*-test analysis revealed that the levels of CCL2 and SNCG, but not Cyr61, CCND3, SERPINE1, IQGAP1, or Galectin3, were significantly higher in the primary tissues of metastasis cases than in those of the control cases (Fig. 3A). In addition, about 8% cases and 13% cases of colon cancers also weakly expressed SNCG and CCL2 in the stromal cells, respectively. Therefore, CCL2 and SNCG were selected for further immunohistochemical analysis with all cases in the training set. The expression of both proteins was higher in the metastasis group than in the nonmetastasis group (Fig. 3B and C). Moreover, clinical factors that included lymph node metastasis, depth of invasion, tumor size, serum carcinoembryonic antigen (CEA) level, and stage showed significant differences between the CRC-M_0 and CRC-M_1 groups. In these cases of the training set, intense CCL2 and SNCG expressions were both correlated with serum CEA level, clinical stage, and distant metastasis. The expression of CCL2 was further correlated with lymph node metastasis and depth of invasion, and the expression of SNCG was further related to tumor size (Table 1).

Additionally, nuclear stain of SNCG was observed in 41 (16.7%) cases among all the 245 cases from the training and test sets (Fig. 3C). This nuclear staining pattern was significantly correlated with stage, and liver and lymph node metastasis

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Table 2. Test course in the independent set of 128 colon cancer patients

	Sensitivity(%)	Specificity(%)
Liver metastasis		
Clinical factors	67.9	74
Two markers	78.2	76
Clinical factors+two markers	89.7	82
Heterochronous liver metastasis		
Clinical factors+two markers	87.5	82

(Table S4). Kaplan-Meier analysis from 245 colorectal cancer cases showed that the intensive expressions of CCL2 and SNCG were both associated with clinical failures (Fig. 3D).

CCL2 and SNCG expression levels predict the risk of metastasis of colon cancer to the liver. Logistic regression was done, according to the above analysis from cases in the training set, to calculate the respective significance of each candidate marker and clinical features for liver metastasis prediction. Expression levels of CCL2 and SNCG in primary tumors, serum CEA level, and lymph node involvement showed predictive significance ($P < 0.05$) and increased the metastatic ratio. The odds ratios of CCL2 and SNCG were 3.026 and 6.894, respectively (Supplementary Table S5). To further evaluate the predictors that reached statistical significance in logistic regression analysis, the four variables were selected for leave-one-out-cross-validation analysis. This analysis revealed that the combination of SNCG, CCL2, serum CEA level, and lymph node involvement yielded the most satisfactory sensitivity (90.5%) and specificity (90.7%) in the training set. Also a discriminant equation was built [f(x) = -2.713 + 1.804*SNCG + 1.041*CCL2 + 0.936*CEA +1.288*N], where the levels of SNCG/CCL2 in primary tumors, serum CEA, and lymph node involvement (N) were standardized for normalized value. According to the discriminant equation, "0" was defined as the cut point. The discriminant equation and cut point were then used for the prediction in an independent test set. Using the two biomarkers, along with lymph node involvement and serum CEA level, the diagnostic assay could correctly classify 70 of 78 CRC-M_1 cases, with 8 CRC-M_0 cases misclassified. The sensitivity and specificity were 89.7% and 82%, respectively. Moreover, of the 48 heterochronous metastatic cases, this combination could correctly classify 42 cases and achieved 87.5% sensitivity and 82% specificity (Table 2). We next calculated the receiver operating characteristic curve for the combination in the test set. The area under the curve for the combination of lymph node involvement and serum CEA level was 0.749 (95 % confidence interval, 0.665-0.834; Fig. 4A). The SNCG/CCL2 combination showed increased discrimination ability; the area under the curve was 0.830 (95% confidence interval, 0.754-0.906; Fig. 4B). The ability of SNCG/CCL2, combined with lymph node involvement and serum CEA level, to discriminate metastasis from control samples was significant ($P = 0.000$), with an area under the curve equal to 0.896 (95% confidence interval, 0.838-0.953; Fig. 4C).

Discussion

Metastasis is a sequential process that includes detachment from the primary site, invasion into vessels, survival in blood, adhesion to the target organ, extravasation, and colonization of a distant site. According to Paget's "seed and soil" hypothesis, we postulated that interactions between tumor cells and the microenvironment of target organs, including endothelium, might have a significant influence on the outcome of the metastatic process (20). We therefore developed two different models that mimicked this interaction and attempted to identify genes that participated in this process.

Cancer cell populations are thought to be heterogeneous. It is possible that highly metastatic subpopulations could be isolated from the original mixture (21, 22). After selection by the model of interaction between colon cancer cells and HLSECs, SW1116p21 cells could develop more observable metastatic foci than SW1116 cells. This result suggested that interaction with HLSECs could allow the selection of cell lines with a higher capacity to form liver metastases. Similarly, using a model based on selection of the *in vivo* mouse liver microenvironment, we

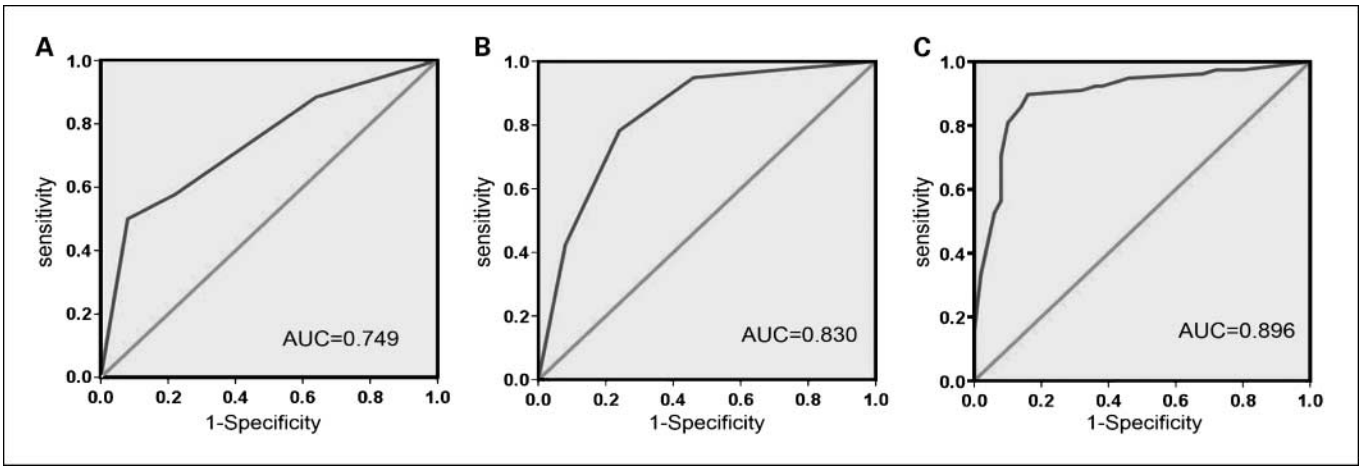

Fig. 4. The receiver operating characteristics curves and the corresponding values of area under the curve (AUC) of CCL2 and SNCG, in combination with clinicopathologic features, in the test set. *A,* clinicopathologic features of lymph node involvement and serum CEA level; *B,* CCL2 and SNCG in combination; *C,* CCL2 and SNCG in combination with lymph node involvement and serum CEA level.

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derived colon cancer cell lines with an elevated potential to metastasize to liver (23).

To help understand the difference between the subpopulations and their parental cells, we compared genome-wide expression profiles. Interestingly, in both the biological aspects and gene expression patterns, the differences between SW1116 and SW1116v3 cells were greater than those between SW1116 and SW1116p21 cells. Most of the genes with significant change in SW1116v3 cells were different from those in SW1116p21 cells. This might be a consequence of the different microenvironments that the cells faced in the *in vitro* or *in vivo* model. For example, Kupffer cells (24) and fibroblasts (25) in the liver, which are obviously absent in the *in vitro* model, are thought to contribute to the hepatic metastasis of colon cancer cells. In addition, the genetic heterogeneity between human and murine liver endothelial cells might have contributed to the difference in gene expression profiles. Therefore, we chose candidate genes according to the features of the selection models. In the *in vitro* model of tumor cell-endothelium interaction, we chose genes that might be involved in the interactions of these two kinds of cells. *Cyr61* is known to promote the adhesion of metastatic gastric cancer cells to the endothelium of the peritoneum through integrin $\alpha 2\beta 1$ (26). *CCL2* is involved in the interaction of malignant prostate cells with bone marrow endothelial cells to create a fertile environment for the metastasis (27). The *in vivo* selection model represented the tumor cells interaction with the microenvironment of liver. Molecules with different functions, such as cell cycle, mobility, adhesion, angiogenesis, and invasion, were considered. *PAI1*, a protease inhibitor, has a role in extracellular matrix turnover and angiogenesis (28). *CyclinD3* expression is clinically correlated with distant metastasis (19). *IQGAP1* has been shown to promote tumor cell motility in cancer metastasis (29). Its expression is up-regulated in colorectal carcinomas and is associated with invasion fronts (30). Recent reports have shown that *Galectin-3* can promote cancer metastasis by altering cell surface glycosylation to increase cancer cell adhesion with the endothelium (31). Moreover, overexpression of *Galectin-3* is observed in liver metastasis foci of colon cancer (32). However, the expression of *Cry61*, *PAI1*, *CyclinD3*, *IQGAP1*, and *Galectin-3* failed to correlate with hepatic metastasis of colorectal cancer in the present immunohistochemical analysis. We deduced that these genes might participate in colon cancer metastasis but not specifically in liver metastasis or do not promote liver metastasis at the initial step in primary tumors, although they were markedly up-regulated in SW1116p21 or SW1116v3, and might promote tumor cell migration to liver in the metastasis models. This might also suggest that the models of colon cancer liver metastasis we used were not able to fully mimic the real situation of human cancer progression.

Immunohistochemical analysis revealed that CCL2 and SNCG were highly expressed in the metastasis group compared with the nonmetastasis group among the seven proteins. Intensive expressions of CCL2 and SNCG were also correlated with less favorable clinical outcome. When were examined the ability to predict colon cancer liver metastasis using logistic regression analysis, the expression of CCL2, SNCG, and the clinicopathologic features of serum CEA level and lymph node involvement were found to be accurate predictors. Cross-validation showed that the combination of these four variables achieved 90.5% sensitivity and 90.7% specificity for distinguishing CRC-M_1 from CRC-M_0. The combination was also verified by an independent test set including 50 cases of CRC-M_0, 30 synchronous cases of CRC-M_1, and 48 heterochronous cases of CRC-M_1. We speculated that heterochronous metastatic cases might have developed micrometastatic foci at the time of initial diagnosis, although it could not be detected by available clinical methods. Otherwise, their cancer cells might have gotten the potential of liver metastasis at the initial diagnostic time although the metastasis had not yet occurred. The local mini foci that were not removed by surgical resection might lead to hepatic metastasis in the coming years. Therefore, the heterochronous metastatic case might have similar dysregulated gene profile with the synchronous metastatic case. In the test set, 111 samples (86.7%) were successfully predicted by the combination. Of 48 heterochronous metastatic cases, 42 cases (87.5%) were correctly classified. In addition, we checked the wrongly predicted cases of the test set. We did not find a tendency that the wrongly predicted heterochronous metastatic cases were mostly from the cases of developing metastases in the first two years after surgical resection or from the cases of developing metastases after two years of surgical resection. And also there was not a tendency that the incorrectly predicted metastatic cases were mostly from the synchronous or heterochronous metastatic cases in the test set.

CCL2 is a member of the CC family of chemokines, and reports about its biological significance in cancer progress are conflictive. In one hand, CCL2 can potentiate both the innate and acquired immune responses that inhibit tumor growth through recruiting immune cells to enter the tumors and enhance contact-dependent cytolysis of tumor cells (33–35). On other hand, CCL2 induces macrophage accumulation and cyclooxygenase-2 expression promoting inflammation in colorectal adenoma epithelium, which has been reported to promote tumor progression in autocrine and paracrine fashions (36–39). Furthermore, CCR2 (receptor of CCL2) can accumulate and regulate hepatic Kupffer cells and stellate cells to secrete matrix metalloproteinase 2, which increases neovascularization and plays a significant role in colon cancer liver metastasis (40). SNCG (synuclein-g) is a member of a neuronal protein family synuclein and is highly expressed in diversified types of cancer primary carcinomas, which might be used as a pathologic indicator to predict the propensity of metastasis to distant organs (41). Our data suggest that a clinical application of the two biomarkers, combined with clinicopathologic features, might provide a valuable index of the probability of liver metastasis before it could be observed in clinical practice, and would result in regular check-up for the prevention of liver metastasis. And the successful prediction of colon cancer liver metastasis by the biomarkers may help doctors design more aggressive treatments to extend survival for patients, such as some kind of more intensive and specific chemotherapy using cytotoxic drugs along with targeted drugs to prevent metastasis formation. In addition, accumulating reports have shown the application of CCL2 and SNCG targeting treatments in cancer prevention. For example, neutralizing antibodies targeting CCL2 inhibit prostate cancer (42) and melanoma (43) progression. A novel peptide (ANK) can associate with SNCG intracellular part and counteract drug resistance in breast cancers (44). Furthermore, our results identified that expression of CCL2 and SNCG was correlated with colon cancer liver metastasis and suggested these two molecules might play important roles in the hepatic

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metastasis. The CCL2 and SNCG targeting therapy may provide an alternative treatment for the liver metastasis of colon cancer.

In summary, we used an *in vitro* and an *in vivo* model of tumor-microenvironment interaction to get colon cancer cells with high metastatic potential, and identified two promising biological markers for predicting liver metastasis of colorectal cancer. Prospective studies are required to assess these novel markers for evaluating the hepatic metastasis risk in the future work.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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