Tumor β-1,4-Galactosyltransferase IV Overexpression Is Closely Associated with Colorectal Cancer Metastasis and Poor Prognosis

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Abstract

Purpose: To elucidate the significance of β-1,4-galactosyltransferase IV (β-1,4-GT-IV) in the clinical presentation and prognostication of colorectal cancer.

Experimental Design: Tissue lysates from paired tumor and nontumor tissues of a colon cancer patient were labeled separately with fluorescent dyes Cy5 and Cy3 for two-dimensional difference in-gel electrophoresis. Subsequent matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and immunoblot analyses identified a down-regulated level of β-1,4-GT-IV in the tumor tissue. In the follow-up study, paired tissue lysates were obtained from 100 colorectal cancer patients with immunoblot analyses done to compare the levels of β-1,4-GT-IV expression in these patients.

Results: Of 100 colorectal patients studied, 48% had down-regulated expression of β-1,4-GT-IV in the tumor tissue but 28% of patients exhibited elevated β-1,4-GT-IV levels. Increased β-1,4-GT-IV in the tumor tissue was significantly coexistent with raised serum level of CA-199 and the presence of tumor metastasis ($P = 0.006$ and $P < 0.001$, respectively) but was independent of age and gender of patient, tumor site, tumor size, serum level of carcinoembryonic antigen, grade of tumor cell differentiation, and depth of tumor invasion. The results of logistic regression analyses suggested that tumor β-1,4-GT-IV overexpression and tumor invasion, but not other patient variables such as tumor size and serum levels of carcinoembryonic antigen and CA19-9, were significantly correlated with the occurrence of metastases ($P < 0.05$). In a multivariate regression analysis, the patient group with tumor β-1,4-GT-IV overexpression strongly predicted for tumor metastasis (odds ratio, 10.009; 95% confidence interval, 2.992-33.484; $P < 0.001$). Likewise, tumor β-1,4-GT-IV overexpression was significantly associated with poor overall survival ($P < 0.01$). By Cox regression analysis, this association remained significant even after adjustment for tumor metastasis ($P = 0.048$).

Conclusion: Increased β-1,4-GT-IV expression in tumor tissue was strongly associated with tumor metastases and poor prognosis in colorectal cancer.

Cancer is a complicated and heterogeneous disease resulting from multiple molecular alterations (1). To explore the breadth and width of protein alterations in cancer, clinical proteomics provides an accessible route that can assist us in the understanding of the process of carcinogenesis, in the development new biomarkers for diagnosis and early detection of cancer, and ultimately in the determination of new therapeutic targets for future therapeutic manipulation (2). For proteomic analysis of tumor tissue proteins, the first and most fundamental step is to efficiently extract tissue proteins from the study specimen. We have previously reported a rapid and efficient tissue lysis protocol compatible with subsequent proteomic and immunoblot analyses (3). In the present study, the tissue lysates prepared by the new protocol were labeled with fluorescent dyes Cy5 and Cy3 and resolved by two-dimensional fluorescence difference in-gel electrophoresis (DIGE). The ensuing mass spectrometric and immunoblot analyses identified a protein, β-1,4-galactosyltransferase IV (β-1,4-GT-IV), to be differentially expressed in the tumor tissue compared with adjacent nontumor tissue of a colorectal cancer patient.

β-1,4-GTs (EC 2.4.1.38) are a family of glycosyltransferases responsible for biosynthesizing *N*-acetyllactosamine by the transfer of a galactose from UDP-galactose to the terminal *N*-acetylgluosamine of acceptor sugars in glycoproteins or glycolipids with a β-1,4-linkage (4–6). There are thus far seven isoforms of β-1,4-GT reported (i.e., types I-VII; ref. 5, 6). Much

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information about β-1,4-GTs was obtained from the studies on β-1,4-GT-I. Expression of β-1,4-GT-I, and in turn the increased level of *N*-acetyllactosamine, has been reported to be associated with occurrence of various malignancies (7–9). An experimental design exploiting antisense DNA to down-regulate β-1,4-GT-I expression has linked β-1,4-GT-I expression to increased metastatic potential of cancer cells (10). The underlying mechanism can be partly attributed to the role served by β-1,4-GT-I as an adhesion molecule for cell-to-cell or cell-to-matrix interaction (11, 12). In addition, β-1,4-GT-I can regulate the glycosylation of sialyl $Lewis^X$–containing proteins (13, 14), which is purported to be a ligand for E-selectin on the vascular endothelium (15). However, studies on the clinical relevance turn out to be lacking to consolidate the association of β-1,4-GT-I with tumor metastasis.

β-1,4-GT-IV shares 41% identity with β-1,4-GT-I (16) but it remains to be determined whether β-1,4-GT-IV plays a similar role in cancer cell metastasis. In this study, we prepared tissue lysates from 100 colorectal cancer patients, followed by immunoblot analyses to detect the expression levels of β-1,4-GT-IV. Forty-eight of 100 colorectal cancer patients exhibited down-regulated β-1,4-GT-IV expression in their tumor tissues whereas 28 patients expressed raised levels of β-1,4-GT-IV in the tumor tissues compared with their adjacent nontumor tissues. Further statistical analyses revealed that tumor β-1,4-GT-IV overexpression was a potent risk factor for occurrence of metastases and a prognostic marker for colorectal cancer.

Materials and Methods

Patients and tumor specimens. This study was to retrospectively investigate the significance of abnormal β-1,4-GT-IV expression in colorectal cancer patients. Assuming a 5-year survival rate of 60% for the control group and 30% for the other group with an expression of 20%, α error of 0.05, power of 80%, and two-sided tests, the sample size was calculated and chosen as 100 patients by using SamplePower 2.0 software (SPSS, Chicago, IL). At first, we collected paired tumor and adjacent nontumor tissues from 100 patients who received surgical resection for colorectal cancer at Taipei Veterans General Hospital during the time period of 1993 to 2004. The informed consent was obtained from each patient. A total of 102 surgical operations were done in these 100 patients, including 2 patients who received both right hemicolectomy and anterior resection due to multiple lesions. The surgical procedures included right hemicolectomy for 21 patients, left hemicolectomy for 12 patients, anterior resection for 21 patients, low anterior resection for 37 patients, abdominoperineum combined resection for 9 patients, and subtotal colectomy for 2 patients. Tissue specimens were frozen in liquid nitrogen immediately after resection and PBS rinse. All were kept in a −80°C freezer until retrieval for study. The nontumor parts, taken from a site at least 10 cm away from the tumor part, were pathologically certified to be free from tumor cells. Our 100 patients consisted of 8 patients of TNM stage I disease, 40 patients of stage II disease, 34 patients of stage III disease, and 18 patients of stage IV disease. All patients of stage IV disease were due to liver metastasis and received surgical resection of primary and metastasizing lesions as well. The patients with tumors unable to be completely removed, such as carcinomatosis or multiple metastases over bilateral lobes of liver, were excluded from this study. All patients of stage III and IV diseases received postoperative chemotherapy with 5-fluorouracil and leucovorin. No patients received any type of research study.

Tissue lysate preparation. We prepared tissue lysates according to the method previously described (3). Briefly, tissue pieces of ~150 mg were added with 1 mL of prechilled T-lysis buffer [30 mmol/L Tris-HCl (pH 9.0), 9 mol/L urea, 4% CHAPS, 1% DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL pepstatin A, 10 μg/mL leupeptin] and subjected to oscillation made by the MagNA Lyser (Roche, Penzberg, Germany) at 6,500 rpm for 80 seconds. The lysate was then centrifuged at 100,000 × *g* for 1 hour at 4°C and the supernatant was saved and assayed for protein concentration by Lowry method (17).

Two-dimensional DIGE. Fifty micrograms of tissue proteins in T-lysis buffer were covalently labeled with 400 pmol of the fluorescent dye Cy5 or Cy3 according to the protocol of the manufacturer (Amersham Biosciences, Piscataway, NJ). After mixing the Cy5- and Cy3-labeled protein samples, 2% of ampholines/pharmalytes (pH 3-10; Amersham Biosciences) was added before two-dimensional gel electrophoresis. Immobilized linear pH gradient strips were rehydrated overnight with tissue protein samples in the dark at room temperature (18). Isoelectric focusing was done at 20°C for a total of 17,500 V-h. The strips were then equilibrated for 15 minutes in 50 mmol/L Tris-HCl (pH 8.8), 6 mol/L urea, 2% (w/v) SDS, 64 mmol/L DTT, 30% (v/v) glycerol, and a trace of bromophenol blue. Equilibrated immobilized linear pH gradient strips were transferred onto 16 × 18-cm 12% uniform polyacrylamide gels. Standard SDS-PAGE was done and the DIGE images were obtained by scanning the gels with Typhoon 8600 Imager (Amersham Biosciences). The gels were poststained with another fluorescent dye, SYPRO Ruby (Molecular Probes, Eugene, OR), before spot picking.

Protein spot identification. The spot of interest (designated as Spot X) was manually excised from two-dimensional gels. After washing twice with 25 mmol/L ammonium bicarbonate in 50% acetonitrile and once with 100% acetonitrile, gel pieces were vacuum-dried and rehydrated with 10 μg/mL of trypsin (Promega, Madison, WI) in 25 mmol/L ammonium bicarbonate (pH 8.0). Proteins in gel pieces were digested overnight at 37°C. The supernatant was collected and the peptides in gel pieces were further extracted with 5% trifluoroacetic acid in 50% acetonitrile. The peptide extract was vacuum-dried and resolubilized in 5 μL of 0.1% trifluoroacetic acid. One microliter of peptide extract was then spotted onto the target plate and α-cyano-4-hydroxycinnamic acid was added as the matrix. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was done using M@LDI-R reflector time-of-flight mass spectrometer (Micromass, Manchester, United Kingdom). All mass spectra were internally calibrated with adrenocorticotropic hormone peptide and trypsinized alcohol dehydrogenase peaks. Peptide mass mapping was carried out using the MS-Fit program (Protein Prospector, University of California, San Francisco, CA).

Preparation of antibody and immunoblot analysis. Anti–β-1,4-GT-IV antibody was prepared by immunization of a rabbit with the peptide ENPKVSRGRYRPQECKA corresponding to the amino acids 104 to 120 of β-1,4-GT-IV. The sequence was suggested by Vector NTI Suite 7 software according to the method previously described (19) and the peptide was conjugated to Keyhole limpet hemocyanin to increase immunogenicity. Immunoblot analyses were done by a canonical procedure (20). In brief, 40 μg of protein samples were resolved in 12% SDS-polyacrylamide gels and then electrotransferred onto polyvinylidene membranes (Amersham Biosciences). After blocking with PBST (PBS plus 0.1% Tween 20) plus 5% nonfat milk, the blots were incubated with anti–β-1,4-GT-IV antibody (in PBST plus 5% milk) at 4°C for 12 hours. The blots were then washed thrice with PBST buffer and incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. Blots were again washed thrice with PBST buffer and the protein bands representing β-1,4-GT-IV were obtained by enhanced chemiluminescence (Amersham Biosciences). The levels of β-1,4-GT-IV were quantitated via measurement of the band intensities by AlphaImager 2000 (Alpha Innotech Co., Avery Dennison, CA). The levels of β-actin in the tissue lysates were also detected as internal reference. The status of β-1,4-GT-IV levels in between tumor and adjacent nontumor tissues was considered as overexpression or down-regulation only when the ratio of difference

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was >2 and <0.5, respectively. In addition, sialyl Lewis^X–containing proteins were also detected from tissue lysates by using anti–sialyl Lewis^X antibody, which was generously provided by Prof. Senitiroh Hakomori (Pacific Northwest Research Institute, Seattle, WA).

Statistical analyses. All statistical analyses were done by the SPSS 11.0 software package (SPSS). The relationships between β-1,4-GT-IV expression and patients' clinicopathologic characteristics were analyzed using the χ^2 test. Differences in the ages and tumor sizes among the patient groups with differential β-1,4-GT-IV expression status were evaluated by one-way ANOVA. Logistic regression analyses were used to assess the association of independent variables with the occurrence of tumor metastasis. Survival rates were calculated by the Kaplan-Meier method and compared by the log-rank test. Cox regression analysis was done to investigate whether tumor β-1,4-GT-IV overexpression with respect to the poor overall survival remained after adjustment for tumor metastasis. Results were considered statistically significant at $P < 0.05$ (two-tailed test).

Results

Identification of β-1,4-GT-IV differentially expressed in colorectal tumor versus nontumor tissue. To search for proteins abnormally expressed in colorectal cancer, tissue lysates from the tumor and adjacent nontumor tissues of a colon cancer patient were labeled with Cy5 and Cy3 for performance of the two-dimensional DIGE. As shown in Fig. 1A, Cy5-labeled proteins were derived from the tumor tissue and Cy3-labeled proteins were from the adjacent nontumor part. The merged image showed a protein spot, called X, to be underexpressed in the tumor tissue (Fig. 1A). Spot X was picked up for trypsin digestion and subjected to MALDI-TOF analysis. Its spectrum is shown in Fig. 1B. The data of the spectrum were searched against the MS-Fit program and β-1,4-GT-IV came up as the most likely candidate. Immunoblot analysis was therefore done to ascertain whether β-1,4-GT-IV was indeed differentially expressed in tumor versus nontumor tissue. Anti–β-1,4-GT-IV antibody was prepared by immunization of a rabbit with the peptide corresponding to the amino acid sequence $_{104}$ENPKVSRGRYRPQECKA$_{120}$ of β-1,4-GT-IV. The resultant immunoblot analysis confirmed that β-1,4-GT-IV was down-regulated in the tumor part of this particular colon cancer patient (Fig. 1C).

Correlation of tumor β-1,4-GT-IV overexpression with colorectal cancer metastasis. To ascertain the differential expression of β-1,4-GT-IV existing in colon cancer patients, we prepared tissue lysates from paired tumor and nontumor tissues of 100 colorectal cancer patients, followed by performance of immunoblot analyses to compare the levels of β-1,4-GT-IV expression in these patients. The results from six patients are shown in Fig. 2A as representative examples. Forty-eight of 100 colorectal cancer patients exhibited down-regulation of β-1,4-GT-IV in their tumor tissues whereas 28 patients had elevated levels of β-1,4-GT-IV in the tumor tissues rather than their adjacent nontumor tissues. The levels of sialyl Lewis^X–containing proteins in tissue lysates of these patients were also detected (Fig. 2B) but we failed to observe a correlation between the β-1,4-GT-IV overexpression and elevated levels of sialyl Lewis^X–containing proteins ($P = 0.350$).

Next, we investigated the clinical relevance of differential β-1,4-GT-IV expression in paired tumor and nontumor tissues. As shown in Table 1, tumor β-1,4-GT-IV overexpression exhibited no correlation with age and gender of patients, tumor site, tumor size, or grade of tumor cell differentiation but was significantly correlated with TNM staging ($P = 0.001$). Further analysis of the stage I and II patients using nonparametric χ^2 test revealed a higher ratio of these patients exhibiting down-regulated expression of β-1,4-GT-IV in their tumor tissues ($P < 0.001$); in contrast, most patients in the stage IV exhibited tumor β-1,4-GT-IV overexpression ($P < 0.001$). Determination of TNM staging is based on the depth of tumor invasion and the occurrence of lymph node or distant metastasis. Our analysis revealed that tumor β-1,4-GT-IV overexpression strongly correlated with tumor metastasis ($P < 0.001$) but not with tumor invasion ($P = 0.636$). Among the 28 patients with raised β-1,4-GT-IV levels in their tumor tissues, 24 (85.7%) cases had documented metastases to the lymph nodes or other visceral organs; in contrast, 60.4% of the patients with tumor β-1,4-GT-IV down-regulation had cancer confined to the primary site without documentation of metastases (Table 1). Additionally, the relationships between tumor β-1,4-GT-IV overexpression and increased serum levels of tumor markers carcinoembryonic antigen (CEA) and CA19-9 were investigated. Serum levels of CEA and CA19-9 were obtained from 91 and 54 of 100 patients, respectively, just before surgical removal of the tumor. In our analysis, 51 of 91 patients had the CEA levels higher than the normal cutpoint value 6 ng/mL in their sera but did not have significantly correlated elevation of β-1,4-GT-IV expression in the tumor tissues ($P = 0.089$). Among them, 20 of 51 (39.2%) patients had tumor β-1,4-GT-IV overexpression but there were also 19 patients exhibiting down-regulated β-1,4-GT-IV levels in the tumor tissues. On the other hand, patients with raised β-1,4-GT-IV expression in tumor tissues were observed to express higher levels of CA19-9 in their sera ($P = 0.006$; Table 2).

Tumor β-1,4-GT-IV overexpression as a risk factor for colorectal cancer metastasis. Next, logistic regression analyses were done to evaluate the predictive value of tumor β-1,4-GT-IV overexpression for metastatic occurrence. The patient variables, including elevated β-1,4-GT-IV expression in tumor tissue, age, tumor size, depth of tumor invasion, and serum levels of CEA and CA19-9, were subjected to univariate analyses (Table 3). Two variables (i.e., elevated β-1,4-GT-IV expression and tumor invasion) had $P < 0.05$ and thus entered into a multivariate regression analysis. The patient group with tumor β-1,4-GT-IV overexpression significantly exhibited presence of tumor metastasis ($P < 0.001$; Table 4). The odds ratio was 10.009 (95% confidence interval, 2.992-33.484) for this patient group compared with the other group without elevated β-1,4-GT-IV expression. The univariate correlation of tumor invasion with metastatic occurrence did not reach a statistical significance after the multivariate analysis ($P = 0.055$).

Tumor β-1,4-GT-IV overexpression as a prognostic factor for colorectal cancer. Metastatic spread of tumor cells is the major cause of colorectal cancer mortality. In 100 studied patients, the patient group with tumor metastases had shorter overall survival when compared with those without metastases (5-year overall survival, 27% versus 63%; $P < 0.001$). As indicated above, tumor β-1,4-GT-IV overexpression served as a potential risk factor for the occurrence of metastases. Likewise, increased β-1,4-GT-IV expression in tumor tissue was significantly associated with patient's poorer overall survival ($P < 0.01$; Fig. 3). Using the SamplePower 2.0 software, the power of this study was 88% to detect a 5-year survival difference with a

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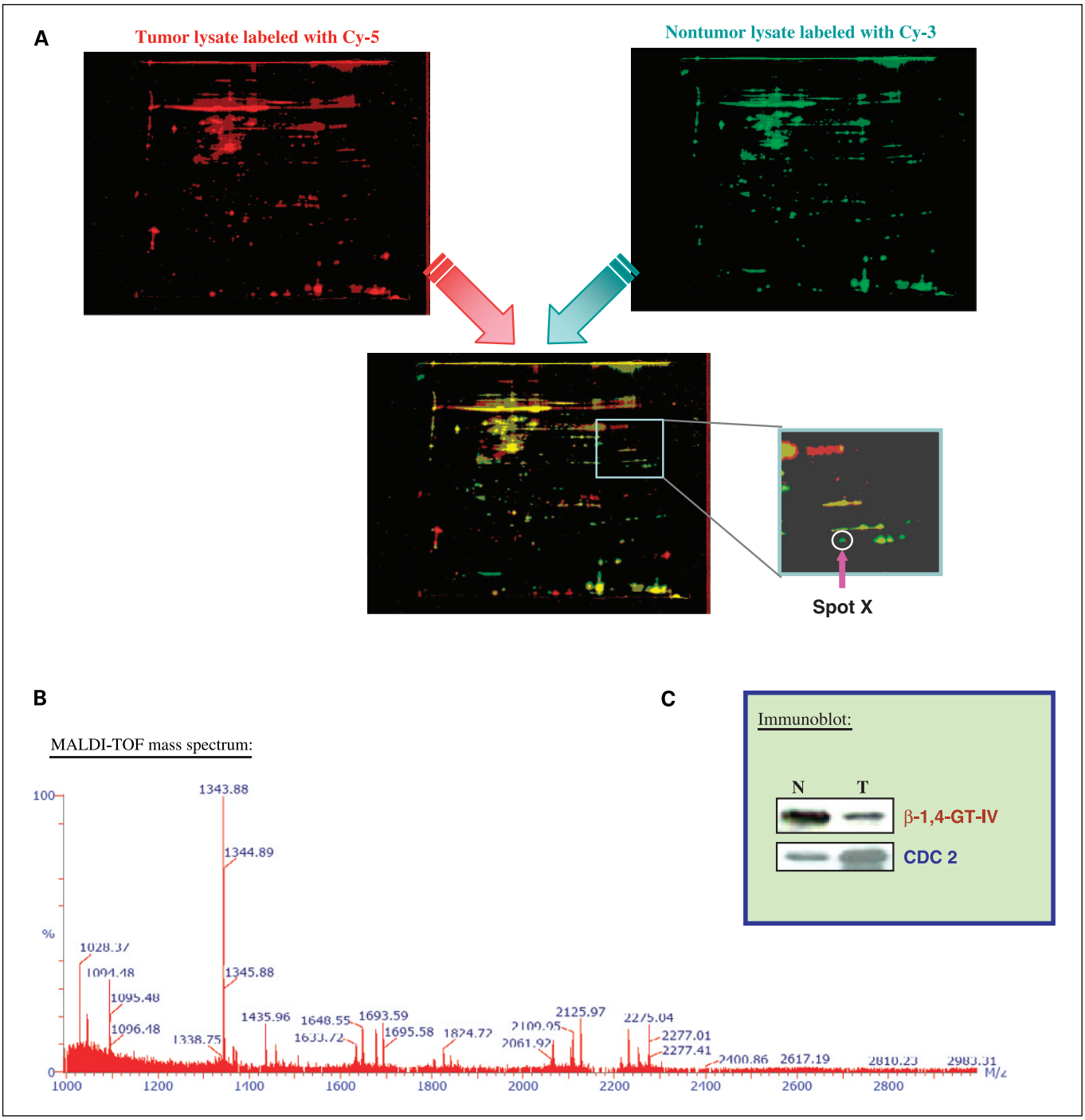

Fig. 1. Identification of the differential expression of β-1,4-GT-IV in the tumor versus nontumor tissue from a colon cancer patient. *A,* differential expression of the protein spot X was observed in the two-dimensional DIGE profiles from tumor and nontumor tissue lysates. Fifty micrograms of protein extracted from tumor tissue were covalently labeled with 400 pmol of Cy5 and proteins extracted from the corresponding adjacent nontumor tissue were labeled with Cy3. After mixing the Cy5- and Cy3-labeled protein samples and analyzing them on the same two-dimensional gel, the DIGE image was obtained by scanning the gel with Typhoon 8600 Imager (Amersham Biosciences). *B,* the MALDI-TOF mass spectrum suggests β-1,4-GT-IV to be the most likely candidate for protein spot X. *C,* immunoblot analysis confirmed the differential expression of β-1,4-GT-IV in the tumor and nontumor tissues from the colon cancer patient. Anti – β-1,4-GT-IV antibody was prepared by immunization of a rabbit with the peptide corresponding to the amino acid sequence $_{104}$ENPKVSRGRYRPQECKA$_{120}$ of β-1,4-GT-IV. The levels of CDC2 in the same-paired tissue lysates were analyzed for comparison.

sample size of 100 and a 20% expression. The hazard rate was 0.1 in one group and 0.26 in the other group. The 5-year survival difference would be detected as 35% (61% versus 27%) when the power was 90% in this study. Cox regression analysis was therefore done to elucidate whether or not tumor β-1,4-GT-IV overexpression could serve as an independent prognostic factor. The potential factors tested for significance in univariate analyses included age of patient (≥68 versus <68 years), tumor size (≥5 versus <5 cm), tumor invasion depth (≥subserosa versus <subserosa), serum CEA level (≥6 versus <6 ng/mL),

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serum CA19-9 level (≥35 versus <35 units/mL), tumor β-1,4-GT-IV overexpression (T > N versus T ≤ N), and lymph node or distant metastasis (yes versus no). Only two factors (i.e., tumor β-1,4-GT-IV overexpression and tumor metastasis) were significantly correlated with patients' poor overall survival ($P = 0.001$ and $P < 0.001$, respectively) and thus entered into Cox regression analysis. The correlation between tumor β-1,4-GT-IV overexpression and poor overall survival remained significant after adjustment for another potential factor tumor metastasis ($P = 0.048$). The odds ratio was 1.894 (95% confidence interval, 1.006-3.559). The strong correlation of tumor metastasis with poor overall survival in the univariate analysis still persisted after multivariate analysis ($P = 0.001$; odds ratio, 3.012; 95% confidence interval, 1.546-5.882).

Discussion

Colorectal cancer is one of the best studied cancer diseases involving multistage carcinogenesis (21). Metastatic formation is the major cause of colorectal cancer mortality. Progression from normal epithelium through adenoma to metastatic carcinoma is characterized by a stepwise accumulation of gene abnormalities. Identification of metastasis-associated genetic abnormalities would assist us to understand the underlying mechanisms, develop new markers for early detection, and ultimately suggest new therapeutic targets. Recently, the advent of microarray technology has greatly enhanced the search for genetic markers and regulators leading to metastasis (22–24). The development of proteomic analyses is also highly expected to disclose molecules involved in the pathogenesis of tumor metastasis (2). In this study, we identified β-1,4-GT-IV differentially expressed in the tumor versus nontumor tissue of a colorectal cancer patient by two-dimensional DIGE and MALDI-TOF mass spectrometry. This differential expression was confirmed by immunoblot analysis. We had prepared the anti–β-1,4-GT-IV antibody by immunization of a rabbit with a peptide sequence derived from β-1,4-GT-IV. Our preliminary data showed that the antibody could major recognize a protein spot that was identified as β-1,4-GT-IV. Although this antibody also detected three faint protein spots with less molecular weight, none of these spots were identified to have any peptide sequence of other β-1,4-GT isoforms. We adopted the immunoblot analysis to detect the β-1,4-GT-IV levels in the paired tumor and nontumor tissues of the colorectal cancer patient. The advantage of using the immunoblot method for tissue protein detection includes higher specificity and more reliable quantitation. Furthermore, immunoblot analyses to detect the β-1,4-GT-IV levels in paired tumor/normal tissue lysates from 100 colorectal cancer patients revealed that 48% of colorectal cancer patients had down-regulated levels of β-1,4-GT-IV in their tumor tissues but 28% of patients exhibited tumor β-1,4-GT-IV overexpression. Tumor β-1,4-GT-IV overexpression did not correlate with age and gender of patient, tumor site, tumor size, grade of tumor cell differentiation, or depth of cancer invasion, but was significantly correlated with the occurrence of tumor metastasis to lymph nodes or distant organs ($P < 0.001$). Our data show for the first time the clinical relevance of β-1,4-GT-IV in colon cancer metastasis.

Surgery is the potentially curative treatment for patients with colorectal cancer. However, the long-term survival results of surgery are still not satisfactory because of frequent tumor

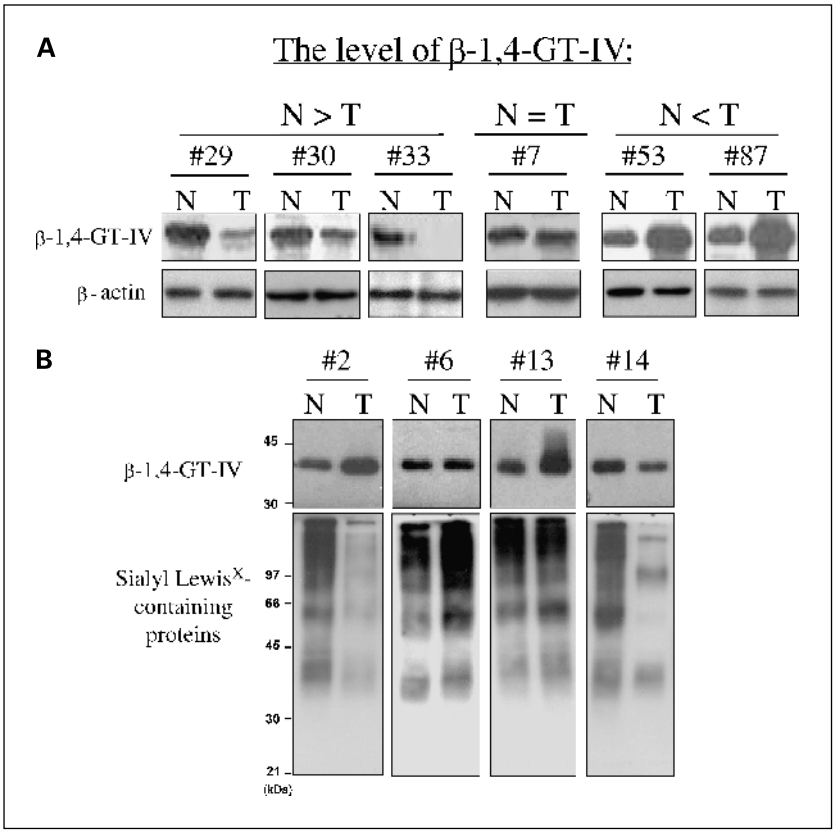

Fig. 2. Examples of differential β-1,4-GT-IV expression levels in human colorectal cancer specimens. *A,* immunoblot analyses were done to analyze the protein levels of β-1,4-GT-IV in the lysates from nontumor (*N*) and tumor (*T*) tissues of colorectal cancer patients #7, #29, #30, #33, #53, and #87. The levels of β-actin in the same-paired tissue lysates were analyzed as internal control. The band intensities of β-1,4-GT-IV and β-actin were quantitated by AlphaImager 2000 densitometer. The status of β-1,4-GT-IV levels between tumor and adjacent nontumor tissues was considered as overexpression or down-regulation only when the ratio of difference was >2 and <0.5, respectively. *B*, no correlation between tumor β-1,4-GT-IV overexpression and elevated levels of sialyl Lewis^X – containing proteins. Immunoblot analyses were done to analyze the levels of sialyl Lewis^X – containing proteins in the lysates from nontumor and tumor tissues of colorectal cancer patients #2, #6, #13, and #14.

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Table 1. Relationships between tumor β-1,4-GT-IV overexpression and clinicopathologic characteristics

	Status of β-1,4-GT-IV expression			*P*
	N > T (*n* = 48)	N = T (*n* = 24)	N < T (*n* = 28)	
Age (mean ± SD), y	66.6 ± 10.3	69.9 ± 10.2	67.6 ± 13.8	0.518
Gender (male/female)	39/9	15/9	23/5	0.153
Tumor size (mean ± SD), cm	5.0 ± 1.9	5.0 ± 2.1	5.1 ± 2.3	0.956
Tumor site (%)				
Cecum	4 (8.3)	2 (8.3)	0 (0.0)	0.575
Ascending colon	5 (10.4)	3 (12.5)	4 (14.3)	
Transverse colon	2 (4.2)	0 (0.0)	0 (0.0)	
Descending colon	4 (8.3)	3 (12.5)	2 (7.1)	
Sigmoid colon	14 (29.2)	2 (8.3)	7 (25.0)	
Rectum or rectosigmoid junction	18 (37.5)	13 (54.2)	13 (46.4)	
Multiple sites	1 (2.1)	1 (4.2)	2 (7.1)	
Tumor cell differentiation (%)				
Well differentiation	1 (2.1)	0 (0.0)	3 (10.7)	0.157
Moderate differentiation	44 (91.7)	24 (100.0)	23 (82.1)	
Poor differentiation	3 (6.3)	4 (16.7)	2 (7.1)	
TNM stage (%)				
I	5 (10.4)	3 (12.5)	0 (0.0)	0.001*
II	24 (50.0)	12 (50.0)	4 (14.3)	
III	16 (33.3)	5 (20.8)	13 (46.4)	
IV	3 (6.3)	4 (16.7)	11 (39.3)	
Tumor invasion depth (%)				
Submucosa	2 (4.2)	0 (0.0)	0 (0.0)	0.636
Muscularis propria	3 (6.3)	3 (12.5)	2 (7.1)	
Subserosa	35 (72.9)	18 (75.0)	19 (67.9)	
Other organs or structures	8 (16.7)	3 (12.5)	7 (25.0)	
Lymph node or distant metastasis	19 (39.6)	9 (37.5)	24 (85.7)	<0.001*

*$P < 0.05$ (two-tailed test).

metastasis. Detection of tissue β-1,4-GT-IV may help the selection of colon cancer patients with high risk of metastasis for more aggressive therapy or investigational therapy. Our Cox multivariate analysis also indicated that β-1,4-GT-IV could serve as an independent predictor for overall survival of colorectal cancer patients after surgical resection. The sensitivity of β-1,4-GT-IV for predicting the 5-year survival was 85% and the specificity was 48%. Due to limited case number in this study, we suggested further larger study to confirm the value of β-1,4-GT-IV. We had successfully prepared the anti–β-1,4-GT-IV antibody and also established a rapid and efficient tissue lysis protocol suitable to a subsequent large-scale immunoblot analyses (3). If the prognostic value of β-1,4-GT-IV in colorectal cancer is confirmed by future studies, it may have important clinical implications and can be tested in a multicenter trial.

β-1,4-GT-IV is a member of the β-1,4-GT family that consists of at least seven isoforms. β-1,4-GT-I was reported to contribute to the metastatic potential of an ovarian cancer cell line (10) but data of its clinical relevance are still lacking. β-1,4-GT-IV shares 41% identity with β-1,4-GT-I (16); however, whether or not β-1,4-GT-IV plays a similar or clinically relevant role in tumor metastasis has not been investigated previously. Our data show the clinical relevance of β-1,4-GT-IV in colon cancer metastasis. Cellular β-1,4-GTs mostly distributes in two subcellular pools, one on the cell membrane and one in the Golgi apparatus (4). On the cell surface, β-1,4-GTs function as an adhesion molecule for cell-to-cell or cell-to-matrix interaction (11, 12). In the Golgi body, the β-1,4-GTs are a family of glycosyltransferases responsible for biosynthesizing *N*-acetyllactosamine by transferring galactose from UDP-galactose to the terminal *N*-acetylgluosamine of acceptor sugars in glycoproteins or glycolipids (4–6). *N*-Acetyllactosamine is a

Table 2. Relationship between tumor β-1,4-GT-IV overexpression and the serum CA19-9 levels

	Serum level of CA19-9 (units/mL)			*P*
	<35* (*n* = 38)	35-200 (*n* = 13)	>200 (*n* = 3)	
Status of β-1,4-GT-IV level (%)				
N > T	20 (52.6)	11 (84.6)	0 (0.0)	0.006†
N = T	9 (23.7)	0 (0.0)	0 (0.0)	
N < T	9 (23.7)	2 (15.4)	3 (100.0)	

*In Taipei Veterans General Hospital, the normal serum level of CA19-9 was measured as <35 units/mL.
†$P < 0.05$ (two-tailed test).

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Table 3. Univariate analysis of the predictive values of tumor β-1,4-GT-IV overexpression and some clinicopathologic variables for the occurrence of metastases

Variable	Odds ratio (95% confidence interval)	*P*
Age (≥68 y)	0.764 (0.345-1.693)	0.508
Tumor size (≥5 cm)	0.725 (0.330-1.594)	0.725
Tumor invasion depth (subserosa or deeper)	5.000 (1.005-24.871)	0.049*
Serum CEA level (≥6 ng/mL)	2.279 (0.978-5.308)	0.056
Serum CA19-9 level (≥35 units/mL)	2.292 (0.690-7.606)	0.175
Tumor β-1,4-GT-IV overexpression (T > N)	9.423 (2.956-30.046)	<0.001*

*$P < 0.05$ (two-tailed test).

precursor carbohydrate of paragloboside and sialyl Lewis^X, which are both significantly elevated and thus taken as tumor-associated carbohydrate antigens in colorectal cancer patients (9, 13, 14). In analyzing the tissue proteins of our patients, tumor β-1,4-GT-IV overexpression did not coincide with increased sialyl Lewis^X level. It was probably because the expression level of sialyl Lewis^X was not determined only by β-1,4-GTs. Abnormal expression of other enzymes such as α-1,3-fucosyltransferase and sialyltransferase might also influence the level of sialyl Lewis^X in tumor tissues (25, 26). Additionally, we observed a significant correlation between the serum CA19-9 levels and the expression of β-1,4-GT-IV. From its carbohydrate structure, which is sialyl Lewis^a, the CA19-9 determinant must be synthesized by a β-1,3-GT but not by any β-1,4-GT. Therefore, the correlation between the serum CA19-9 levels and the expression of β-1,4-GT-IV may be merely coincidental.

Tumor β-1,4-GT-IV overexpression was closely associated with colorectal cancer metastasis; however, we also noted that 48% of colorectal cancer patients exhibited down-regulated expression of β-1,4-GT-IV in their tumor tissues. Our statistical analysis revealed that expression of β-1,4-GT-IV significantly decreased in cancers compared with nonmalignant epithelial cells in the TNM stage I and II diseases, whereas it apparently increased in the stage IV disease. A two-phased alteration model can be proposed to fulfill this observation; i.e., β-1,4-GT-IV was down-regulated at the beginning of colon carcinogenesis and turned out to be overexpressed during the later stages of tumor progression to facilitate metastatic carcinoma formation. After a series of genetic alterations, gain of metastatic phenotype by colon tumor cells is long supposed to be an outcome of environmental selection late in malignant progression (27). In addition, it would be of interest to find out whether or not these patients also expressed abnormal levels of other β-1,4-GT members. Seven β-1,4-GT members have been isolated thus far and the catalytic domain is largely conserved in all seven members. To clearly delineate the role of each individual β-1,4-GT protein becomes complicated because it depends on the fluctuating status in which these proteins may compete or complement each other. β-1,4-GT-I was the first isoform to be isolated and, therefore, much information about β-1,4-GT has been obtained from the β-1,4-GT-I studies. β-1,4-GT-I–deficient (β-1,4-GT-I$^{-/-}$) mice exhibited growth retardation, semilethality, hyperplasia of the small intestine, and impaired differentiation of intestinal villus cells, suggesting that β-1,4-GT-I plays an essential role in the regulation of proliferation and differentiation of epithelial cells (28). Some investigators have suggested that β-1,4-GT-I exhibited a positive effect on cell proliferation because mouse parotid gland hyperplasia showed a marked increase in β-1,4-GT-I activity as well as a significantly higher proliferation potential (29), and consistently, inhibitors of β-1,4-GT-I suppressed hyperplasia of the parotid gland (30). On the other hand, other investigators have suggested a cell growth–inhibiting activity exerted by β-1,4-GT-I because excessive β-1,4-GT-I expression indeed suppressed cell proliferation (31). Down-regulation of β-1,4-GT-I expression by antisense DNA was also reported to affect the metastatic potential, but not the tumor-forming ability, of ovarian cancer cells (10). Therefore, there is no consensus about the

Table 4. Multivariate analysis of the predictive values of tumor β-1,4-GT-IV overexpression and invasion depth for the occurrence of metastases

Variable	Odds ratio (95% confidence interval)	*P*
Tumor invasion depth (subserosa or deeper)	5.882 (0.961-36.004)	0.055
Tumor β-1,4-GT-IV overexpression (T > N)	10.009 (2.992-33.484)	<0.001*

*$P < 0.05$ (two-tailed test).

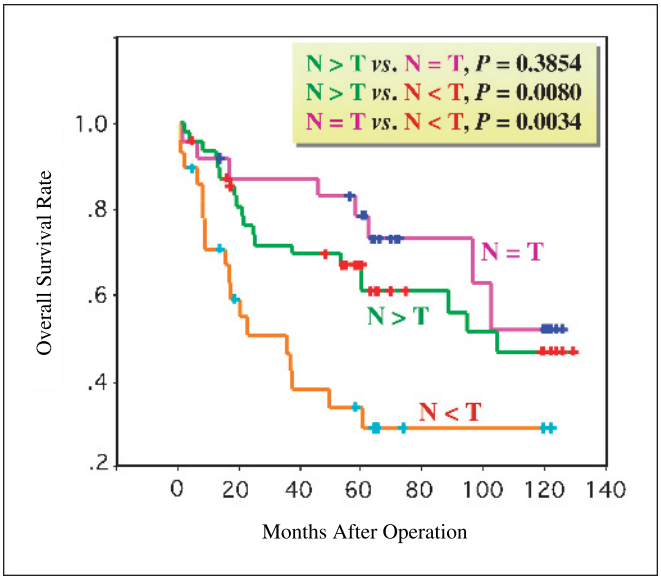

Fig. 3. Kaplan-Meier curve for overall survival. Patient group with down-regulated β-1,4-GT-IV level in tumor tissues: patients entered, 48; died, 20. Patient group without changes in β-1,4-GT-IV level in tumor tissues: patients entered, 24; died, 8. Patient group with elevated β-1,4-GT-IV level in tumor tissues: patients entered, 28; died, 18. Time scale stops at 129.1 months.

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relationship between β-1,4-GT-I and proliferation potential of cancer cells. With regard to the β-1,4-GT-II studies, β-1,4-GT-II has been viewed as a potential tumor marker but it has not been put into clinical use because of its high false-positive rates in normal or benign tissues (32, 33). In this study, the full significance of down-regulation of β-1,4-GT-IV in colorectal carcinogenesis remains to be investigated.

In conclusion, we first identified abnormal β-1,4-GT-IV expression to be associated with colorectal cancer by currently prevalent proteomic technology. The statistical analyses furthermore provided data showing the tight clinical relevance of tumor β-1,4-GT-IV overexpression to colorectal cancer metastasis. Tumor β-1,4-GT-IV overexpression can serve as a hazard factor for colorectal cancer prognosis.

References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
2. Petricoin EF, Zoon KC, Kohn EC, Barrett JC, Liotta LA. Clinical proteomics: translating benchside promise into bedside reality. Nat Rev Drug Discov 2002;1:683–95.
3. Chen W-S, Chang H-Y, Chang J-T, et al. A novel rapid tissue lysis method to evaluate cancer proteins: correlation between elevated Bcl-X_L expression and colorectal cancer cell proliferation. World J Gastroenterol 2005;11:5162–8.
4. Shur BD. Cell surface β1,4 galactosyltransferase: twenty years later. Glycobiology 1991;1:563–75.
5. Amado M, Almeida R, Schwientek T, Clausen H. Identification and characterization of large galactosyltransferase gene families: galactosyltransferases for all functions. Biochim Biophys Acta 1999;1473:35–53.
6. Furukawa K, Sato T. β-1,4-galactosylation of *N*-glycans is a complex process. Biochim Biophys Acta 1999;1473:54–66.
7. Ichikawa T, Nakayama J, Sakura N, et al. Expression of *N*-acetyllactosamine and β1,4-galactosyltransferase (β4GalT-I) during adenoma-carcinoma sequence in the human colorectum. J Histochem Cytochem 1999;47:1593–601.
8. Nakayama J, Ota M, Katsuyama T, et al. Immunohistochemical demonstration of glycoconjugates bearing the type 2 chain-backbone structure in human fetal, normal and neoplastic gastrointestinal tract. Histochemistry 1992;97:303–10.
9. Myoga A, Taki T, Arai K, et al. Detection of patients with cancer by monoclonal antibody directed to lactoneotetraosylceramide (paragloboside). Cancer Res 1988;48:1512–6.
10. Yamashita H, Kubushiro K, Ma J, et al. Alteration in the metastatic potential of ovarian cancer cells by transfection of the antisense gene of β-1,4-galactosyltransferase. Oncol Rep 2003;10:1857–62.
11. Evans SC, Lopez LC, Shur BD. Dominant negative mutation in cell surface β-1,4-galactosyltransferase inhibits cell-cell and cell-matrix interactions. J Cell Biol 1993;120:1045–57.
12. Lopez LC, Youakim A, Evans SC, Shur BD. Evidence for a molecular distinction between Golgi and cell surface forms of β-1,4-galactosyltransferase. J Biol Chem 1991;266:15984–91.
13. Fukuda M. Possible roles of tumor-associated carbohydrate antigen. Cancer Res 1996;56:2237–44.
14. Hakomori S. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. Cancer Res 1996;56:5309–18.
15. Rosen SD, Bertozzi CR. The selectins and their ligands. Curr Opin Cell Biol 1994;6:663–73.
16. Lo N-W, Shaper JH, Pevsner J, Shaper NL. The expanding β4-galactosyltransferase gene family: messages from the data-banks. Glycobiology 1998;8:517–26.
17. Lowry OH, Rosebrough NJ, Lewis Farr A, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.
18. Li C-P, Huang J-H, Chang A-C, et al. A G-quadruplex ligand 3,3′-diethyloxadicarbocyanine iodide induces mitochondrion-mediated apoptosis but not decrease of telomerase activity in nasopharyngeal carcinoma cells. Pharm Res 2004;21:93–100.
19. Welling GW, Weijer WJ, van der Zee R, Welling-Wester S. Prediction of sequential antigenic regions in proteins. FEBS Lett 1985;188:215–8.
20. Wu C-W, Chi C-W, Huang T-S. Elevated level of spindle checkprotein MAD2 correlates with cellular mitotic arrest, but not with aneuploidy and clinicopathological characteristics in gastric cancer. World J Gastroenterol 2004;10:3240–4.
21. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759–67.
22. Bernards R, Weinberg RA. A progression puzzle. Nature 2002;418:823–4.
23. Trojan L, Schaaf A, Steidler A, et al. Identification of metastasis-associated genes in prostate cancer by genetic profiling of human prostate cancer cell lines. Anticancer Res 2005;25:183–91.
24. Chen JJW, Lin Y-C, Yao P-L, et al. Tumor-associated macrophages: The double-edged sword in cancer progression. J Clin Oncol 2005;23:953–64.
25. Lowe JB. Glycan-dependent leukocyte adhesion and recruitment in inflammation. Curr Opin Cell Biol 2003;15:531–8.
26. Wang P-H. Sialylation changes in cancers of female reproductive organs. J Cancer Mol 2005;1:73–84.
27. Fidler IJ, Kripke ML. Metastasis results from preexisting variant cells within a malignant tumour. Science 1977;197:893–5.
28. Asano M, Furukawa K, Kido M, et al. Growth retardation and early death of β-1,4-galactosyltransferase knockout mice with augmented proliferation and abnormal differentiation of epithelial cells. EMBO J 1997;16:1850–7.
29. Humphreys-Beher MG, Schneyer CA, Zelles T. α-Lactalbumin acts as a bimodal regulator of rat parotid acinar cell growth. Biochem Biophys Res Commun 1987;147:174–81.
30. Humphreys-Beher MG, Schneyer CA, Kidd VJ, Marchase RB. Isoproterenol-mediated parotid gland hypertrophy is inhibited by effectors of 4-β-galactosyltransferase. J Biol Chem 1987;262:11706–13.
31. Hinton DA, Evans SC, Shur BD. Altering the expression of cell surface β-1,4-galactosyltransferase modulates cell growth. Exp Cell Res 1995;219:640–9.
32. Kim YS, Perdomo J, Whitehead JS, Curtis KJ. Glycosyltransferase and *N*-acetylglucosaminyltransferase in patients with liver disease. J Clin Invest 1972;51:2033–9.
33. Kessel D, Sykes E, Henderson M. Glycosyltransferase levels in tumor metastatic to liver and in uninvolved liver tissue. J Natl Cancer Inst 1977;59:29–32.

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