·Colerectal Cancer-related Research·

Expression and clinical significance of metastasis-related tumor markers in colorectal cancer

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[Abstract] **Background and Objective:** Besides current clinicopathologic staging system extensively used in clinic, more information of molecular staging is need for more accurate staging of colorectal cancer (CRC). This study was to evaluate the prognostic value of metastasis-related tumor markers in CRC. **Methods:** The expression of CD44v6, matrix matalloproteinase-2 (MMP-2), cyclooxygenase-2 (COX-2), epidermal growth factor (EGF), epidermal growth factor receptor (EGFR) and vascular epidermal growth factor (VEGF) in a tissue microarray containing 95 specimens of CRC were detected by immunohistochemistry (IHC). The correlations of these tumor markers to the prognosis of CRC patients were analyzed. **Results:** In patients with Dukes' A/B disease, the 5-year recurrence rates were significantly higher in CD44v6-, EGF- and EGFR-positive groups than in negative groups (30.9% vs. 8.3%, P=0.045; 38.1% vs. 8.8%, P=0.022; 27.5% vs. 11.8%, P=0.047, respectively). In patients with Dukes' C disease, the 5-year recurrence rates were significantly higher in MMP-2-, COX-2- and VEGF-positive group than in negative groups (73.3% vs. 37.5%, P=0.045; 69.2% vs. 25.0%, P=0.017; 62.5% vs. 25.0%, P=0.03, respectively). In patients with Dukes' A/B disease, there were a significantly higher 5-year recurrence rate and a lower 5-year survival rate in those with more than three positive markers than in those with 1-3 positive markers (P=0.019, P=0.03). However, there was no significant difference in patients with Dukes' C disease in such condition. **Conclusions:** Over-expression of CD44v6, EGF and EGFR are related to poor prognosis of Dukes' A/B CRC, while over-expression of MMP-2, COX-2 and VEGF are related to poor prognosis of Dukes' C CRC. For patients with Dukes' A/B CRC, the more positive markers, the higher 5-year recurrence rate and the poorer 5-year survival.

Key words: colorectal neoplasm, tumor marker, tissue microarray, immunohistochemistry

The therapeutic efficacy of colorectal cancer (CRC) is unsatisfactory, which has not been improved significantly in the past 30 years. Cutler, a member of American Cancer Society, analyzed 25 000 patients with colorectal cancer received operation during 1940-1960, and found that the 5-year survival rate of colon cancer patients increased from 48% to 56% and that of rectal

cancer patients increased from 44% to 50%.

Dukes staging system is extensively used in evaluation of prognosis and selection of treatment approaches. However, the outcomes of some patients with the same Dukes stage diseases and similar characteristics and have received the same treatment are various. Hence, molecular staging (such as motility factors, adhesive molecules, molecules inhibit or promote apoptosis, molecules induce angiogenesis and signal transduction) of colorectal cancer is investigated extensively, and some tumor markers were found to have correlation with prognosis of colorectal cancer.

Materials and Methods

Patients characteristics. Clinical data of 95 patients with colorectal cancer who underwent radical operation from January 1st, 1997 to December 30th, 1998 in Sun Yat-sen University Cancer Center were analyzed. All tumors were histologically and clinically diagnosed. Of the 95 patients, 50 (52.6%) were men and 45 (47.4%) were women, aged 22-79 years (median, 56 years), with 9 aged of <35, 15 aged of 35-45, 23 aged of 45-55, 26 aged of 55-65, and 22 aged of >65. Of these patients, 29 (30.5%) had colon cancer, and 66 (69.5%) had rectal cancer; 68 (71.6%) had tumor diameter of ≤ 5 cm, 27 (28.4%) had tumor diameter of > 5 cm. Of the 95 cases of colorectal cancer, 39 (41.1%) were mass type, 53 (55.7%) were ulcer type, and 3 (3.2%) were infiltrating type; 59 (62.1%) were well and moderately differentiated, 36 (37.9%) were poorly differentiated and undifferentiated; 9 (9.5%) were at stage T1, 45 (47.3%) at stage T2, 34 (35.8%) at stage T3, and 7 (7.4%) at stage T4; 63 (66.3%) were at stage Dukes A/B, 32 (33.7%) at Dukes C.

The patients were followed up for 24-96 months till December 30th, 2004, with a median follow-up of 63 months; 18 patients died during follow-up.

Preparing colorectal cancer tissue array. Tissue array was produced with Personal Tissue Arrayer (Beecher, USA). The 95 paraffin-embedded specimens of colorectal cancer were collected to prepare one array for immunohistochemistry. In the process of silde preparation and immunohistochemistry, some tissue sections shifted or fell off, and 83-86 cases remained for statistical analysis.

Immunohistochemistry assay of colorectal cancer tissue array. Reagents and methods. SP immunohistochemistry was performed according to the instructions to examine the expression of CD44v6, matrix metalloproteinase-2 (MMP-2), cyclooxygenase-2 (COX-2), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) in colorectal cancer tissues. Supersensitive kits and all monoclonal antibodies were purchased from Maixin Bio-Tech Development Company (Fuzhou, China).

Immunohistochemical staining evaluation. As Kawasaki et al.[2] reported, the degree of immunohistochemical staining was scored for intensity of staining and proportion of positive cells. The intensity of staining was scored on a scale of 0 (no staining), 1 (light yellow), 2 (yellowish brown), 3 (brown); the proportion of positive cells was scored on a scale of 0 (0%), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), 4 (76%-100%). The sum of two scores determined the result: 0 score as negative (-), <3 as (positive (+), 3-6 as moderately positive (++), >6 as intensively positive (+++).

MMP-2, EGF, EGFR and VEGF was expressed in cytoplasm; COX-2 was expressed in endoplasmic reticulum and on nuclear membrane; CD44v6 was mainly expressed on cytomembrane, with minor in cytoplasm.

PBS instead of primary antibody was used for negative controls; the known positive colorectal slides were applied as positive controls.

Statistical analysis. All data were analyzed with SPSS10.0 statistical software package. The χ^2 test were used to analyze the relationship between marker expression and clinicopathologic characteristics of colorectal cancer, while survival curves were plotted by Kaplan-Meier method. $P< 0.05$ was considered significant.

Recurrence and metastasis were diagnosed with pathologic evidences, or typical imaging with corresponding clinical manifestations. Recurrence

and survival time were defined as the period from operation day to recurrence, death or the last visit (follow-up). The lost, non-tumor-related death and survival cases at endpoint were analyzed as censored data statistically.

Results

In the process of slides preparation and immunohistochemistry, some tissue specimens shifted or fell off, only 85 remained for CD44v6, 86 for MMP-2, 85 for COX-2, 83 for EGF, 83 for EGFR, and 83 for VEGF detection. Twenty-four patients had recurrence or metastasis,. Of these people, including 11 (19.6%) of the 63 patients at stage Dukes A/B and 13 (46.4%) of the 32 patients at stage Dukes C.

Relationships of tumor marker expression to recurrence and metastasis of colorectal cancer. Among patients with Dukes A/B disease, the recurrence and metastasis rates were significantly higher in CD44v6-, EGF- and EGFR-positive groups than in negative groups (30.9% vs. 8.3%, P=0.045; 38.1% vs. 8.8%, P=0.022; 27.5% vs. 11.8%, P=0.047), the rates between MMP-2-, COX-2- and VEGF-positive groups and negative groups had no significant difference (P=0.189, 0.434, 0.762, respectively). Among patients with Dukes C disease, the recurrence and metastasis rates were significantly higher in MMP-2-, COX-2- and VEGF-positive groups than in negative groups (73.3% vs. 37.5%, P=0.045; 69.2% vs. 25.0%, P=0.017; 62.5% vs. 25.0%, P=0.049), the rates between CD44v6-, EGF- and EGFR-positive groups and negative groups had no significant difference (P=0.934, 0.390, 0.152, respectively) (Table 1).

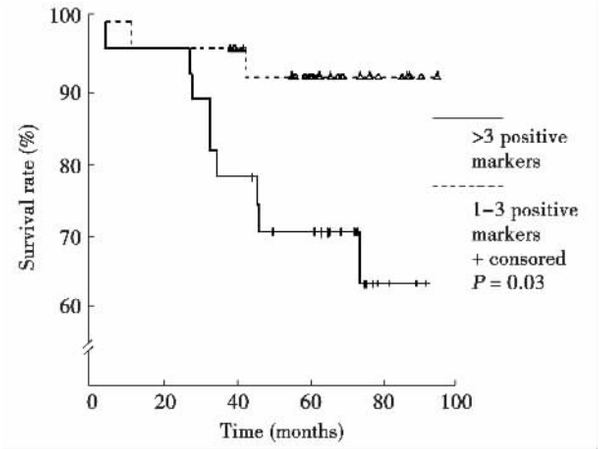
Relationship of multiple tumor marker overexpression to prognosis of colorectal cancer. Among patients with Dukes A/B disease, the 5-year recurrence and metastasis rates were significantly higher in those with more than three positive markers than in those with 1-3 positive markers (28.6% vs. 7.1%, P=0.019), the 5-year survival rates were significantly lower in the former patients than in the latter (P=0.03) (Fig. 1). Among patients with Dukes C disease, the 5-year recurrence and metastasis rates between those with more than three positive markers and those with 1-3 positive markers showed no significant difference (57.1% vs. 35.7%, P=0.256), the 5-year survival rates were significantly lower in the former patients than in the latter (P=0.03) (Fig. 2).

Discussion

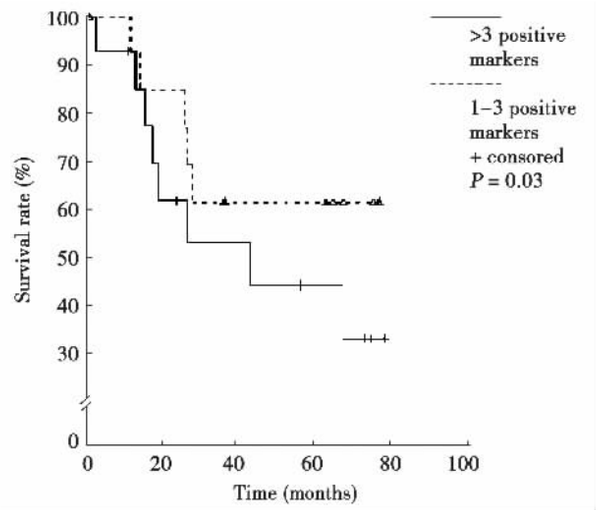
Tumor metastasis is a sequential and continuous process including detachment, dissemination and growth that involved multiple gene interactions and differential expressions of regulatory proteins. With analysis of gene expression profiles of colorectal carcinoma cell

Table 1 Recurrence rate of patients with Dukes' A/B or Dukes' C colorectal cancer

Marker	Recurrence rate of patients at Dukes' A/B stage (%)	P_1 value	Recurrence rate of patients at Dukes' C stage (%)	P_2 value
CD44v6 Positive	30.3(10/33)		47.1(8/17)	
Negative	8.3(2/24)	0.045	45.5(5/11)	0.934
MMP-2 Positive	25.8(8/31)		37.5(6/16)	
Negative	8.3(2/24)	0.189	73.3(11/15)	0.045
COX-2 Positive	22.8(8/35)		69.2(9/13)	
Negative	14.3(3/21)	0.434	25.0(4/16)	0.017
EGF Positive	38.1(8/21)		36.4(4/11)	
Negative	8.8(3/34)	0.022	52.9(9/17)	0.390
EGFR Positive	27.5(11/40)		60.0(12/20)	
Negative	11.8(2/17)	0.047	33.3(2/6)	0.152
VEGF Positive	19.4(7/36)		62.5(10/16)	
Negative	21.1(4/19)	0.762	25.0(3/12)	0.049



Finger 1 Survival curves of stage A/B colonrectal cancer patients with 1–3 or >3 positive markers



Finger 2 Survival curves of stage C colonrectal cancer patients with 1–3 or >3 positive markers

lines and tissues, Sun et al.[3] found that more than 30 kinds of genes (including motility factors, adhesive molecules, molecules inhibit or promote apoptosis, molecules induce angiogenesis and signal transduction) were up-regulated or down-regulated constantly.

Growth factor family is a hot-spot in research of tumor progression and metastasis, which mainly includes EGF, transforming growth factor (TGF) and VEGF. EGF promotes mitosis of epithelial cells and other cells. EGFR, the receptor of EGF, is the protein product of proto-oncogene c-erbB-1 and plays a important role in malignant transformation and proliferation of cells. Si et al.[4] suggested that over-expression of EGF and EGFR were closely related to the tumorigenesis and development of colon cancer. VEGF is one of the most important angiogenesis factors produced by tumor cells. In addition to mitogenic effect on vascular endothelial cells, VEGF could also increase vasopermeability and cause extracellular matrix changes. The binding ot EGF and EGFR leads to a series of biochemical processes of cell differentiation and proliferation. For example, it causes the activation of regulatory oncogenes or the inactivation of tumor suppressor genes, then promote the growth, local invasion and metastasis of tumor cells. Our study showed that the recurrence and metastasis rates of patients with Dukes A/B disease were significantly higher in EGF- and EGFR-positive groups than in negative groups (P=0.022, P=0.047); the rates of patients with Dukes C disease were significantly higher in VEGF-positive group than in negative group (P=0.049). It appeared that overexpression of EGF, EGFR and VEGF may contribute to high recurrence and metastasis rate, and they may be predictors for invasion and metastasis of colorectal cancer. Radinsky et al.[5] had also found that EGFR expression was correlated to high invasiveness and poor differentiation of tumors, and the 5-year survival rate was significantly lower in EGFR-positive patients than in negative patients. Cascinu et al.[6] found that the recurrence rates were 50% in VEGF-positive colorectal cancer patients and 11.7% in negative patients.

CD44v6 and MMP-2 are correlated to adhesion and migration of tumor cells. CD44v6 could connect extracellular matrix components, bind with cytoskeletal proteins and participate in cell pseudopodia formation, thus can lead to alterations of cell morphology and dissemination, and contribute to tumor invasion and metastasis. CD44v6-positive tumor cells could be fixed in distant lymphatic vessels and blood vessels through ligand-receptor interaction, then lodge in and grow steadily to form metastatic tumors. Wang et al.[7] found that CD44v6 was correlated to the tumorigenesis, development and metastasis of colorectal cancer. It is the biochemical principal of tumor peripheral infiltration that MMP-2 could degrade type Ⅳ collagen selectively, which is the major component of

basement membrane, thus, out the confinement of extracellular matrix, highly proliferative tumor cells could disseminated easily. As previously reported, MMP-2 expression was higher in colorectal cancers than in normal colorectal mucosa tissues, and closely correlated to infiltration depth and differentiation degree of tumors.[8] Wu et al.[9] suggested that E-cadherin deletion, MMP-2 and VEGF expression were correlated to invasion and metastasis of middle and inferior segment rectal cancer.

In our study, the recurrence and metastasis rate of patients with Dukes A/B disease was significantly higher in CD44v6-positive group than in negative group (P=0.045), indicating the relationship between overexpression of CD44v6 and high recurrence rate. In patients with Dukes C disease, the rate was significantly higher in MMP-2-positive group than in negative group (P=0.045), suggesting that MMP-2 may be a prognostic marker of colorectal cancer, and patients with MMP-2 overexpression have high risk to recurrence and metastasis. Ring et al.[10] also reported that the higher the expression of MMP-2, the worse the prognosis.

COX-2 is involved in local infiltration of cells. Tumor cells with COX-2 overexpression have enhanced adhesiveness to the matrix and down-regulated E-cadherin expression. Saukkonen et al.[11] found that COX-2 overexpression could promote tumor angiogenesis which facilitated the tumorigenesis, development and metastasis of cancer. Our study showed that the recurrence and metastasis rate of patients with Dukes C disease was significantly higher in COX-2-positive group than in negative group (P=0.017). Tomozawa et al.[12] found that COX-2 overexpression was correlated with recurrence and hematogenous metastasis of colorectal cancer, and disease-free survival time was significantly shorter in those with high expression of COX-2 than in those with low expression of COX-2; further more, COX-2 was the only independent factor correlated with survival time among 8 prognostic factors (including age, histological type, tumor size, and Dukes stage). Therefore, COX-2 overexpression was considered to be correlated with recurrence and metastasis of colorectal cancer. Tsujii et al.[13] also found that metastasis potential increased in colon cancer with COX-2 overexpression.

Researches on multiple tumor marker detection and its correlation to the prognosis of colorectal cancer are mostly seen in serological diagnosis. Chen et al.[14] reported that any combination of multiple markers with high seropositivity rate can not improve the diagnosis rate of colorectal cancer. Researches on combined detection of multiple tumor markers in colorectal cancer tissues are rare. We found that in patients with Dukes A/B disease, the 5-year recurrence and metastasis rate was significantly higher and the 5-year survival rate was significantly lower in those with more than three positive markers than in those with 1-3 positive markers (P=0.019, P=0.030); in patients with Dukes C disease, no significant difference was found in the rates between those with more than three positive markers and those with 1-3 positive markers. Therefore, for patients with Dukes A/B disease, overexpression of more than three markers is related to high recurrence rate, and chemotherapy should be administrated.

In this study, tissue array containing 95 specimens of colorectal cancer were produced, and immunohistochemistry was performed to examine the expression of CD44v6, MMP-2, COX-2, EGFR, EGF and VEGF. Our results showed that these markers may be molecular factors in estimation of development and prognosis of colorectal cancer, and their significances are different in tumors at different stages.

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