SPANDIDOS PUBLICATIONS

Clinical significance of fibronectin expression in colorectal cancer

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Abstract. We measured the serum and urinary levels of fibronectin (FN) in 113 patients with colorectal cancer (CRC) - 15 with synchronous hepatic metastases, 21 with metachronous hepatic metastases and 77 with no hepatic metastases - as well as in 40 controls, with the aim of determining if FN can be used as a marker of CRC invasion or metastasis and its clinical significance. Urinary FN levels were significantly higher in patients with CRC than in the controls, and both urinary and serum FN levels rose with cancer progression. Patients positive for FN tended to have a more advanced disease. High levels of FN expression in both urine and serum showed a sensitivity of 80%, specificity of 33.3%, accuracy of 66.6% and positive predictive value of 75% for the diagnosis of metachronous hepatic metastases. These results indicate that FN levels increase with the progression of CRC, that FN expression in urine and serum is a useful marker of the degree of disease advancement, and that FN may play a part in cancer growth and development.

Introduction

Of all malignancies, CRC is one of those most likely to metastasise to the liver, which commonly occurs in approximately 20% of patients. We consider cell adhesion and cell proliferation to be the key mechanisms of cancer invasion and metastasis, and have thus sought to elucidate the responsible factors, such as those that comprise the extracellular matrix (ECM) or that are involved in intercellular and intravascular adhesion and implantation of circulating metastastic cells, and the growth factors that control their activities. Fibronectin (FN) is present mainly in the blood, but is also found in the ECM (1). A variety of the physiological functions of FN, other than cell adhesion, have been identified, including cell migration, cell differentiation, tissue repair, cell proliferation and blood coagulation. FN has also been implicated in cancer development and growth, although there are few studies on the serum and urinary FN levels of patients who have undergone surgery for CRC. The merits of various surgical treatments have been investigated and the assessment of the malignant potential of metastases, the major determinant of outcome, is no longer a matter of debate. Therefore, in this study we investigated the clinical significance of the serum and urinary levels of FN in relation to invasion and metastasis in CRC, and as predictive markers of tumour staging.

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Patients and methods

Patients. The subjects of this study were 113 patients who had undergone surgical resection of CRC at the Department of Surgery II, Tokyo Women's Medical University School of Medicine, between June 1997 and November 2001 (68 males, 45 females; age range 43-92, mean 64.5±10.8 years). The primary lesion was located in the caecum in 3 patients, the ascending colon in 15, the transverse colon in 13, the descending colon in 6, the sigmoid colon in 25 and the rectum in 51. Histological grading was well-differentiated adenocarcinoma in 54 patients, moderately-differentiated adenocarcinoma in 53 patients and poorly-differentiated adenocarcinoma in 6 patients. Dukes' staging was A in 23 patients, B in 19, C in 33, and D in 7. Synchronous hepatic metastases were present in 15 patients, 21 had metachronous hepatic metastases (defined as onset within 3 years after surgery) and 77 had no hepatic metastases. The follow-up continued until December 2004.

The control group comprised 40 patients (23 males, 17 females; age range 51-88, mean 65.3±11.7 years) with benign conditions, 21 with inguinal hernia, 12 with cholelithiasis, 3 with internal haemorrhoids and 4 with other conditions.

Serum and urine samples. Serum was separated from preoperative peripheral blood samples by centrifugation and stored at -80˚C, to be thawed for later testing. Random urine samples were also collected preoperatively, frozen, and then promptly weighed. Serum samples were tested at a 1:250 dilution, whereas urine samples were measured in their original state, though creatinine (Cre) correction was required.

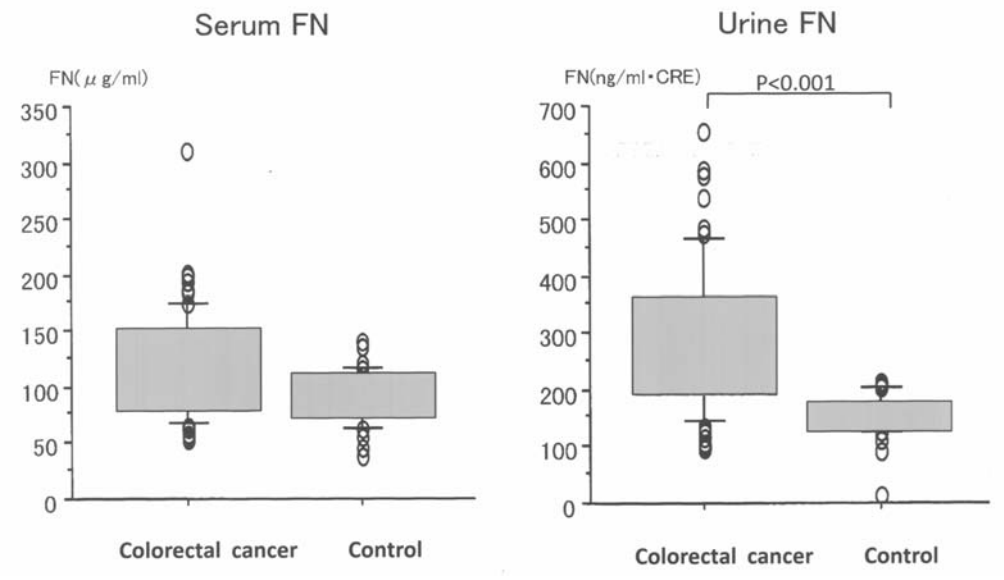

Figure 1. Serum and urinary fibronectin levels in colorectal cancer patients. Significantly higher urinary levels were present in the CRC patients than in the controls (p<0.001).

Testing methods. Both the serum and urinary FN levels were measured using a two-step sandwich enzyme immunoassay (Fibronectin EIA Kit; Takara Bio Inc., Shiga, Japan). Briefly, 50 μl of buffer solution was placed in each well of the antibody plate, then 200 μl of test sample was added and the plate was sealed and then agitated using a shaker for 2 h at room temperature. Following suction removal of the reaction fluid, the plates were washed three times using cleaning fluid, then 200 μl of precipitant solution was added to each well and the plate was sealed and agitated using a shaker for 20 min at room temperature. Finally, 50 μl of stop solution was added to each well and the absorbance wavelength of each well was measured using a microplate reader. The concentration of each antibody was obtained from the standard curve.

All terms used in this report conformed to the Japanese Classification of Colorectal Carcinoma (JSCCR) (2). The χ^2 test (Fisher's exact method) was used for analysis of correlations between groups, and $p<0.05$ was considered a significant difference.

Study parameters

Serum and urinary biochemistry. We investigated possible correlations between serum and urinary FN levels and histological stage and Dukes' classification, reflecting the following clinicopathological factors: depth of tumour invasion, lymph node metastasis, lymphatic invasion, venous invasion, hepatic metastasis and peritoneal metastasis.

Relationship between serum and urinary FN expression. We examined the relationship between serum and urinary FN expression and the diagnostic utility of the combination.

Results

Serum FN levels. The mean serum FN level was 115.1± 47.30 μg/ml in the patients with CRC and 88.55±22.16 μg/ml in the controls (Fig. 1). There was no significant correlation with histological grade or depth of invasion. The mean serum FN level of 109.5±49.42 μg/ml in patients with histological stages I-III was significantly lower than the FN level of 145.8±17.57 μg/ml in patients with histological stage IV disease (p=0.041). Similarly, serum FN expression was significantly lower at 110.4±48.40 μg/ml in patients with Dukes' A-C disease than in patients with Dukes' D disease (149.4±17.26 μg/ml, p=0.042) (Fig. 2). The mean serum FN level of 89.37±27.51 μg/ml in patients with no hepatic metastases was significantly lower than the mean serum FN level of 159.3±37.37 μg/ml in patients with hepatic metastases (p<0.001) (Fig. 3).

Urinary FN levels. The mean urinary FN (UFN) level in patients with CRC was 293.03±141.08 ng/mg·Cre, significantly higher than the UFN level of 151.98±37.21 ng/mg·Cre in the controls (p<0.001) (Fig. 1). There was no significant correlation with histological grade or depth of invasion. UFN expression was significantly lower at 271.2±133.50 ng/mg·Cre in patients with histological stages I-III than in patients with histological stage IV disease (376.2±93.31 ng/mg·Cre, p=0.033). Similarly, UFN expression was significantly lower at 272.3±132.66 ng/ mg·Cre in patients with Dukes' A-C disease than in patients with Dukes' D disease (378.6±95.89 ng/ mg·Cre, p=0.037) (Fig. 2). A significantly lower UFN level of 252.12±131.61 ng/ mg·Cre was observed from the preoperative stage onwards in patients with no hepatic metastases compared with the UFN level of 372.73±94.77 ng/mg·Cre in patients with hepatic metastases (p<0.001) (Fig. 3).

Relationship between serum and urinary FN levels in patients with hepatic metastases. We set the cut-off level for serum FN at 160 μg/ml (mean ± SD) and for urinary FN at 430 ng/mg·Cre (mean ± SD), and defined high expression for both variables as levels greater than the cut-off. Upon examination of the group of 36 patients with hepatic metastases, high expression of both

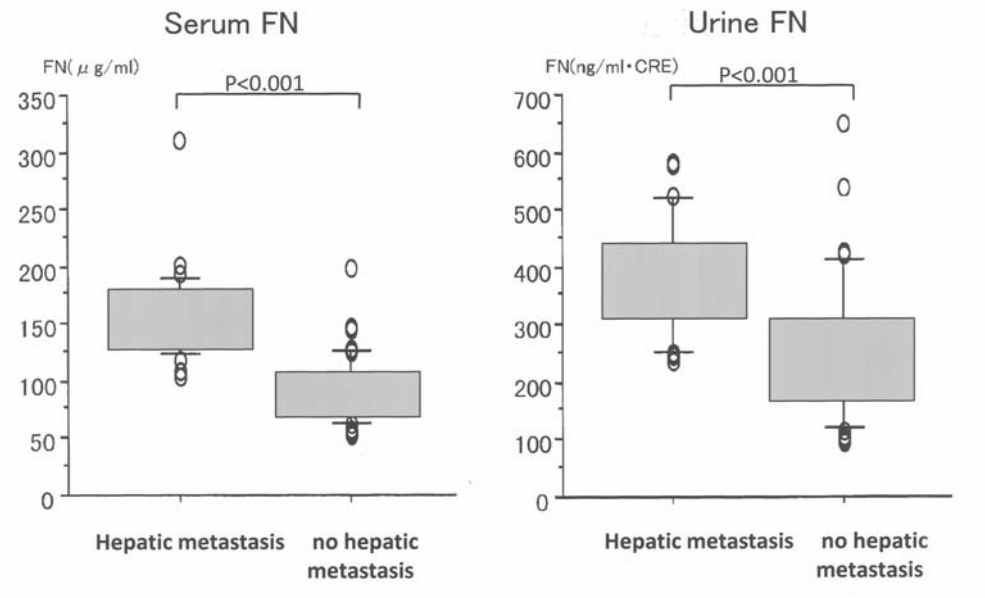

Figure 2. Serum and urinary fibronectin levels in the hepatic metastasis group. Significantly higher fibronectin levels were present in the hepatic metastasis group compared to the group with no hepatic metastases (p<0.001).

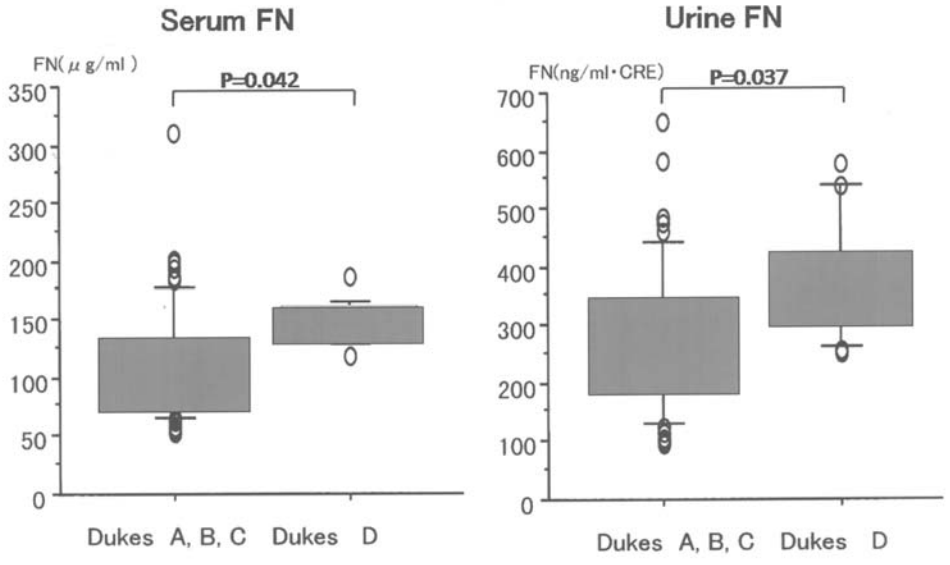

Figure 3. Serum and urine fibronectin levels according to Dukes' classification. Significantly higher fibronectin levels were present in patients with Dukes' D disease than in patients with A-C classification.

serum and urinary FN was detected in 17 patients, giving a sensitivity of 73.9%, specificity of 30.7%, accuracy of 58.3%, and positive predictive value of 65.3% (Table I).

Discussion

FN was first discovered in 1948 by Morrison *et al* as a circulating cold-insoluble globulin (1). It was separately identified in 1973 as a glycoprotein that disappeared from the surface of cultured animal cells as they became malignant and was given various names, such as galactoprotein a and LETS protein. In 1975, these proteins were identified as the same entity and, in 1978, the name was standardized as fibronectin. It is broadly classified, according to location, as plasma FN, which is secreted by the liver into the circulation, cellular FN, which covers the cell surface, and amniotic FN, with minor structural differences between types. There is an A chain with a molecular weight of 230 kDa and a B chain with a molecular weight of 220 kDa, and FN exists and functions as a dimer with an AB unit. FN bioactivity is mediated through binding to integrins, which are receptor proteins on the cell surface. It controls all functions of the cell it contacts, but is mainly involved in guiding cell migration, regulating cell differentiation, tissue repair and cell proliferation.

The current theory of carcinogenesis is that an autocrine mechanism operates whereby tumour cells themselves produce

Table I. Combined test of preoperative serum and urinary fibronectin levels in relation to hepatic metastases.

Hepatic metastases (n=36)		Urine (ng/mg·Cre)	
		FN >430	FN ≤430
Serum (μg/ml)	FN >160	17 (47%)	9 (25%)
	FN ≤160	6 (17%)	4 (11%)

High serum and urine FN levels are both significant ($p<0.05$).

Table II. Combined test of preoperative serum and urinary fibronectin levels in relation to metachronous hepatic metastases.

Metachronous hepatic metastases (n=21)		Urine (ng/mg·Cre)	
		FN >430	FN ≤430
Serum (μg/ml)	FN >160	12 (57%)	4 (19%)
	FN ≤160	3 (14%)	2 (9%)

High serum and urine FN levels are both significant ($p<0.05$).

growth factors that act via receptors to cause autonomous proliferation. For example, the stimulation of cell proliferation by epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor ß (TGF-ß) and hepatocyte growth factor (HGF) may play an important role. On the other hand, ECM constituents, such as laminin, type IV collagen and FN, are located on the basement membrane and are thought to dissociate with the destruction of the basement membrane that occurs with cancer invasion.

We conducted a number of studies based on the premise that cell adhesion and growth factors play a vital role in the mechanism of cancer invasion and metastasis. Of the ECM constituents, we have in particular studied the relationship between serum laminin and CRC. We measured serum laminin levels in 205 patients undergoing surgery for CRC, finding an increased level in the group with hepatic metastases in comparison with their preoperative levels. In addition, multivariate analysis of the correlation between laminin and outcome identified the serum level of laminin as an independent prognostic factor, together with hepatic, pulmonary and peritoneal metastases (3). Much consideration has also been given to the importance of matrix metalloproteinases (MMP), the enzymes that break down the basement membrane. We measured the preoperative serum levels of MMP-2 and -9, investigating their relationship to CRC, and our results suggested that high preoperative levels of circulating MMP-2 are associated with tumours that have a high invasive and metastatic potential (i.e. to the liver) (4).

Investigating cellular growth factors, we found that high preoperative levels of circulating EGF are associated with highly malignant tumours that are likely to invade and metastasise, with a significant difference between positive and negative immune staining specimens (5). Investigating the relationship between CRC and HGF and mesenchymal epithelial transition factor (c-Met), we found higher preoperative levels of expression of circulating HGF/c-Met in patients with hepatic metastases. The results of the immune staining were, however, unpredicted, demonstrating increased expression of HGF and decreased expression of c-Met as disease staging progressed (6). Our present understanding of this phenomenon suggests that the production of HGF by both paracrine and endocrine mechanisms leads to the supersaturation and downregulation of c-Met (6). In another study, we found that TGF-ß1 expression within CRC tissue was significantly higher in patients with lymphatic metastases and high-grade venous infiltration, and that serum TGF-ß1 levels were also significantly higher in patients with CRC than in the controls. Serum TGF-ß1 levels were also significantly higher in the positive than in the negative staining group, with a positive correlation between the two (7).

We also conducted studies with other malignancies, and have commenced studies of EGF, HGF and TGF-ß1 in patients with gastric cancer. There, our results with EGF have differed from those observed with CRC, with lower preoperative serum EGF levels found in more advanced disease (8). This is thought to occur because EGF uptake by tumour cells increases as the cancer is more poorly-differentiated and more locally invasive, although further studies are obviously required. At present, we devised a molecular mimicry hypothesis in which cancer cells break down the ECM and cover themselves with cellular adhesion factors as they circulate within the vasculature, thus masquerading as normal cells and evading the immune system (3).

Few previous studies of ECM constituents have concentrated on FN in addition to laminin. Haglund *et al* measured the circulating levels of cellular FN (cFN), comparing it with other tumour markers, including carbo-hydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) (9). They found that the highest frequencies (50-67%) of elevated cFN values were observed in patients with hepato-pancreato-biliary malignancies, and the greatest advantage over CA 19-9 and CEA was seen in patients with local CRC and in hepatocellular carcinomas. They reported that a combination of cFN and CA 19-9 showed the highest overall sensitivity, of 47%, compared with 31% for cFN and 33% for CA 19-9. The corresponding specificities were 76% for cFN ± CA 19-9, 85% for cFN and 83% for CA 19-9. The accuracy of a combination of cFN and CA 19-9 or CEA (60%, respectively) was higher than that of cFN (55%), CA 19-9 (55%) or CEA (45%) alone.

Studies on urinary tract tumours comprise the majority of reports concerning UFN (10-17). In a rare study on other types of cancer, Katayama *et al* found that UFN levels were significantly elevated in patients with various types of cancer, and were extremely elevated in certain patients with distant metastasis. They suggested that UFN fragments which increase in cancer patients are generated by ECM destruction (18).

While all of the abovementioned studies measured serum or urinary FN levels in patients with colorectal and other types of cancer, few studies measured both levels at the same time.

present study, we measured preoperative serum and urinary FN levels in patients undergoing surgery for CRC, and investigated whether either or both can be used as indicators of the degree of disease progression. In addition, we followed the postoperative course of the patients to investigate a possible correlation between hepatic metastases and the serum and urinary FN levels.

We found that serum FN levels were significantly higher in the CRC patients than in the controls. Significant differences were also observed in the clinicopathological findings, between histological stages I-III and IV disease, and between Dukes' A-C and D disease. From the preoperative stage onwards, serum FN levels were significantly higher in patients with hepatic metastases than in patients with no hepatic metastases. UFN levels were also significantly higher in CRC patients than in the controls and, similar to the serum FN results, significant differences were also seen between histological stages I-III and IV disease, and between Dukes' A-C and D disease. From the preoperative stage onwards, UFN levels were significantly higher in patients with hepatic metastases than in patients with no hepatic metastases. Upon examination of the group of patients with metachronous hepatic metastases, high expression of both serum and urinary FN gave a sensitivity of 80%, specificity of 33.3%, accuracy of 66.6% and positive predictive value of 75% (Table II).

These results indicate that the FN levels increase with the progression of CRC, that FN expression in urine and serum is a useful marker of the degree of disease advancement, and that FN may play a part in cancer growth and development.

We are also investigating the use of FN in antimetastasis therapies, in addition to our evaluation of malignant potential, and are developing a possible anti-adhesive therapy that targets the metastatic mechanism mediated by the cell-binding domain of FN (19). In the future, we hope to identify biomarkers that can be used to evaluate the degree of progression of a number of malignancies, in addition to CRC, that can be measured in blood samples, and also in the more readily-collected urine samples.

References

1. Morrison PR, Edsall JT and Miller SG: The separation of purified fibrinogen from Fraction I of human plasma. J Am Chem Soc 70: 3103-3108, 1948.
2. Japanese Society for Cancer of the Colon and Rectum: Japanese Classification of Colorectal Carcinoma. 1st English edition, Kanehara & Co. Ltd, Tokyo, 1977.
3. Saito N and Kameoka S: Serum laminin is an independent prognostic factor in colorectal cancer. Int J Colorectal Dis 20: 238-244, 2005.
4. Nakazawa S, Saito N, Kameoka S and Kobayashi M: Clinical significance of matrix metalloproteinase (MMP)-2, -9 and E-cadherin expression for assessing the spreading of human colorectal cancer. J Tokyo Womens Med Univ 74: 26-32, 2004.
5. Hirosawa T, Saito N, Kameoka S and Kobayashi M: Clinical significance of epidermal growth factor (EGF) expression for assessing the spreading of human colon cancer. J Jpn Soc Coloproctol 55: 402-412, 2002.
6. Hashimoto T, Saito N, Kameoka S, Shibata N and Kobayashi M: Clinical significance of hepatocyte growth factor and its specific receptor c-Met expression in colorectal cancer progression. Acta Histochem Cytochem 37: 139-146, 2004.
7. Daiko W, Saito N and Kameoka S: Clinical significance of TGF-ß1 expression in evaluation of the malignancy of colorectal cancer. J Jpn Soc Coloproctol 58: 377-382, 2005.
8. Yamada A, Saito N, Kameoka S and Kobayashi M: Clinical significance of epidermal growth factor (EGF) expression in gastric cancer. Hepatogastroenterology 54: 1049-1052, 2007.
9. Haglund C, Ylatupa S, Mertaniemi P and Partanen P: Cellular fibronectin concentration in the plasma of patients with malignant and benign diseases: a comparison with CA 19-9 and CEA. Br J Cancer 76: 777-783, 1997.
10. Hegele A, Heidenreich A, Varga Z, *et al*: Cellular fibronectin in patients with transitional cell carcinoma of the bladder. Urol Res 30: 363-366, 2003.
11. Menendez V, Fernandez-Suarez A, Galan JA, Perez M and Garcia-Lopez F: Diagnosis of bladder cancer by analysis of urinary fibronectin. Urology 65: 284-289, 2005.
12. Webb KS and Lin GH: Urinary fibronectin: potential as a biomarker in prostatic cancer. Invest Urol 17: 401-404, 1980.
13. Malmstrom PU, Larsson A and Johansson S: Urinary fibronectin in diagnosis and follow-up of patients with urinary bladder cancer. Br J Urol 72: 307-310, 1993.
14. Sanchez-Carbayo M, Urrutia M, Gonzalez de Buitrago JM and Navajo JA: Evaluation of two new urinary tumor markers: bladder tumor fibronectin and cytokeratin 18 for the diagnosis of bladder cancer. Clin Cancer Res 6: 3585-3594, 2000.
15. Kirkali G, Tuzel E, Guler C, Gezer S and Kirkali Z: Significance of tissue laminin P(1) elastase and fibronectin levels in transitional cell carcinoma of the bladder. Eur Urol 39: 292-299, 2001.
16. Wunderlich H, Reichelt O, Zermann DH, Schubert J, Berndt A and Kosmehl H: Fetal fibronectin: a new screening-marker for bladder cancer? Oncol Rep 8: 669-672, 2001.
17. Eissa S, Swellam M, Sadek M, Mourad MS, El Ahmady O and Khalifa A: Comparative evaluation of the nuclear matrix protein, fibronectin, urinary bladder cancer antigen and voided urine cytology in the detection of bladder tumors. J Urol 168: 465-469, 2002.
18. Katayama M, Kamihagi K, Nakagawa K, *et al*: Increased fragmentation of urinary fibronectin in cancer patients detected by immunoenzymometric assay using domain-specific monoclonal antibodies. Clin Chim Acta 217: 115-128, 1993.
19. Saito N, Mitsuhashi M, Hayashi T, *et al*: Inhibition of hepatic metastasis in mice treated with cell-binding domain of human fibronectin and angiogenesis inhibitor TNP-470. Int J Clin Oncol 6: 215-220, 2001.