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## 與大腸直腸癌細胞在轉移過程中進入血管可能有關的因子

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# Intravasation-Related Metastatic Factors in Colorectal Cancer

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**Short Title**: Intravasation-Related Metastatic Factors

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## Abstract

Alterations in adhesion molecules, angiogenesis, and matrix metalloproteinases have been associated with metastasis and intravasation. The present study investigated the role of these metastatic factors in the context of primary colorectal tumor. Intravasated colorectal epithelial cells were detected by an RT-PCR assay, and expression of E-cadherin, α-catenin, or β-catenin as well as the vascularity of tumor was assessed by immunohistochemical staining. Activity of matrix metalloproteinase was assessed by gelatin zymography. The tumor venous blood was positive for GCC mRNA expression in 40 of 68 patients, but alteration in expression of E-cadherin, α-catenin, or β-catenin was not significantly associated with the presence of colorectal epithelial cells in paired portal venous blood. Further, matrix metalloproteinase activity did not correlate with the presence of intravasated colorectal epithelial cells. Multivariate analysis demonstrated that the only factor associated with intravasated colorectal tumor cells was vascularity of the tumor. Thus, metastasis of colon cancer may result from passive entry into the circulation secondary to angiogenic factors and does not appear to involve other metastatic factors studied in our experiments.

## Introduction

The metastatic spread of cancer cells from a primary tumor to distant sites in the body is responsible for most morbidity and mortality in cancer patients. Metastasis involves a cascade of linked, sequential steps, including invasion of extracellular matrix, neovascularization, invasion of blood vessel wall (intravasation), exit from the circulation (extravasation), and establishment of secondary growth (1, 2). While most aspects of cancer dissemination have been extensively studied, very little biochemical information related to the process of intravasation is available. This may be due to the paucity of experimental models capable of mimicking the cellular and molecular interactions required for a successful completion of intravasation. Further, most “experimental metastases” in xenogenic hosts are produced after intravascular delivery of a large number of cancer cells, a route that bypasses the need of intravasation. Another obstacle in the study of intravasation is the nature of most metastatic assays. These assays tend to involve end-point factors in which nature of the primary tumor and outcome (survival rate and ratio of metastasis) are known while mechanistic properties are based on inference.

Postulated intravasation-related metastatic factors include neovascularization, mutation of adhesion molecules and matrix metalloproteases (3-5). Although many studies report a significant correlation between metastasis formation and these

postulated intravasation-facilitating factors (3-5), these associations are based on inferential, end point assay rather than by direct observation.

The current study attempts to more directly investigate intravasation by assessing for the presence of tumor cells in the blood. PCR-based assays of mutated DNA or tissue-specific RNA are highly sensitive methods for detecting circulating tumor cells. However, DNA-targeted PCR assay may detect DNA derived from degraded tissue instead of viable tumor cells (6). In contrast, RNA identification implies that RNA is intact (extracellular RNA is rapidly degraded if it is not within an intact cell) and functional (only viable cells produce the protein). Therefore, an RNA-targeted PCR assay was selected to detect intravasated viable tumor cells. Detection of cytokeratin ($CK^4$)- 20 mRNA (7), carcinoembryonic antigen ($CEA^4$) mRNA (8), and guanylyl cyclase C ($GCC^4$ ) mRNA (9) have been used for the detection of circulating viable colorectal tumor cells. Of note, GCC transcripts have greater specificity than transcripts of CK -20 or of CEA in detecting circulating colorectal tumor cells (10).

In our previous study, vascularity of primary colorectal tumor was a valuable predictor of the presence of circulating colorectal epithelial cells in portal venous blood (11). Thus, we investigated whether vascularity and the presence of other postulated intravasation-related factors (MMP2, MMP9, E-cadherin, α-catenin, and β-catenin) was associated with the presence of circulating colorectal epithelial cells

in the portal venous blood from patients with colorectal carcinomas.

## Patients and Methods:

## Patients

Informed consent was obtained from all patients, and the ethics committee of the National Taiwan University approved the study protocol.

From January 1997 to June 1998, 68 patients (35 males and 33 females; ages 28–89 years; mean age of 65 years) with histologically confirmed colorectal adenocarcinoma (32 patients with colon carcinoma, and 36 patients with rectal carcinoma) were treated at our institution. Tumor stage and grading were classified as per the Astler-Coller system. Potential curative resections were performed in 53 patients; and palliative resections of the colorectal tumor due to multiple liver metastases were elected in 15 patients.

**Blood and Tumor Sample Collection**

Immediately after entering the peritoneal cavity and prior to manipulation of the tumor, 10 cc of blood was collected from the drainage vein of the tumor-bearing colorectal segment and used as portal venous blood sample. A piece consisting of at least 2 $cm^3$ of freshly harvested tumor tissue from each resected specimen was snap frozen in liquid nitrogen at the time of operation and stored at –70°C.

In addition, peripheral blood samples from 11 healthy volunteers were obtained as control.

**RNA Extraction and Nested Duplex RT-PCR**

RNA extraction (from peripheral mononuclear blood cells and frozen tissue) and GCC RT-PCR was performed as previously described (11). In brief, cDNA was synthesized in a 20-μl reaction mixture containing 2 μg of total RNA. For the first round of the nested polymerase chain reaction (PCR), 20-μl reactions were prepared with 4 μl of the cDNA preparation and primer (antisense, nucleotides 1197-1218; and sense, nucleotides 685-708). The second PCR was performed with 10 μl of this reaction mixture and antisense (nucleotides 1000-1021) and sense (nucleotides 759-781) primers. RT-PCR products were analyzed by agarose gel (2.5%) electrophoresis and visualized by UV transillumination after staining with ethidium bromide (0.5 μg/ml). The nested GCC PCR yielded a 262-bp product. The amplified products were sequenced using the ABI Model 373A DNA Sequencer (Perkin Elmer Biosystems, U-S-A) as specified by the manufacturer. The DNA sequences were aligned and analyzed using an Acer computer.

**Sensitivity of GCC RT-PCR**

The sensitivity of the GCC RT-PCR assay was determined in cell spiking experiments as previously described, allowing the detection of 10 CCL 220 cells in 10 ml of blood (11).

**Gelatinase Zymography**

Up to four 10-μm sections (between 10 and 20 mg of tissue) from each cryopreserved tumor were homogenized in protein extraction buffer (500 μl). Ten minutes later, the sample was centrifuged at 4°C at maximum r.p.m. for 10 min, and the supernatant was collected and stored in -20°C until protein assay was performed. Using a Bio-Rad protein assay reagent, the protein content of each sample was measured against bovine serum albumin standards.

Gelatin zymography was performed according to the method described by Parsons *et al.* (12). Briefly, each sample (20 μg of extracted protein) was run in parallel with a molecular weight marker, and 20 μg of extracted protein from patient 1 was included as an internal standard on SDS-polyacrylamide gels (7.5%) containing 0.1% gelatin as the substrate. This method can detect the inactive proforms of collagenases, because SDS causes activation of the enzymes without proteolytic cleavage of the inhibitory N-terminal sequence (13).Western blotting using monoclonal antibodies for latent MMP-9, active MMP9, latent MMP-2, and active MMP-2 was performed to verify that the bands seen on zymography were as described.

**Control Gels for MMPs**

Control gels contained the MMP inhibitor EDTA in the MMP incubation buffer to confirm that the lysis bands were due to MMPs.

**Quantification of the gels**

Quantification was performed using laser densitometry and Quantity One software (Discovery Series, Pharmacia Biotech, UK). The relative gelatinolytic activity was determined for each proteinase by multiplying the area of each band by its optical density. The following four lysis bands were observed on the gelatin zymography in all the patients' samples: 92 kDa, corresponding to latent MMP-9 (Gelatinase B); 82kD, active MMP-9; 72 kDa, latent MMP-2 (Gelatinase A); and 62 kDa, active MMP-2. The total gelatinolytic activity (expressed in arbitrary units / 20 μ g of protein) was obtained by summing the activities of the 92-kDa latent MMP-9, 82-kD active MMP-9, 72-kDa latent MMP-2 and 62-kDa active MMP-2. To correct the variation in background staining of the gel (intergel variation), the total gelatinolytic activity of 20 μg of protein from patient 1 on each gel was defined as 20 arbitrary units of gelatinolytic activity and served as internal standard. Separate (latent or active MMP2 or MMP9) and total gelatinolytic activity of each specimen on the same gel was then expressed in arbitrary units / 20μg of protein.

**Immunohistochemistry**

The indirect avidin-biotin immunoperoxidase method was used for immunostaining. All tissue samples were fixed in 10% buffered formalin and routinely processed for light microscopy. Sections (4-mm thick) were dewaxed in

xylene and dehydrated in ethanol. The sections were pre-digested with protease for 20 min at 37$^{o}$C and then immersed in 3% hydrogen peroxide ($H_2O_2$) for 30 min to inhibit endogenous peroxidase. After washing with PBS, sections were incubated in normal rabbit serum for 30 min, followed by incubation overnight with either anti-E-cadherin monoclonal mouse antibody (R & D System Europe, Abingdon, UK) diluted 1:200 in Tris-buffered saline pH 7.6 (TBS); anti- β -catenin monoclonal IgG (Transduction Laboratories, Lexington, KY, USA ) diluted 1: 100 in TBS; anti- α -catenin monoclonal IgG (Zymed Laboratories Inc., South San Francisco, CA, USA) diluted 1:20 in TBS; or anti-CD31 monoclonal antibody (Union Biotech Inc.) at a 1:50 dilution. The sections were then thoroughly washed with TBS followed by addition of a biotinylated rabbit anti-mouse immunoglobulin G for 15 minutes (Amersham Life Science, UK). After incubation with ABC reagent (Dako, UK), the slides were developed by immersion into 0.01% $H_2O_2$ and 0.05% diaminobenzidine tetrahydrochloride (DAB) for 2 minutes. Normal mouse IgG was substituted for the primary antibody in the negative control. The sections were counterstained with hematoxylin.

After staining, blood vessels appeared intensely brown in color, which facilitated identification and quantification. Expression of E-cadherin and catenins in cancer cells was compared with that of normal epithelial cells in the same samples, which

always expressed these molecules. Two observers without knowledge of the clinical and histological parameters evaluated these slides independently. Slides were scored as ‘normal’ when more than 80% of the tumor epithelial cells showed linear intercellular staining, and ‘reduced’ when less than 80% of the tumor cells expressed intercellular staining (Fig.1).

**Vascular Counting**

Vascular counting was performed as previously described (11). In brief, slides were examined at low power magnification (X40 and X100) to identify the areas of highest vessel density. For each slide, the three most vascular areas within the tumor mass were chosen. A 200X field in each of these three areas was counted. The average counts of the three fields were recorded. Two pathologists without knowledge of the corresponding clinicopathologic data counted all of the immunostained slides.

**Statistical Analysis**

Because intravasation was either present or absent, logistic regression was used to analyze our data. Associations between the presence or the absence of GCC mRNA expression in portal venous blood and potential prognostic factors, such as tumor size, tumor grade, tumor stage, or those postulated intravasation-related metastatic factors were determined. This method provided odds ratios or estimates of the relative risk of the presence of intravasated colorectal epithelial cells. Univariate relations were

investigated to determine which factors were related to intravasation, and stepwise multivariate logistic regression was used to determine whether some combination of variables provided a better estimate of the relative risk of intravasation than any single variable.

Probability values $< 0.05$ were considered significant; all reported p values are two-sided.

## Results

### Expression of GCC mRNA in portal venous blood

Peripheral blood samples from 11 all healthy volunteer were negative for GCC mRNA. In contrast, GCC transcripts were detected in portal venous blood from 40 of 68 patients (59%) with colon caner. As shown in Table 1, tumor stage was the only predictor of the presence of intravasated colorectal epithelial cells.

### Gelatinolytic activity in primary colorectal tumor tissue

Patients were classified into one of two groups according to low or high individual type gelatinolytic activity of their colorectal cancer tissue. For example, in analyzing active MMP2, patients were classified according to low or high active MMP2 gelatinolytic activity. The cutoff level corresponded to the median value of the entire population of each type MMP for this classification scheme. However, none of any individual type or total gelatinolytic activity was significantly related to the presence of intravasated colorectal epithelial cells (Table 2).

### E-cadherin, α-catenin, and β-catenin Expression in colorectal tumor

Noncancerous epithelium of the large bowel expressed E-cadherin, α-catenin, and β-catenin at cell-cell boundaries. As summarized in Table 3, the expression of E-cadherin was maintained in 39 (57%) of the 68 tumors; the expression of α-catenin was maintained in 36 (53%) of the 68 tumors; and the expression of β

-catenin was maintained in 39 (57%) of the 68 tumors. However, decreased expression of any of these proteins was not significantly associated with the presence of intravasated colorectal epithelial cells (table 3).

**Microvessel density in colorectal tumor**

Considerable intratumor heterogeneity was observed in the distribution of stained microvessels. Mean microvessel density (MVD) for anti-CD 31 antibody was 75.79 ± 24. In analysis of angiogenesis, patients were classified into one of two groups according to low or high microvessel density in their colorectal cancer tissue. The cutoff level corresponded to the median value of the entire population of microvessel density for this classification scheme.

**Correlation between postulated intravasation-related factors and the presence of intravasated colorectal epithelial cells**

The relation of each postulated intravasation factor to the presence of intravasated colorectal epithelial cells is summarized in Table 4. Only high MVD in the primary tumor was positively related to the presence of intravasated colorectal epithelial cells. High MVD in the primary tumor was associated with a nearly four fold increase in detection of intravasated colorectal tumor cells (hazard ratio 3.52; 95% confidence interval [CI] 1.3-9.750, Table 4). Multivariate analysis of postulated intravasation-related metastatic factors demonstrated that MVD was the only factor

associated with detection of intravasated colorectal tumor cells (Table 4).

## Discussion

Previous studies using animal models have demonstrated that radiolabelled tumor cells injected intravenously were entrapped in the capillary beds of the first target organ and only a few were detectable in the peripheral blood (14). Theoretically, intravasated colorectal tumor cells are detected more frequently in portal venous blood than in peripheral venous blood. Thus, detection of intravasated tumor cells in the drainage veins of tumor-bearing colorectal segments may be a better reflection of the presence of intravasated colorectal tumor cells.

The use of RT-PCR system for detecting intravasated tumor cells may result in the detection of tissue-specific mRNA instead of tumor cell specific mRNA. However, normal colon epithelia or liver cells rapidly underwent apoptosis after having been released into the circulation (15). We further minimized contamination by direct puncture of drainage vein of tumor bearing segment. The metastatic cascade is postulated to start with a breakdown of the epithelial integrity, thereby enabling tumor cells to leave epithelial structures and invade the surrounding stroma. The loss of E-cadherin expression has been associated with tumor cell de-differentiation and correlates with an increased likelihood of distant metastases (16, 17). Presumably, down-regulation of E-cadherin and/or associated catenins reduces the ability of cells to adhere to each other and facilitates detachment from the primary tumor and

advancement into the surrounding tissue and vessels. However, these presumptions were also obtained by end point assay and have not been definitely proven.

Among various adhesion molecules, the cadherin family of transmembrane glycoproteins (responsible for calcium-dependent intercellular adhesion by homophilic interaction) is of particular importance (18). The family includes many subtypes, including E-cadherin (E-cad), P-cadherin, and N-cadherin, which are distinct in immunologic specificity and distribution. Among these molecules, E-cadherin is especially noteworthy as it maintains the epithelial structure (19). The cytoplasmic domain of E-cadherin interacts with intracellular proteins called α-, β-, and γ-catenins, which make contact with the microfilament network (18). The interaction of these molecules is the prerequisite for the proper formation of functionally intact adherens junctions. Thus, in tumors with normal expression of E-cadherin, perturbations of the cadherin cell adhesion system may be due to abnormal expression or function of the associated catenins (20). However, the present study showed no association of down regulation of E-cadherin, α or β catenin with increased risk of colorectal epithelial cell intravasation.

After detachment from the primary tumor mass, tumor cells must create passageways for migration via enzymatic degradation of the ECM components. The first barrier for an invading epithelial tumor is the basement membrane, which

consists of type IV collagen and gelatin. Type IV collagenase is a metalloproteinase that cleaves type IV collagen of epithelial and vascular basement membranes and that is involved in tumor cell invasion (21). The present data failed to show an association between high individual or high total gelatinolytic activity and presence of intravasated colorectal epithelial cells. In fact, microvessel density was the only predictor of the presence of intravasated colorectal epithelial cells.

Metastasis results from selective competition that favors survival of a subpopulation of metastatic tumor cells that preexist within the heterogeneous primary tumor (22). Kerbel determined that the metastatic subpopulation dominates the primary tumor mass early in its growth (23), while many studies establish metastasis as the final stage in tumor progression from a normal cell to a fully malignant cell (24).

Some animal studies have demonstrated that the primary tumor may actually suppress the growth of metastatic lesions (25). This is consistent with the evolutionary viewpoint that the primary tumor's main goal is to assure its own growth rather than establish secondary growth in other organ. Promotion of primary tumor growth is greatly dependent on angiogenesis, which results from the net balance between positive and negative regulators of neovascularization (26). Endothelial cells stimulated by mitogens such as basic fibroblast growth factor or vascular endothelial

cell growth factor , secrete metalloproteinase-2 (gelatinase A) which contributes to degradation of basement membrane in microvessel walls (27). This breakdown in the vascular basement membrane may facilitate extravasation of endothelial cells during formation of neovascular sprouts, as well as intravasation of tumor cells into the lumen (28). If tumor vessel formation is rapid and haphazard and endothelial proliferation is insufficient or endothelial junctions are unstable, cancer cells may be exposed to the lumen and passively enter the circulation during the angiogenic process (29). This presumption is compatible with our data that angiogenesis was the only predictor of the presence of intravasated colorectal epithelial cells. Supporting evidence is provided by studies of mosaic blood vessels in tumors that demonstrated a subpopulation of tumor cells that coexist with endothelial cells and are progressively shed into the circulation (42).

In conclusion, vascularity of the tumor was a significant predictor of the presence of intravasated colorectal epithelial cells. In contrast, expression or activity of E-cadherin, α-catenin, β-catenin, MMP-2, and MMP9 had no significant association with the presence of intravasated colorectal epithelial cells. These data may indicate that tumor cells enter the circulation passively during the process of angiogenesis.

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**Table 1 Association of clinicopathologic variables with presence of intravasated colorectal epithelial cells**

| Features | No. of Cases | Positive intravasated Colorectal epithelial cells (%) | P Value |
|---|---|---|---|
| Age | | | 0.202 |
| <65 years | 33 | 22 (67%) | |
| ≧65 years | 35 | 18 (51%) | |
| Sex | | | 0.202 |
| Male | 33 | 22 (67%) | |
| Female | 35 | 18 (51%) | |
| Depth of wall invasion | | | 0.061 |
| Mucosa/submucosa | 2 | 0 (0%) | |
| Muscle layer/subserosa | 25 | 12 (48%) | |
| Serosa exposed or invasion to adjacent organ | 41 | 28 (68%) | |
| LN metastasis | | | 0.305 |
| Present | 39 | 25 (64%) | |
| Absent | 29 | 15 (52%) | |
| Lymphatic invasion | | | 0.772 |
| Present | 33 | 20 (61%) | |
| Absent | 35 | 20 (57%) | |
| Vascular invasion | | | 0.036 |
| Present | 37 | 26 (70%) | |
| Absent | 31 | 14 (45%) | |
| Duke’s stage | | | 0.008 |
| A | 2 | 0 (0%) | |
| B | 23 | 11 (48%) | |
| C | 28 | 15 (54%) | |
| D | 15 | 14 (93%) | |

**Table 2 Correlation of gelatinolytic activity with the presence of colorectal epithelial cells in paired portal venous blood.**

| | Intravasated colorectal epithelial cells, Present | Intravasated colorectal epithelial cells, Absent | P-value |
|---|---|---|---|
| Active MMP2 | | | 0.622 |
| High | 19 | 15 | |
| Low | 21 | 13 | |
| Latent MMP2 | | | 0.622 |
| High | 19 | 15 | |
| Low | 21 | 13 | |
| Active MMP9 | | | 0.139 |
| High | 23 | 11 | |
| Low | 17 | 17 | |
| Latent MMP9 | | | 0.622 |
| High | 21 | 13 | |
| Low | 19 | 15 | |
| Total MMP | | | 0.096 |
| High | 21 | 9 | |
| Low | 19 | 19 | |
| Sum of MMP2 | | | 0.622 |
| High | 21 | 13 | |
| Low | 19 | 15 | |
| Sum of MMP9 | | | 0.324 |
| High | 22 | 12 | |
| Low | 18 | 16 | |

**Table 3 Correlation of E-cadherin, α-catenin , or β-catenin with the presence of colorectal epithelial cells in paired portal venous blood**

| | Intravasated colorectal epithelial cells, Present | Intravasated colorectal epithelial cells, Absent | P-value |
|---|---|---|---|
| E-cadherin expression | | | 0.128 |
| Normal | 26 | 13 | |
| Reduced | 14 | 15 | |
| α-catenin expression | | | 0.283 |
| Normal | 19 | 17 | |
| Reduced | 21 | 11 | |
| β-catenin expression | | | 0.128 |
| Normal | 26 | 13 | |
| Reduced | 14 | 15 | |

**Table 4 Analyses using the Multivariate Cox Proportional Hazard Regression Model**

| Factor | Univariate Analysis | | Adjusted for MVD | |
|---|---|---|---|---|
| | Hazard ratio (95% CI) | P value | Hazard ratio(95% CI) | P value |
| Active MMP2 | 0.784 (0.298~2.064) | 0.622 | 0.968 (0.347~2.705) | 0.951 |
| Latent MMP2 | 0.828 (0.300~2.282) | 0.715 | 0.828 (0.300~2.282) | 0.715 |
| Active MMP9 | 2.091 (0.782~5.592) | 0.142 | 2.258 (0.798~6.390) | 0.125 |
| Latent MMP9 | 1.275 (0.484~3.357) | 0.622 | 1.306 (0.473~3.605) | 0.606 |
| Sum of MMP2 | 1.275 (0.484~3.357) | 0.622 | 1.545 (0.549~4.347) | 0.410 |
| Sum of MMP9 | 1.630 (0.615~4.315) | 0.326 | 1.358 (0.487~3.785) | 0.558 |
| Sum of MMP | 2.091 (0.782~5.592) | 0.142 | 2.076 (0.741~5.816) | 0.165 |
| E-cadherin | 2.143 (0.921~7.932) | 0.130 | 2.703 (0.921~7.932) | 0.07 |
| α -catenin | 0.585 (0.220~1.560) | 0.284 | 0.649 (0.233~1.809) | 0.409 |
| β -catenin | 2.143 (0.799~5.748) | 0.130 | 1.533 (0.530~4.434) | 0.430 |
| MVD or mean | 3.519 (1.270~9.750) | 0.016 | | |

Legends for figure 1:

Figure 1. Immunostaining for α -catenin and E-Cadherin. (A) Reduced expression of α -catenin [left], compared with normal colorectal epithelial cells; (B) Strong and diffuse α -catenin expression in another case; (C) Loss of E-cadherin expression in cancer cells; (D) Expression of E-cadherin in cancer cells (200X).

Dear Dr. Phil D. Rye:

It is our great pleasure to submit the paper entitled, "**Intravasation-Related Metastatic Factors in Colorectal Cancer**" for consideration for publication in Tumor Biology. Neither the submitted paper nor any similar paper, either in whole or in part, other than an abstract or preliminary communication, has been or will be submitted to or published in any other primary scientific journal. All of the listed authors are aware of and agree to the content and submission of the paper. There are no financial or other interests with regard to the submitted manuscript that might be construed as a conflict of interest.

While most aspects of cancer dissemination have been extensively studied, very little direct biochemical information related to the process of intravasation is available. In the present study, we utilized the endpoint of the presence of intravasated colorectal tumor cells detected by GCC RT-PCR rather than metastasis formation for more direct studies of the phenomenon. Of the studied variables, only vascularity of the tumor was a significant predictor of the presence of intravasated tumor cells. Thus, tumor intravasation may occur in a passive process related to angiogenesis rather than an active process related to adhesion molecules or other mechanisms. We hope you'll like it.

Sincerely yours,

Yu-Wen Tien

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