

RESEARCH ARTICLE

Multivariate Analysis of Molecular Indicators for Postoperative Liver Metastasis in Colorectal Cancer Cases

Li-Yuan Qian¹, Ping Li², Xiao-Rong Li¹, Dao-Jin Chen¹, Shai-Hong Zhu^{1*}

Abstract

Aims: To explore the relationship between various molecular makers and liver metastasis of colorectal cancer (CRC). **Method:** Using immunohistochemistry, protein expression of CEA, nm23, c-met, MMP2, COX-2, VEGF, EGFR, and CD44 was assessed in 80 CRC cases. The Chi-square test and logistic regression were performed to analyze the relationship between these indicators and CRC liver metastasis. **Results:** There were significant differences in expression of CEA, MMP2, CD44, VEGF and EGFR between the liver metastasis and non metastasis groups ($P < 0.05$); no significant differences were noted for nm23, c-met, and COX-2 expression. Logistic regression analysis showed that only CEA, VEGF, and EGFR entered into the regression equation, and had significant correlations with CRC liver metastasis (α inclusion = 0.10, α elimination = 0.15, $R^2 = 0.718$). **Conclusions:** Combination detection of CEA, VEGF, and EGFR may be an effective means to predict CRC liver metastasis. Nm23, c-met, MMP2, COX-2, and CD44, in contrast, are not suitable as prognostic markers.

Keywords: Colorectal cancer - post-operative liver metastasis - molecular indicators - multivariate analysis

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Introduction

Colorectal cancer (CRC) is a clinically common seen malignant tumor and the second leading cause of cancer death in the western country (Chung-Faye et al., 2000). In China, CRC is the fifth-leading cause of cancer mortality, with age-adjusted mortality rates of 5.29 and 3.86 per 100,000 for males and females, respectively (Zheng et al., 2003). Currently, the primary cause of CRC death relates to the development of distant metastases to organs, particularly to liver. More than 50% of patients will ultimately develop liver metastasis (Porte, 2009), including synchronous liver metastasis (15-25%) and metachronous liver metastasis (20%-25%) (Liu et al., 2003). There was a strong evidence that surgical resection of liver metastasis lesion may remit tumor burden of the patients and prolong disease-free survival to the greatest extent (Bentrem et al., 2005). However, liver metastasis could be recognized only at a very late stage of the disease so that surgical resection may not be an appropriate option. Therefore, it is essential to explore mechanism and early diagnosis of liver metastasis in colorectal cancer.

Recent studies indicate that many molecular factors play important roles in CRC, such as carcinoembryonic antigen (CEA) (Bakalakos, 1999), tumor suppressor nm23 (Yamaguchi et al., 1993), tyrosine kinase receptor c-met (Zeng et al., 2008), matrix metalloproteinases 2 gene (MMP2) (Okada et al., 2001), cyclooxygenase 2 (COX-2) (Yao et al., 2004), vascular endothelial growth

factor (VEGF) (Tokunaga et al., 1998), epidermal growth factor receptor (EGFR) (Kuramochi et al., 2010), and cell-adhesion molecule CD44 (Nanashima et al., 1999). Their expressions were all associated with liver invasiveness and metastasis. Nonetheless, the most desired molecular markers or molecular combination for liver metastasis is still unclear. In this study, we aim to explore the expression of those eight molecular factors in colorectal liver metastasis and further evaluate their clinical diagnose value by multivariate analysis.

Materials and Methods

Patients

All persons have given their informed consent prior to their inclusion in the study, and all human studies have been approved by China Ethics Committee and performed in accordance with the ethical standards. From the May 2001 to March 2002, a total of 80 CRC patients underwent radical surgical treatment in the Third Xiangya Hospital, Central South University. Four years after the surgery, 34 patients (19 male and 15 female) suffered from liver metastasis, but 46 (28 male and 18 female) patients without liver metastasis. The age of liver metastasis patients ranged from 27 to 80 years old, with the median age of 51.0. The age of patients without liver metastasis ranged from 30 to 78 years old, with the median age of 54.2. Our criteria were as follows: (1) Radical resection for primary CRC (2) Intact clinical and pathological records

¹Department of General Surgery, ²Department of Burn and Plastics Surgery, Xiangya Third Hospital, Central South University, Changsha, China *For correspondence: zhushaih@gmail.com

(3) Without preoperative tumor complication and liver metastasis (4) Without preoperative radiochemotherapy history (5) All cancers were graded according to the modified Dukes' stage: stage A, tumor limited to colonic wall; stage B, extension through serosa into pericolic fat; stage C, as for stage B, but with positive findings of local lymphadenopathy; stage D, distant metastases (6) Postoperative standard chemotherapy with FOLFOX (7) Following up once for three months by clinical visits based on pathological parameters, intraoperative exploration, CT, and ultrasonic B-scan imaging.

Immunohistochemistry assay

Immunohistochemistry streptavidinperoxidase (SP) method was used to detect the expression of CEA, nm23, c-met, MMP2, COX-2, VEGF, EGFR, and CD44 in 34 cases of liver metastasis and 46 cases of non metastasis. The CRC tissue was fixed in 10% formalin, dehydrated with gradient ethanol and embedded in paraffin. The paraffin-embedded sections (4 μ m) were mounted onto APES-coated glass slides and dried overnight at 60°C. For antigen retrieval, slides were immersed in sodium citrate buffer (pH 6.0) and boiled for 15 min in a 700W microwave oven. The slides were then washed with PBS three times for 5 min each and treated with 3% H₂O₂ for 10 min to quench endogenous peroxidase activity. The slides were incubated in normal non-specific goat serum for 10 min at room temperature, followed by overnight incubation with primary antibodies (Zhongshan, Beijing) at 37 °C. The dilution was 1:50. The sections were then rinsed in PBS three times each for 3 min, incubated with biotin-conjugated secondary antibody for 30 min, again rinsed in PBS for three times each for 3 min. SP complex (ZYMED SP kit) was added and then 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used for the color reaction. HDAC1 was used as positive control. For negative control, the slides were treated with PBS in place of primary antibody.

Positive cell counting

All the slides were assessed by two pathologists blinded for all patients under a light microscope. In the case of differing opinions, the definitive assessment was obtained by consensus. The percentage of positive cells was scored as follows: 0, no positive cells; 1, \leq 1% labeled tumor cells; 2, 11%-50% labeled tumor cells; 3, 51%-75% labeled tumor cells; 4, >75% labeled tumor cells. The intensity of peroxidase deposits, ranging from light beige to dark brown, was assessed visually as indicating the tumor cell cytoplasm and was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). A composite score was obtained by multiplying the grade by the intensity, that is, - (0, 1, 2); + (3, 4); ++ (6, 8); +++ (9, 12). If the difference was more than 3 in liver metastasis and non-metastatic CRC tissue, we considered it significant.

Statistics analysis

All the statistics analysis was performed by SPSS13.0 software. Immunohistochemical indicators between liver metastasis and non-metastatic CRC tissue were evaluated with chi-square statistics. $P < 0.05$ was considered as

statistically significant. Furthermore, multivariate analysis was performed using unconditioned logistic regression with a backwards elimination strategy.

Results

Immunohistochemistry assay

The expression level of CEA, nm23, c-met, MMP2, COX-2, VEGF, EGFR, and CD44 protein in 34 cases of liver metastasis and 46 cases of non-metastatic CRC tissue could be seen in the following Table 1 and Figure 1 (Table 1, Figure 1). Of them, there was a significant difference in the expression level of CEA, MMP2, CD44, VEGF, and EGFR protein in liver metastasis and non-metastatic CRC tissue ($P < 0.05$). No significant difference could be observed in nm23, c-met, and COX-2 protein ($P > 0.05$).

Multivariate logiatic regression

The assigned values for above eight risk factors of liver metastasis were listed in Table 2 (Table 2). And then

Table 1. The Expression Level of Various Molecular Indicators in Liver Metastasis and Non-metastatic CRC Tissue Liver Metastasis/Non-metastasis

Index	-	+	++	+++	χ^2 value	p-value
CEA	0/10	4/17	12/20	10/7	19.209	0
nm23	1/4	3/5	19/26	11/11	1.625	0.654
C-met	3/11	11/16	19/18	1/1	3.81	0.283
MMP2	18/37	11/17	5/2	0/0	7.098	0.029
COX-2	5/12	18/20	11/14	0/0	1.583	0.453
VEGF	2/16	6/23	5/21	5/2	30.881	0
EGFR	3/35	10/15	1/16	0/0	40.829	0
CD44	4/22	15/19	5/13	2/0	17.072	0.001

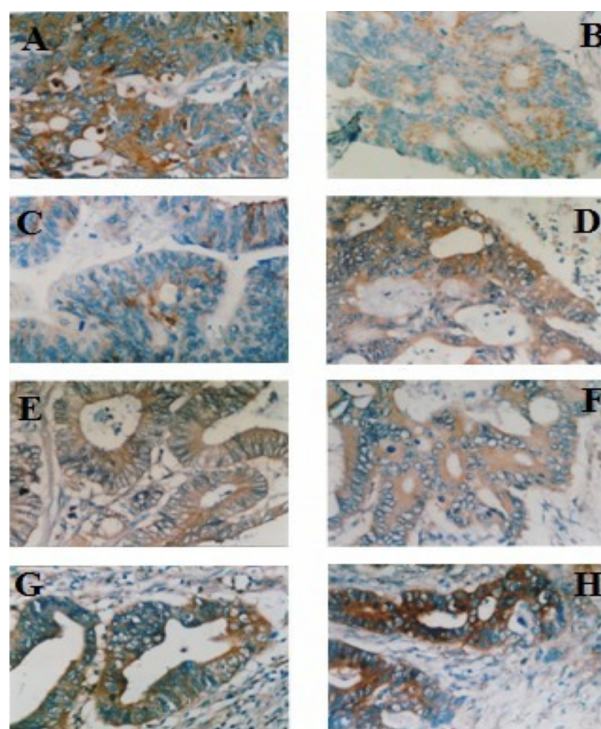


Figure 1. Immunohistochemistry Assay for Positive Molecular Indicators. Brown color in cytoplasm indicated positive result. A: CEA (10 \times 40); B: MMP2 (10 \times 40); C: CD44 (10 \times 40); D: VEGF (10 \times 40); E: EGFR (10 \times 40); F: COX-2 (10 \times 40); G: c-met (10 \times 40); H: nm23 (10 \times 40)

Table 2. Value Assignment for Above Eight Risk Factors for Liver Metastasis

Index	Variable	Value assignment			
CEA	X1	=-0	+=1	=2	=3
nm23	X2	=-0	+=1	=2	=3
C-met	X3	=-0	+=1	=2	=3
MMP2	X4	=-0	+=1	=2	
COX-2	X5	=-0	+=1	=2	
VEGF	X6	=-0	+=1	=2	=3
EGFR	X7	=-0	+=1	=2	
CD44	X8	=-0	+=1	=2	
metastasis	Y	metastasis =1		non-metastasis =0	

Table 3. Unconditioned Logistic Regression Analysis

Index	b	Sb	WaldX ²	P	OR	OR 95% confidence
CEA	0.833	0.472	3.112	0.078	2.299	0.912-5.798
VEGF	1.059	0.472	5.043	0.025	2.884	1.144-7.270
EGFR	2.642	0.663	15.901	0	14.041	3.832-51.450
Constant	-5.154	1.229	17.598	0		

Table 4. The Sample Backward Substitution Test

Original group	Sample size	Proved liver metastasis		Proved non-metastasis	
		Number	Percentage	Number	Percentage
Non-metastasis	46	3	6.50%	43	93.50%
Liver metastasis	34	30	88.20%	4	11.80%

logistic regression was performed using with a backwards elimination strategy with the $\alpha = 0.10$ as the criterion for inclusion and $\alpha = 0.15$ as the criterion for elimination. Our results indicated only three factors were entered into regression equation, including CEA ($P = 0.078$), VEGF ($P = 0.025$), and EGFR ($P = 0.000$), which could explain 71.8% etiology of liver metastasis ($R^2=0.718$) (Table 3). The sample backward substitution test found the accuracy rate was 88.2% and 93.5% for estimating liver metastasis and non metastasis, respectively (Table 4).

Discussion

Colorectal liver metastasis is a highly selective, non-random process in which a subpopulation of cells within a tumor express genes that allow them to progress through distinct steps and spread to distant organs. Alteration of gene expression in these cells leads to transformation, growth, angiogenesis, invasion, dissemination, survival in the circulation, and attachment in the organ of metastases. Once the tumor cell has attached in the liver, it must respond appropriately to the new microenvironment, which includes being able to use growth factors and blood vessels from the liver for the benefit of the tumor mass. This then enables the tumor cells to undergo further invasion, angiogenesis, and subsequent growth (Ellis, 2003; Bird et al., 2006). Therefore, it is important to study the relationship between tumor expressed genes and CRC liver metastasis. In this study, we selected eight relevant genes, such as CEA, nm23, c-met, MMP2, COX-2, VEGF, EGFR, and CD44 to further explore the relationship between their expression and liver metastasis.

CEA is a member of cell surface glycoproteins family that was first described by Gold as specific for digestive carcinomatous and foetal tissues (Thomson et al., 1969). It

has also been demonstrated as one of the most widely used tumor markers worldwide (Wang et al., 2007). In CRC patients, CEA may be involved in regulating the sensibility of tumor cells to cytotoxic lymphocyte and causes the tumor cell to evade the host immune defense. Besides, it could be distributed in any sites of tumor cell, including space between cell and cell or cell and extracellular matrix, and thus, it could disturb cell normal connection formed by other adhesive molecules and reduce the adhesion ability, resulting in cell connection breakage, tumor cell shed and metastasis (Pessaux et al., 2006). In this study, we found CEA was significantly higher expressed in liver metastasis than that in non metastasis colorectal cancer, with 100% positive in the 34 cases of liver metastasis and 78.2% positive in the 46 cases of non metastasis colorectal cancer. This result indicated that CEA positive patients may be prone to liver metastasis.

MMP2 was initially called 72-kd type IV collagenases because it had the ability to degrade this abundant basement membrane component in vitro (Gill and Parks, 2011). Degradation of extracellular matrix by MMP-2 facilitates cell migration and proliferation and thus may play an important role in tumor invasion and metastasis (Zeng et al., 1999). Dong et al found the expression of MMP2 was significantly higher in CRC tissues than in the colorectal tissues. In addition, high levels of MMP2 protein were positively correlated with the status of distant metastasis and tumor invasion (Dong et al., 2011). In this study, we found MMP2 was significantly higher expressed in liver metastasis than that in non metastasis colorectal cancer, with 47% positive in liver metastasis group and 15.2% positive in non metastasis group. This result indicated that MMP2 was associated with liver metastasis, but the positive expression percentage was not very high. Therefore, it was undetermined indicator to predict the liver metastasis in colorectal cancer.

CD44 is a broadly distributed cell surface transmembrane protein thought to mediate cell attachment to extracellular matrix components or specific cell surface ligands (Aruffo et al., 1990). Recent research showed CD44 expression was also associated with CRC invasiveness and liver metastasis (Huang et al., 2011). High expressed CD44 may promote tumor cell attachment to vascular endothelial cells or extracellular matrix and further promote tumor cell to invade into cell matrix. In addition, high expressed CD44 may affect tumor cell migration and motor ability through affecting cytoskeleton protein aggregation and distribution and finally led to metastatic tumor formation. In this study, we identified CD44 was significantly higher expressed in liver metastasis than that in non metastasis colorectal cancer, with 88.2% positive in liver metastasis group and 52.1% positive in non metastasis group.

Angiogenesis is an important step in the outgrowth of a primary tumor and a key source for hematogenous tumor dissemination, progression, and metastasis. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and it has been extensively studied for its role in the invasion and metastasis of cancer cells (Wang et al., 2008). Previous research suggested expression of VEGF was positively observed in the CRC patients, and VEGF

status was significantly associated with tumor stage, lymph nodes and liver metastases (Zafirellis et al., 2008; Cao et al., 2009). Identically, our results demonstrated VEGF was significantly higher expressed in liver metastasis than that in non metastasis colorectal cancer, with 94.1% positive in liver metastasis group and 65.2% positive in non metastasis group.

Epidermal growth factor receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases (RTKs), is highly expressed in many human cancers, including CRC where increased EGFR expression levels in tumors are associated with promoting tumor cell growth, metastasis, angiogenesis (Siena et al., 2009) and inhibiting cell apoptosis (Kaulfuß et al., 2009). EGFR was significantly higher expressed in liver metastasis than that in non metastasis colorectal cancer in our work, with 91.2% positive in liver metastasis group and 23.9% positive in non metastasis group.

COX-2, a rate-limiting enzyme in arachidonic acid metabolism, is induced specifically expressed in plenty of abnormal status, such as inflammation (Seibert et al., 1997) and tumor. It can promote tumor cell growth, invasiveness and metastasis by inhibiting tumor cell apoptosis, stimulating neovascularization, and influencing cell cycle, etc. Study showed COX-2 was also high expressed in CRC tissue indicating an important role in CRC development and progression (Cox et al., 2004). However, in this study, we found there was no significant difference of COX-2 expression between liver metastasis group and non metastasis group. Therefore, further study is needed.

The met proto-oncogene encodes a tyrosine kinase receptor for hepatocyte growth factor and plays an important role in many physiological and pathological processes. Over-expression of the c-met oncogene may give a selective advantage for the acquisition of metastatic potential of colorectal cancer (Di Renzo et al., 1995). However, in our study, no significant difference of c-met protein was present between liver metastasis group and non metastasis group, which was in accordance with previous report 32. Therefore, we suggested c-met was not suitable for predicting liver metastasis.

NM23 is one of the most attracting tumor suppressor genes for investigators because it not only suppresses the tumor metastasis, but also tumor formation. There was evident that nm23 play a negative regulation role in CRC development, progression and metastasis (Yamaguchi et al., 1993). Reduced nm23 expression was related with advanced tumor stages, high angiolymphatic invasion, nodal metastasis and liver metastasis potential (Dursun et al., 2002). However, our study found no significant difference of nm23 expression was present between liver metastasis group and non metastasis group.

In conclusion, univariate analysis indicated CEA, MMP2, CD44, VEGF, and EGFR could be as underlying makers for clinical diagnose of colorectal liver metastasis, but not COX-2, met, and nm23.

Furthermore, multivariate logistic regression analysis was performed to confirm optimal molecular makers for colorectal liver metastasis. The results showed that only three factor were entered into regression equation,

including CEA (P=0.078), VEGF (P=0.025), and EGFR (P=0.000), which seemed to be in line with our univariate analysis. This indicated combined detection of CEA, VEGF, and EGFR may be an effective predictive model for liver metastasis. Although MMP2 and CD44 were highly associated with liver metastasis by univariate analysis, they didn't enter into the regression equation, indicating other factors may weak their relationship for liver metastasis. The sample backward substitution test found the prediction accuracy rate was very high when combined detection of CEA, VEGF, and EGFR, with 88.2% and 93.5% for estimating liver metastasis and non metastasis, respectively.

Taken together, we suggested combined detection of CEA, VEGF, and EGFR have important clinical significance for predicting liver metastasis of CRC. We anticipate our study could lay a basis for further early diagnose of colorectal liver metastasis.

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