Modulation of uPA, MMPs and their inhibitors by a novel nutrient mixture in human colorectal, pancreatic and hepatic carcinoma cell lines

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Abstract. Colorectal, pancreatic and hepatic carcinomas are characterized by high levels of matrix metalloproteinase (MMP)-2 and -9 secretions, allowing cancer cells to spread to distal organs. These and other proteases, such as uPA, play a key role in tumor cell invasion and metastasis by digesting the basement membrane and ECM components. Strong clinical and experimental evidence demonstrates association of elevated levels of uPA and MMPs with cancer progression, metastasis and shortened patient survival. MMP activities are regulated by specific tissue inhibitors of metalloproteinases (TIMPs). Our main objective was to study the effect of a nutrient mixture (NM) on activity of uPA, MMPs and TIMPs in colon HCT-116, pancreatic MIA PaCa-2 and hepatic carcinoma SK-Hep-1 cell lines. These cell lines (ATCC) were cultured in their respective media and treated at confluence with NM at 0, 50, 100, 250, 500 and 1000 μg/ml. Analysis of uPA activity was carried out by fibrin zymography, MMPs by gelatinase zymography and TIMPs by reverse zymography. All three cancer cell lines expressed uPA, which was inhibited by NM in a dose-dependent manner. On gelatinase zymography, SK-Hep-1 showed bands corresponding to MMP-2 and MMP-9 with enhancement of MMP-9 with PMA (100 ng/ml) treatment. HCT-116 and MIA PaCa-2 showed strong bands corresponding to MMP-9 but no MMP-2 band. NM inhibited their expression in a dose-dependent manner. Activity of TIMPs was upregulated by NM in all cancer cell lines in a dose-dependent manner. Analysis revealed a positive correlation between uPA and MMP-9 and a negative correlation between uPA/MMP-9 and TIMP-2. These findings suggest the therapeutic potential of NM in treatment of colon, pancreatic and hepatic carcinomas.

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Introduction

According to the American Cancer Society, ~136,830 people were expected to be diagnosed with colorectal cancer in the US in 2014, and ~50,310 people were predicted to die of the disease. In both men and women, colorectal cancer is the third most commonly diagnosed cancer and the third leading cause of cancer death (1). While colorectal cancer is very treatable upon early detection, 5-year survival is <10% once the cancer metastasizes to the lymph nodes, liver or other areas (2). The American Cancer Society estimates that, in 2015, 48,960 people (24,840 men and 24,120 women) will be diagnosed with pancreatic cancer and 40,560 people (20,710 men and 19,850 women) will die of pancreatic cancer. Rates of pancreatic cancer have been fairly stable over the past several years, accounting for 3% of all cancers in the US and 7% of cancer deaths (3). Hepatocellular carcinoma (HCC) is the most common (80% of cases) form of liver cancer in the US and is the third leading cause of cancer-related death worldwide (4). For 2014, 33,190 new cases and 23,00 deaths were estimated from liver and intrahepatic duct cancer (5). The most prevalent causes of death in patients with HCC include uncontrolled metastasis and recurrence; only 16.6% of liver cancer patients survive 5 years (5).

Tumor metastasis results from cancer cell detachment from the primary tumor, invasion through degraded basement membrane into the surrounding stroma, and entry into and transport through the vascular or lymphatic system to distal sites such as the liver, lungs, and brain, and extravasation, tumor cell proliferation and angiogenesis at distal sites (6-10). Tumor cell invasion depends upon integrity of the extracellular matrix (ECM), which, when intact, acts as a barrier to block cancer cell invasion. The ECM is composed of collagen, proteoglycans, fibronectin, laminin and other glycoproteins (11-13). Matrix metalloproteinases (MMPs) and urokinase plasminogen activators (uPA) are involved in tumor invasion and metastasis. Numerous clinical and experimental studies have demonstrated that elevated levels of uPA and MMPs are associated with tumor growth, cancer progression, metastasis and shortened survival in patients (14,15).

MMPs, especially MMP-2 and MMP-9 play key roles in tumor cell invasion and metastasis due to their ability to degrade type IV collagen, a major component of the ECM (13,16,17). Secreted as inactive pro-enzymes, MMP-2

and -9 are activated by other MMPs or proteases. Proteolytic activities of MMP-2 and MMP-9 are inhibited by specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs). The balance between MMP and TIMP levels is a critical determinant of net proteolytic degradation. Clinical studies note the association of MMP expression with progression and poor prognosis of colon (18), pancreatic (19), and hepatic carcinomas (20).

The serine protease uPA converts plasminogen to plasmin, which is capable of promoting tumor growth and angiogenesis, degrading the ECM and basement membrane and activating pro-MMPs (21). Components of the uPA system such as uPA, plasminogen activator inhibitor-1 (PAI-1), and urokinase-type plasminogen activator receptor (u-PAR) are overexpressed in a variety of cancer types, including colon cancer (22), pancreatic cancer (23), and HCC (24) and correlate with cancer progression, metastasis and poor prognosis. Thus, the uPA system represents a potential target for anticancer strategies.

Rath and Pauling (25) proposed using nutrients such as lysine and ascorbic acid to target plasmin-mediated connective tissue degradation as a universal approach to tumor growth and expansion. Lysine blocks plasminogen activation into plasmin by tissue plasminogen activator (t-PA) by binding to plasminogen active sites, modulating the plasmin-induced MMP activation cascade (21). Subsequent studies confirmed this approach and lead to formulating a novel mixture composed of lysine, ascorbic acid, proline and green tea extract and other micronutrients (NM), which has shown significant anticancer activity against a large number (~40) of cancer cell lines, blocking cancer growth, tissue invasion and MMP expression both *in vitro* and *in vivo* (26). In this study, we focused on the modulating effect of NM on the activities of uPA, MMP-2 and -9, and TIMPs in human colon, pancreatic and hepatic carcinoma cell lines.

Materials and methods

Materials. Human colon cancer HCT-116, pancreatic cancer MIAPaCa-2, and hepatocellular carcinoma SK-Hep-1, along with their culture media were obtained from ATCC. Antibiotics, penicillin, and fetal bovine serum (FBS), were obtained from Gibco (BRL, Long Island, NY, USA). Twenty-four-well tissue culture plates were obtained from Costar (Cambridge, MA, USA). Gelatinase zymography was performed in 10% Novex pre-cast SDS polyacrylamide gel (Invitrogen) with 0.1% gelatin in non-reducing conditions. The nutrient mixture (NM), prepared by VitaTech (Hayward, CA, USA) was composed of the following ingredients in the relative amounts indicated: vitamin C (as ascorbic acid and as Mg, Ca and palmitate ascorbate) 700 mg; L-lysine, 1000 mg; L-proline, 750 mg; L-arginine, 500 mg; N-acetyl cysteine, 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 μg; copper, 2 mg; manganese, 1 mg. All other reagents used were of high quality and were obtained from Sigma, unless otherwise indicated.

Cell cultures. The cell lines were grown in their respective media: colon cancer and hepatocellular carcinoma in MEM, and pancreatic cancer in DME, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in 24-well tissue culture plates. The cells were plated at a density of $1x10^5$ cells/ml and grown to confluency in a humidified atmosphere at 5% CO_2 at 37˚C. Serum-supplemented media were removed and the cell monolayer was washed once with PBS with the recommended serum-free media. The cells were treated with the nutrient mixture, dissolved in media and tested at 0, 50, 100, 250, 500 and 1000 μg/ml in triplicate at each dose for uPA and TIMP-2 studies. For MMP analysis, cells were treated with NM at 0, 10, 50, 100, 500 and 1000 μg/ml. Parallel sets of cultures were treated with PMA (100 ng/ml) for induction of MMP-9. Control and PMA treatments were done in triplicates. The plates were then returned to the incubator. The conditioned media were collected separately, pooled and centrifuged at 4˚C for 10 min at 3000 rpm to remove cells and cell debris. The supernatant was collected and used to assess uPA activity (by fibrin zymography on 10% SDS-PAGE gels containing fibrinogen and plasminogen), MMP-2 and -9 (by gelatinase zymography), and TIMPs (by reverse zymography).

Fibrin zymography. Fibrin zymography was used to analyze uPA activity on 10% SDS-PAGE gels containing fibrinogen (5.5 mg/ml) and plasminogen (50 μg/ml). After electrophoresis, the gels were washed twice with 2.5% Triton X-100 for 30 min. The gels were then incubated overnight at 37˚C with 0.1% glycine buffer pH 7.5 and then stained with 0.5% Coomassie Brilliant Blue R250 and destained. Electrophoresis of uPA and t-PA were conducted for comparison. Fibrin zymograms were scanned using CanoScan 9950F Canon Scanner.

Gelatinase zymography. Gelatinase zymography was performed in 10% Novex pre-cast SDS polyacrylamide gel (Invitrogen) in the presence of 0.1% gelatin under non-reducing conditions. Culture media (20 μl) were mixed with sample buffer and loaded for SDS-PAGE with Tris glycine SDS buffer as suggested by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gels were then incubated at 37˚C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Upon renaturation of the enzyme, the gelatin is digested by gelatinases in the gel and gives clear bands against an intensely stained background. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Reverse zymography. TIMPs were analyzed by reverse zymography on 15% SDS gels containing serum-free conditioned medium from cells. After electrophoresis the gels were washed twice with 2.5% Triton-X for 30 min at room temperature to remove SDS. The gels were then incubated at 37˚C overnight in 50 mM Tris-HCl and 10 mM $CaCl_2$ at pH 7.6 and stained with 0.5% Coomassie Blue R25, destained and scanned.

Scanning of gelatinase, reverse and fibrin zymograms. Gelatinase, reverse and fibrin zymograms were scanned using CanoScan 9950F Canon scanner at 300 dpi. The intensity of the bands was evaluated using the pixel-based densitometer

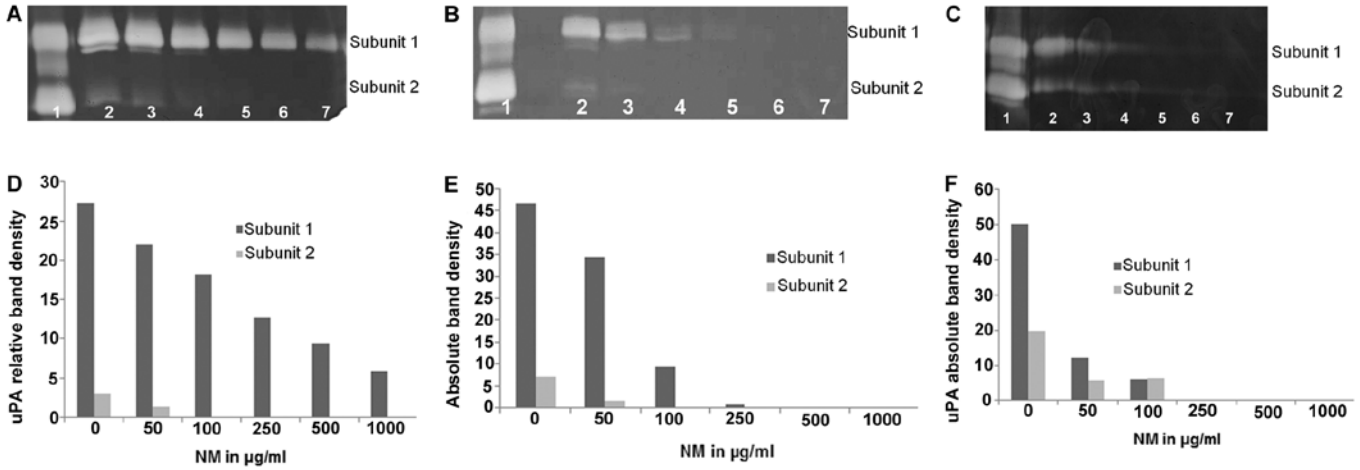

Figure 1. Effect of NM on colon HCT-116, pancreatic MIA PaCa-2 and hepatocellular carcinoma SK Hep-1 uPA expression. Fibrin zymograms of HCT-116 (A), MIA PaCa-2 (B), SK Hep-1 (C) uPA expression. Lane 1, uPA; 2, Markers; 3, Control; 4-7, NM 50, 100, 250, 500, 1000 μg/ml, respectively. Densitometric analyses of HCT-116 (D), MIA PaCa-2 (E) and SK Hep-1 (F) uPA expression.

Table I. Overview of MMP-2 and -9, uPA and TIMP-2 expression of colon, pancreatic and hepatocellular carcinoma cell lines.

Cancer cell line	MMP-2	MMP-9	uPA	TIMP-2
Colon HCT-116	-	+	+	+
Pancreatic MIA PaCa-2	-	+	+	+
Hepatocellular carcinoma SK-Hep-1	+	+	+	+

program Un-Scan-It, version 5.1, 32-bit, by Silk Scientific Corp. (Orem, UT, USA), at a resolution of one scanner unit (1/100 of an inch for an image that was scanned at 100 dpi). The pixel densitometer calculates the optical density of each pixel (values, 0-255) using the darkly stained background of the gel as a pixel value of 0. A logarithmic optical density scale was used since the optical density of the film and gels is logarithmically proportional to the concentration. The pixel densitometer sums the optical density of each pixel to give the band density.

Statistical analysis. Pearson's correlation coefficient was determined between NM effect on mean MMP-9, uPA and TIMP-2 expression levels of colon, pancreatic and HCC cell lines using MedCalc Software (Mariakerke, Belgium).

Results

Table I provides an overview of the tested cell line uPA, MMP and TIMP-2 activities.

Effect of NM on uPA activity in human colorectal carcinoma, pancreatic adenocarcinoma and hepatocellular carcinoma cell lines. Activity of uPA was detected in HCT-116, MIA PaCa-2, and SK-Hep-1 cell lines. HCT-116, MIA PaCa-2 and SK-Hep-1 showed 2 bands corresponding to subunits 1 and 2 at 55 and 33 kD. NM inhibited HCT-116 uPA in a dose-dependent manner to 21.2% of control at 1000 μg/ml (linear trend R^2=0.994) for subunit 1 and virtual block of subunit 2 at 100 μg/ml (linear trend R^2=0.678). NM exerted dose response inhibition with virtual block of uPA activity at 500 μg/ml (linear trend R^2=0.827) and 100 μg/ml (linear trend R^2=0.583) for subunits 1 and 2, respectively in MIA-PaCa-2. SK-Hep-1 cell uPA activity was inhibited by NM in a dose-dependent manner with virtual total inhibition at 250 μg/ml (linear trends R^2=0.639 and 0.732 for subunits 1 and 2, respectively). See Fig. 1 for respective fibrin zymograms and densitometry analyses.

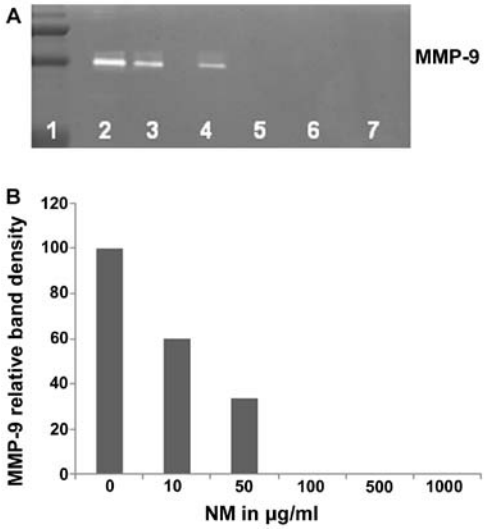

Figure 2. Effect of NM on colon HCT-116 MMP-2 and -9 expression. Gelatinase zymogram of untreated HCT-116 (A) MMP-2 and MMP-9 expression. Lane 1, Markers; 2, Control; 3-7, NM 10, 50, 100, 500, 1000 μg/ml, respectively. Densitometric analysis of untreated (B) HCT-116 MMP-2 and -9 secretion.

Effect of NM on MMP-2 and MMP-9 expression by colon cancer cell line HCT-116. On gelatinase zymography, HCT-116 cells demonstrated no MMP-2 and strong expression of MMP-9, which was inhibited by NM in a dose-dependent fashion with virtual total inhibition of MMP-9 at 100 μg/ml

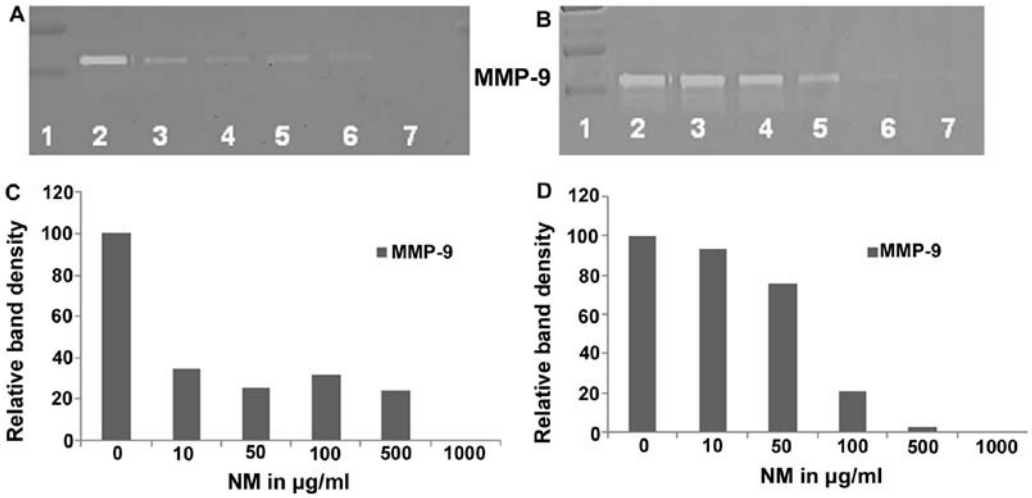

Figure 3. Effect of NM on pancreatic MIA PaCa-2 MMP-2 and -9 expression. Gelatinase zymograms of normal MIA PaCa-2 (A) and PMA-treated MIA PaCa-2 (B) MMP-2 and MMP-9 expression. Lane 1, Markers; 2, Control; 3-7, NM 10, 50, 100, 500, 1000 μg/ml, respectively. Densitometric analyses of normal (C) and PMA-treated (D) MIA PaCa-2 MMP-2 and -9 secretion.

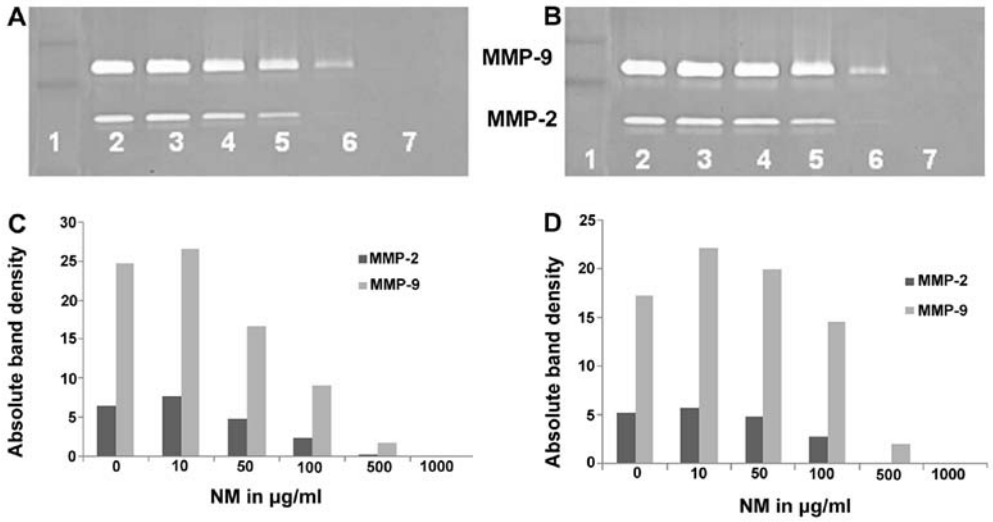

Figure 4. Effect of NM on HCC SK Hep-1 MMP-2 and -9 expression. Gelatinase zymograms of normal SK Hep-1 (A) and PMA-treated SK Hep-1 (B) MMP-2 and MMP-9 expression. Lane 1, Markers; 2, Control; 3-7, NM 10, 50, 100, 500, 1000 μg/ml, respectively. Densitometric analyses of normal (C) and PMA-treated (D) SK Hep-1 MMP-2 and -9 secretion.

(linear trend R^2=0.858) See Fig. 2 for gelatinase zymogram and densitometry analysis.

Effect of NM on MMP-2 and MMP-9 expression by pancreatic cancer cell line MIA PaCa-2. On gelatinase zymography, MIA PaCa-2 cells demonstrated no MMP-2 secretion but moderate MMP-9 secretion which was enhanced with PMA (100 ng/ml) treatment. NM inhibited untreated cellular MMP-9 secretion in a dose-dependent fashion with virtual total inhibition of MMP-9 at 500 μg/ml (linear trend R^2=0.695) and PMA-treated MMP-9 secretion at 1000 μg/ml (linear trend R^2=0.920). See Fig. 3 for gelatinase zymograms and densitometry analyses.

Effect of NM on MMP-2 and MMP-9 expression by HCC cell line SK-Hep-1. Zymography demonstrated strong expression of MMP-9 and slight expression of MMP-2 by normal and PMA-treated (100 ng/ml) SK-Hep-1 cells that were inhibited by NM in a dose-dependent fashion with virtual total inhibition of MMP-2 and MMP-9 at 1000 μg/ml (linear trends R^2=0.934 and 0.891 for MMP-9 and -2, respectively). See Fig. 4 for gelatinase zymograms and densitometry analyses.

Effect of NM on TIMP-2 activity in colon HCT-116, pancreatic MIA PaCa-2 and HCC SK-Hep-1 cells. Reverse zymography revealed upregulation of TIMP-2 activity with NM treatment in all cancer cell lines in a dose-dependent manner. Minimum activity was expressed at 50 and maximum at 1000 μg/ml NM. See Fig. 5 for respective reverse zymograms and densitometry analyses.

Correlation between colon, pancreatic and hepatic carcinoma cell line uPA, TIMP-2 and MMP expression levels. Analysis revealed a positive correlation between uPA and MMP-9 expression levels of NM-treated colon, pancreatic

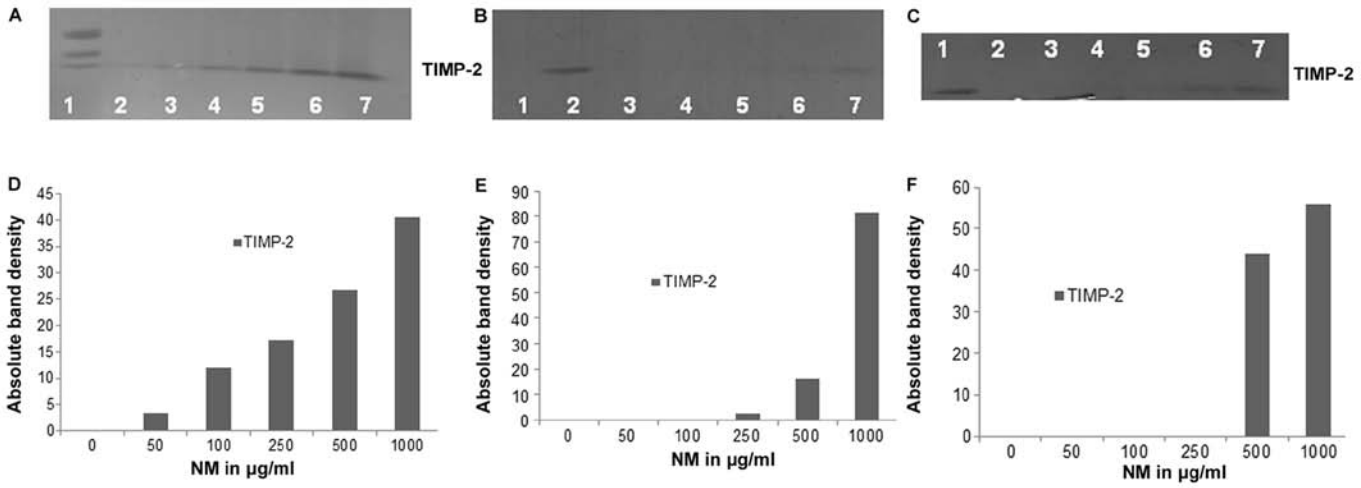

Figure 5. Effect of NM on colon HCT-116, pancreatic MIA PaCa-2 and hepatocellular carcinoma SK Hep-1 TIMP-2 expression. Reverse zymograms of HCT-116 (A), MIA PaCa-2 (B) and SK Hep-1 (C) TIMP-2 expression. Lane 1, Markers; 2, Control; 3-7, NM 50, 100, 250, 500, 1000 μg/ml, respectively. Densitometric analyses of HCT-116 (D), MIA PaCa-2 (E) and SK Hep-1 (F) TIMP-2 expression.

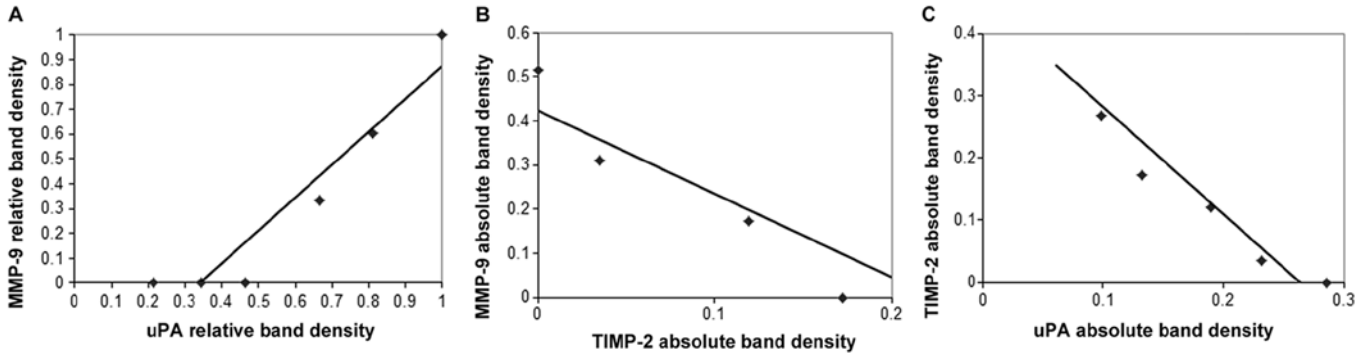

Figure 6. (A) Correlation between the effects of NM on colon HCT-116 uPA and MMP-9 expression (correlation coefficient r=0.998). (B) Correlation between the effects of NM on colon HCT-116 MMP-9 and TIMP-2 expression (correlation coefficient r= -0.976). (C) Correlation between the effects of NM on colon HCT-116 uPA and TIMP-2 expression (correlation coefficient r= -0.964).

Table II. Correlation between effects of NM on colon, pancreatic and hepatocellular uPA, MMP-9 and TIMP-2.

Cell line	uPA and MMP-9	MMP-9 and TIMPs	uPA and TIMP-2
Colon HCT-116	r=0.998	r= -0.976	r= -0.964
Pancreatic MIA PaCa-2	r=0.776	r= -0.374	r= -0.466
Hepatocellular carcinoma SK-Hep-1	r=0.688	r= -0.832	r= -0.446

and hepatocellular carcinoma, as shown in Table II. Fig. 6A shows the correlation graph for colon uPA and MMP-9 with a correlation coefficient r=0.998. Negative correlations were found between the expression levels of MMP-9 and TIMP-2 in all three cell lines treated with NM as shown in Table II. The correlation (r= -0.976) between MMP-9 and TIMP-2 is shown for colon in Fig. 6B. Negative correlations were found between expression levels of TIMP-2 and uPA in all NM-treated cell lines studied. The correlation (r= -0.964) between uPA and TIMP-2 is shown for colon in Fig. 6C.

Discussion

Tumor cell invasion occurs secondary to cell attachment, degradation of the ECM and migration through the disrupted matrix. Two families of proteases, matrix metalloproteinases (MMPs) and urokinase plasminogen activators, play critical roles in tumor cell invasion. Experimental studies have demonstrated the role of urokinase plasminogen, especially cell surface uPA, as an initiator of ECM proteolysis and associated tumor cell invasion (27). The protease uPA converts plasminogen to plasmin, which is capable of promoting tumor growth and angiogenesis, degrading the ECM and basement membrane and activating pro-MMPs (21). The urokinase-type plasminogen activator (uPA) system, which includes the serine protease (uPA), its receptor (uPAR), and the endogenous inhibitors, plasminogen activator inhibitors 1 and 2, plays an important role in invasion and subsequent metastasis of various tumors including colorectal tumors (15).

Multivariate survival analysis of patients undergoing curative surgery confirmed that the uPA cancer: normal ratio was related to outcome (relative risk, 2.67; P=0.02) and was independent of tumor stage (relative risk, 2.26; P=0.03). Their study suggests that a high ratio of cancer to normal mucosal uPA

indicates an increased risk of colorectal cancer progression. Measurement of uPA may provide useful prognostic information in patients undergoing curative surgery for colorectal cancer. The aggressive behavior of colorectal cancers with a high uPA ratio suggests that the protease might be a suitable target for the development of therapeutic agents to prevent invasion and metastasis (22).

Xue *et al* found that overexpression of uPA in patients with pancreatic ductal adenocarcinoma to be correlated with poorer survival rate (23). Harvey *et al* found that uPA was a prognostic indicator in pancreatic cancer (19). uPA activity has been found to be a strong predictor for the recurrence and prognosis of HCC (24). MMPs, especially MMP-2 and MMP-9, play pivotal roles in tumor cell invasion and metastasis due to their ability to degrade type IV collagen, a major component of the ECM. Overproduction of MMPs, especially MMP-2 and -9 and low levels of tissue inhibitors of metalloproteinases (TIMPs) have been shown to be associated with a more aggressive behavior of colon (18), pancreatic (19), and hepatic carcinomas (20).

Our study demonstrated that the specific mixture of nutrients tested significantly inhibited uPA secretion in colon HCT-116, pancreatic MIA PaCa, and HCC SK-Hep-1 cell lines. Furthermore, the NM demonstrated dose-dependent decrease in MMP secretion and increase in TIMP-2 secretion by all these cell lines. As expected, a significant positive correlation was found between the secretion of uPA and MMP-9 and a significant negative correlation between uPA and TIMP-2 and between MMP-2 and TIMP-2 secretion by NM treatment of colon HCT-116, pancreatic MIA PaCa-2, and HCC SK-Hep-1 cell lines. Furthermore, a previous study demonstrated significant correlation between NM inhibition of Matrigel invasion and NM modulation of the MMP-2 and -9 activities of these cell lines (28). A significant negative correlation was found between NM modulation of Matrigel invasion inhibition and MMP-9 secretion with colon HCT-116 (r= -0.936), pancreatic MIA PaCa (r= -0.834) and HCC SK-Hep-1 (r= -0.880). Previous *in vivo* studies of the effects of NM 0.5% dietary effect on xenograft tumor growth in nude mice support these results in that they demonstrated significant inhibition of xenograft tumor growth: 63%, P=0.0002 in colon HCT-116 xenografts (29) and 42%, P=0.09 in HCC SK-Hep-1 xenografts (30).

In contrast to the associated toxicity and limited efficacy of standard cancer chemotherapy and radiation therapy, the efficacy and safety of dietary and botanical natural compounds in cancer prevention has been extensively documented (31). The nutrient mixture was formulated by selecting nutrients that act on critical physiological targets in cancer progression and metastasis, as documented in both clinical and experimental studies. Combining these micronutrients expands metabolic targets, maximizing biological impact with lower doses of components. A previous study of the comparative effects of NM, green tea extract and EGCG on inhibition of MMP-2 and MMP-9 secretion of different cancer cell lines with varying MMP secretion patterns, revealed the superior potency of NM over GTE and EGCG at equivalent doses (32). These results can be understood from the more comprehensive treatment offered by the combination of nutrients in NM over individual components of NM since MMP-2 and MMP-9 are mediated by differential pathways.

Optimal ECM structure depends upon adequate supplies of ascorbic acid and the amino acids lysine and proline to ensure proper synthesis and hydroxylation of collagen fibers. In addition, lysine contributes to ECM stability as a natural inhibitor of plasmin-induced proteolysis (25,33). Manganese and copper are also essential for collagen formation. There is considerable documentation of the potency of green tea extract in modulating cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression (34-40). N-acetyl cysteine and selenium have demonstrated inhibition of tumor cell MMP-9 and invasive activities, as well as migration of endothelial cells through ECM (41-43). Ascorbic acid demonstrates cytotoxic and antimetastatic actions on malignant cell lines (44-48) and cancer patients have been found to have low levels of ascorbic acid (49,50). Low levels of arginine, a precursor of nitric oxide (NO), can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis (51).

In conclusion, the NM demonstrated potent anticancer activity by targeting primary mechanisms responsible for the aggressive spread of colon, pancreatic and hepatic carcinomas. In this *in vitro* study, the NM significantly inhibited secretion of uPA and MMP-2 and/or -9 and increased secretion of TIMP-2 in colon, pancreatic and hepatic carcinoma cells, suggesting its potential in modulating cancer invasion and metastasis. NM inhibition of MMP-9 secretion was found to be correlated significantly with Matrigel invasion of these cell lines. Furthermore, use of the nutrient mixture would not pose any toxic effect clinically, especially in the relevant doses, as *in vivo* safety studies demonstrate. An *in vivo* toxicology study showed that NM had no adverse effects on vital organs (heart, liver and kidney), or on the associated functional serum enzymes (52).

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