Article published online: 2022-07-07

ARTICLE

The Role of Angiogenic Biomarkers in Gastrointestinal Cancer

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Published: 08 July 2013
Ibnosina J Med BS 2013,5(4):196-205
Received: 31 December 2012
Accepted: 09 March 2013
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Abstract

Background: Angiogenesis is a key issue in the carcinogenesis progression. Gastrointestinal tract (GIT) cancer is a multi-stage disease, multifaceted, concerning convention of different signaling cascades. Abnormal angiogenesis have been related to pathogenesis of tumor. **Objectives:** We hypothesized inter-relationships between indices of angiogenesis biomarkers across the spectrum of GIT cancer and its relation to the pathogenesis and prognosis of the diseases. We also evaluated the consequence of estimating these indices in untimely tumor recognition. **Methods:** Forty patients were studied and divided into two groups: Group 1 (n=30) with GIT cancer and group 2 (n=10) with benign lesion. The cancer group subdivided into patients with gastric cancer (n= 12) and patients with colorectal cancer (n=18). 20 healthy controls (C) were involved in the study. Serum levels of all biochemical indices were estimated. **Results:** Significantly high serum levels of studied angiogenic biomarkers were detected in patients with GIT cancer compared to C and benign group ($p<0.000l$ and $p<0.001$ for each respectively). Their levels being significantly higher in late stage of the disease versus early stage and in patients with high tumor burden versus patients with low tumor burden with the exception of Cathpsin-B and HA that showing no significant difference. Positive Significant correlations was present between all indices in cancer groups ($p<0.001$). Also, there was significant positive correlations between these biomarkers and stage of the tumor ($p<0.01$). **Conclusions:** There was a gradation in angiogenesis biomarkers in patients with GIT cancer which related to stage and size of the tumors. This may reflect the relative roles of these biomarkers in the biology of GIT cancer. Their estimation may have implications for our understanding of the path-physiology of tumor, play a novel beneficial roles in early tumor detection and targeted tumor therapy by using ant-angiogenic therapy

Key words: Angiogenesis, Gastric cancer and Colorectal cancer.

Introduction

Neoplasms are the second leading cause of death after vascular diseases, despite the huge progress in medical

sciences in the last 20 years. Recently, gastric cancer morbidity has decreased, but mortality is still high (1). It is considered the fourth most common cancer and the second most frequent cause of cancer-related death, accounting for 10.4% of cancer death worldwide (2). Also, colorectal cancer represents the fourth commonest malignancy, and constitutes a major cause of significant morbidity and mortality among other diseases (3). More than 1.2 million new cases of colorectal cancer are reported each year worldwide (4). Despite the improvements, estimated cure rates for patients with advanced stages of cancer remain poor. Therefore, it is important to understand the nature of these tumors to investigate new therapeutic strategies for treatments (2). Moreover, innovative screening tools could help detect cancer at early stages and allow curative treatment interventions (4). Angiogenesis, the formation of new blood vessels from the endothelium of the existing vasculature, is fundamental in tumor growth, progression and metastasis. The complex network of tumor blood microvessels guarantees adequate supply of tumor cells with nutrients and oxygen and provides efficient drainage of metabolites (5).

Numerous pro- and antiangiogenic molecules, their ligands, and intracellular signaling pathways have been identified. Several initial clinical trials gave no convincing evidence for efficient antitumoral therapy by classical antiangiogenic agents as monotherapy. This has led to the development of new combinations of antiangiogenic compounds with chemotherapy (5,6). Tumor cells and stromal cells produce various angiogenic factors, including, vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF). VEGF is a potent angiogenic agent that acts as a specific mitogen for vascular endothelial cell through specific cell surface receptors (9). VEGF suppression leads to retrogression of neoplastic vessels and tumor growth restriction (10). PD-ECGF is a non glycosylated single chain polypeptide that stimulates chemotaxis for endothelial cells in vitro and angiogenesis in vivo and plays a direct role in promoting lymphangiogenesis and metastatic spread to lymph nodes (11).

Cathepsins are lysosomal proteinases are associated with tumor invasion and metastasis. Elevated expressions of cathepsins and diminished levels of their inhibitors have been observed in several human cancer (12). IL-8, a chemokine, is known to possess tumorigenic and proangiogenic properties. Over-expression of IL-8 is associated with tumor growth, metastasis, chemoresistance, angiogenesis and poor prognosis, implying IL-8 to be an important therapeutic target (13,14).

Hyaluronan (HA), a major macropolysaccharide in the extracellular matrix of connective tissues, is intimately involved in the biology of cancer, It accumulates into the stroma of various human tumors and modulates intracellular signaling pathways, cell proliferation, motility, invasion, tumor growth and metastasis (15). NO has been the focus of cancer study for its function in tumorigenesis, tumor progression and death. The effects of NO on tumor cells are multifaceted, but many details underlying these effects are not yet well understood (16,17). We hypothesized the changes in serum levels of VEGF, PD-ECGF, Cathpsin-B, HA, IL-8 and NO across the spectrum of GIT cancer and their relationships to the pathogenesis of cancer. We also clarified the consequence of estimating these indices in untimely tumor recognitions.

Pathological angiogenesis is a hallmark of cancer and various ischaemic and inflammatory diseases. Concentrated efforts in this area of research are leading to discovery of a growing number of pro- and anti- angiogenic molecules. The complex interaction among these molecules are now beginning to be elucidated and understanding this integration is leading to the development of a number of exciting and bold approaches to treat cancer (6). The aim of the present study was to evaluate the angiogenic factors (VEGF, PD-ECGF, Cathpsines, IL-8, HA and NO) in patients with gastric and colorectal cancer and determine their relationship with the clinico-pathological parameters, early tumor detection and targeted tumor therapy by using antiangiogenic therapy which thought to be a promising approach to cure cancer.

Material and Methods

Study protocol

The study was approved by the ethics committee of Faculty of Medicine, Assiut University, Egypt and informed consents were obtained from patients and controls. The case control study includes 40 patients with gastro-intestinal tumors who were selected from South Egypt Cancer Institute in Assiut University, Assiut, Egypt. They were divided into 2 groups according to the type of the tumor: Group 1: patients with malignant tumor (n=30). Group 2: patients with benign tumor (n=10). The malignant group subdivided into two subgroups: patients with gastric cancer (n=12), and patients with colorectal cancer (n=18). In addition,

20 healthy controls of matched age and sex were matched from subjects attended to annual physical check up without evidence of any tumors. Complete medical history, physical examination, and routine investigations were done for all participants. All patients were subjected to abdominal computer tomography, magnetic resonance imaging and upper and lower endoscopy when required. The GIT cancer was classified according to TNM staging system (18). TNM combinations correspond to one of five stages: Stage 0: carcinoma in situ, stage I: cancer is limited to the organ in which it began, stage II: indicate large tumor size spread to the muscle layer of the organ, stage III: indicate large tumor size which spread beyond the organ to nearby lymph node or adjacent organs, Stage IV: The cancer has spread through blood or lymph to another organs. In addition they were classified according to the size of cancer into high tumor burden (>6x6 cm) and low tumor burden (≤6x6 cm). Primary tumors in other organs, hereditary cancer syndrome, previous malignancy, previous radiotherapy or chemotherapy were excluded from the study.

Estimation of Biochemical Indices

Fasting 10 ml of blood sample were collected by vein puncture under complete aseptic condition. Samples were allowed to clot for 30 minutes and centrifuged at 1000 g for 10 minutes. Serum was collected and stored at -70 ºC in 6 aliquots till time of assay. Serum VEGF levels were measured with Qauantikine ELISA Kite, (Cat. no. DVEOO, R and D system, Minneapolis, MN), according to the method of He et al., (19). Serum PD-ECGF levels were determined chemically according to the method of Scocca, (20). Also, serum Cathepsin-B levels were determined chemically according to the method of Barrett (21,22). Serum levels of IL-8 were measured with ELISA kit (cat. No. 2237, Immunotech, France). Serum levels of HA were determined chemically by the methods of Greiling, (23). In this method, hylauronate lyase degrades hyaluronan qualitatively to an unsaturated disaccharide leading to the liberation of N-acetyl-glucosamine end groups. These can be determined colorimetrically by the Morgan-Elson reaction, which depends on the formation of a red dye. When N-acetyl-glucosamine is heated in alkali, it produces furan

Table 1. Clinical and demographic data in patients with malignant tumor (G2), benign tumor (G1) and healthy controls (C).

Biomarkers	Healthy controls C (n=20)	Benign tumor G1 (n=10)	Malignant tumor G2 (n=30)	P value
Age (years)	44 (15)	41 (19)	47(18)	0.26
Sex (% male)	9 (45%)	6 (60%)	14 (46.7%)	0.322
Family history (%)	No	1 (10%)	3 (10%)	<0.01
Smoking (%)	0	3 (30%)*	5 (16.7%)Ω	0.001>
Diabetes (%)	0	2 (20%)*	5 (16.7%) Ω	<0.001
Hypertension (%)	0	3 (30%)*	7 (23.3%) Ω	<0.001

*Data presents as mean (standard deviation) or as percentage. P value by ANOVA or chi-squared (sex, diabetes, etc). ΩP <0.05 (Tukey's test compared to G2). Ω G2 significantly higher compared to G1and C (P<0.001). *G1 significantly higher compared to C (P<0.05).*

Table 2. Serum levels of various biochemical indices in patients with malignant tumor (G2), benign tumor (G1) and healthy controls (C).

	Healthy Controls C N=20	Benign group G1 N=10	Cancer group G2 N=30	Significance P=value
VEGF (pg/ml)	193 ± 17	298 ± 35	595 ±168	C versus G1 0.00** C versus G2 0.00** G1 versus G3 0.00**
IL8 (ng/ml)	82 ± 11	141 ± 14	296 ± 82	C versus G1 0.00** C versus G2 0.00** G1 versus G2 0.00**
NO (Mmol/l)	5 ± 1	7 ± 9	16 ± 4	C versus G1 0.04* C versus G2 0.00** G1 versus G2 0.00**
PD/ECGF (nmol/ml)	27 ± 9	71 ± 7	154 ± 41	C versus G1 0.00** C versus G2 0.00** G1 versus G2 0.00**
Cathepsin B (nmol/L)	7 ± 2	8 ± 1	20 ± 6	C versus G2 0.00** G1 versus G2 0.00** G1 versus G2 NS$^{\beta}$
HA(mg/L)	23 ± 5	27 ± 4	73 ± 25	C versus G2 0.00** G1 versus G2 0.00** G1 versus G2 NS$^{\beta}$

*Data presented as (mean ±SD), P value by Kruskall-Wallis.: Comparisons between the 3 groups by one-way ANOVA with Tukey's test .P** value in G2 significantly higher compared to G1 and C (p<0.0001), P$^{\beta}$ value in G2 compared to G1 in HA and Cathepsin B (p=NS).*

derivatives that react with p-dimthylamine benzaldhyde producing a red dye. The concentration was calculated by reference to a standard hyaluronan. NO levels were determined colorimetrically as total nitrite and nitrate as described by van Bezooijen et al. (24). The samples were deproteinized with ZnSO4(30%), and colour developed by reaction with Griess reagent (1% sulfanlamide, 0.1% naphthylenediamine dihydrochloride, w/v in 2.5% H3PO4) was recorded at 550 nm against reagent blank using sodium nitrite 10-100 Mmol as standard.

Statistical analysis

It was performed using SPSS statistical program Version 14. Following application of the Shapiro-Wilkes test to determine a normal distribution, non-categorical data distributed normally are expressed as mean (standard deviation) and data distributed non-normally are expressed as median (interquartile range). Categorical data are analysed by the chi-squared test was used to compare between qualitative data. Continuously variable data are analysed by ANOVA or the Kruskal-Wallis test. Mann Whitney test was used to compare between two groups. Spearman correlation coefficient was used to study the relation between variables. P< 0.05 was considered as statistically significant.

Results

Demographic characteristics

There were no significant difference in regard to the age and sex between patients and controls (Table 1). Patients with malignant and benign tumors showed high incidence rates of smoking, diabetes mellitus and hypertension.

Table 3. Serum levels of various biochemical indices in different stages of cancer

	Early stage (I, II) N=13	Late stage (III, IV) N=17	Significance P-value
VEGF (pg/ml)	458 ± 100	701 ± 142	0.001***
IL8 (ng/ml)	228 ± 48	347 ± 69	0.01**
NO (Mmol/l)	13 ± 3	18 ± 3	0.01**
PD/ECGF (nmol/ml)	122 ± 28	178 ± 35	0.001***
Cathepsin B (nmol/L)	15 ± 3	23 ± 6	0.01**
HA(mg/L)	47 ± 20	86 ± 24	0.01**

*Data presented as (mean ±SD), P value by Kruskall-Wallis. Comparisons between the two groups by Mann–Whitney test. P***value of PD/ECGF and VEGF are significantly higher in late stage compared to early stage (P<0.0001). P**value of NO, Cathepsin B, IL8 and HA are significantly higher compared to early stage (P<0.01).*

Table 4. Serum levels of biochemical indices in gastric and colorectal cancer

	Gastric cancer N=12	Colorectal cancer N=18	Significance P-value
Age (years)	43 ± 22	38 ± 17	0.67
VEGF (pg/ml)	612 ±162	582 ± 162	0.56
IL8 (ng/ml)	297 ± 87	295 ± 81	0.89
NO (Mmol/l)	16 ±3	16 ± 4	0.87
PD/ECGF (nmol/ml)	158 ± 40	153 ± 40	0.34
Cathepsin D (nmol/L)	21 ± 6	20 ± 6	0.77
HA(mg/L)	80 ± 26	69 ± 23	0.46

Data presented as (mean±SD). Comparison between the two groups by Mann Whitney test. No significant difference between two groups of cancer (P>0.05).

Concentration of biochemical indices in GIT cancer

All biochemical parameters were significantly higher in patients with benign and malignant tumors compared with controls (Table 2). Also, these parameters were significantly higher in malignant compared to benign group with the exception of cathpsin-B and HA that showing no significant difference. The serum levels of various biochemical indices in different stages of cancer are summarized in table 3. Stage I and II were considered as early stage, and stage III and IV as late stage. The levels of VEGF, IL-8, NO, PD-ECGF, Cathpsin-B, and HA were significantly elevated in late stage as compared with early stage of the tumor. The

Table 5. Serum levels of biochemical indices in high and low tumor burden.

Parameters	High tumor burden HB (N=9)	Low tumor burden LB (N=7)	P value
VEGF (pg/ml)	686.0 ± 76.6	559.5 ± 99.4	< 0.05
PD-ECGF nmol/ml)	172.5 ± 23.8	145.3 ± 23.9	< 0.05
Cathepsins (nmol/L)	21.2 ± 4.5	18.2 ± 4.5	0.56
IL-8 (ng/ml)	335.6 ± 56.6	280.6 ± 63.1	< 0.05
HA (mg/L)	79.4 ± 25.2	67.2 ± 18.1	0.76
NO (Mmol/L)	17.2 ± 2.2	16.7 ± 3.4	< 0.05

Data presented as (mean ±SD), Comparisons between the2 groups by Mann Whitney. P value in LB group significant compared to HB group (P<0.05). No significant difference between LB group compared to HB group (p>0.05)

Table 6. Correlations between the studied parameters in cancer groups.

Cancer group N=30	STAGE	VEGF (pg/ml)	IL8 (ng/ml)	NO (Mmol/l)	PD/ECGF (nmol/ml)	CATHEPSIN (nmol/ml)	HA (mg/L)
VEGF (pg/ml)	r=.651(b) p=.000						
IL8 (ng/ml)	r=.624(b) p=.001	r=.945(b) p=.0001					
NO (Mmol/L)	r=.611(b) p=.001	r=.763(b) p=.0001	r=.801(b) p=.0001				
PD/ECGF (nmol/ml)	r=.579(b) p=.002	r=.945(b) p=.000	r=.937(b) p=.000	r=.727(b) p=.0001			
CATHEPSIN B (nmol/L)	r=.474(a) p=.015	r=.858(b) p=.000	r=.897(b) p=.000	r=.708(b) p=.000	r=.939(b) p=.000		
HA (mg/L)	r=.509(b) p=.008	r=.799(b) p=.000	r=.800(b) p=.000	r=.728(b) p=.000	r=.841(b) p=.000	r=.877(b) p=.000	
BURDEN	N.S	r=.629(a) p=.028	N.S	N.S	N.S	N.S	r=.757(b) p=.004

Spearman colorations between all indices, a Correlation is significant at the 0.05 level, b Correlation is significant at the 0.01 level.

serum levels of all biochemical parameters show no significant differences between gastric and colorectal cancer (Table 4). In addition, there were significant elevation of the serum levels of VEGF, PD-ECGF, IL-8 and NO, in patient with high tumor burden as compared with patients with low tumor burden (Table 5).

Correlation Between Biochemical Indices and Tumor Characteristics

There was significant positive correlation between the serum levels of VEGF, PD-ECGF, cathepsin-B, IL-8, HA and nitric oxide (Table 6). Also, there was significant positive correlation between the above biochemical parameters and stages of tumors. On the other hand, there was no significant correlation between these parameter and patients with high or low tumors burden with the exception of VEGF and HA that showing significant positive correlation (Table 6).

Discussion

In the present study, serum levels of various biochemical indices were significantly higher in GIT malignant tumors in comparison with controls and benign tumors group. Moreover, their levels being significantly higher in late stage versus early stage and in patients with high tumor burden versus low tumor burden with the exception of Cathpsin-B and HA that showing no significant difference.

Higher levels of VEGF have been reported in patients with gastric cancer than in healthy controls (25,26). In agreement with our findings, VEGF serum levels correlated with tumor grade and size (25). Similarly, serum levels of VEGF were significantly higher in patients with colorectal cancer than controls (27) and this marker and its receptors were expressed at high levels in metastatic human colon carcinomas (28). Consequently, VEGF is recognized as a prominent angiogenic factor in colon carcinoma. Assessment of VEGF expression may be useful for predicting metastasis and high levels of VEGF expression were associated with advanced cancer stage and related to unfavorable prognosis (28). VEGF expression was reported to be significantly higher in tumor area than non-tumor area and this levels reflected tumor stage and used as a poor prognostic parameter (29). Preoperative estimation may be used for evaluating the biological behavior, invasion and metastasis of gastric and colorectal carcinoma and may help for identify patients with poor prognosis (30).

PD-ECGF has been reported as significantly higher in malignant than control group and the increased expression was present with increase in depth of invasion (31). This could be attributed to the insufficient perfusion to areas of the tumor resulting in the existence of hypoxia, glucose depletion and low PH. Secretion of PDGF by gastric carcinoma cells and expression of its receptors by tumor-associated stromal cells are associated with lymphatic metastasis (11). Therefore, blocking the receptors signaling pathway may inhibit node metastasis in gastric cancer. In case of colorectal carcinoma, De Bruin et al (32) reported that, there was increased expression in colorectal tumor tissue compared to normal tissue and high levels are a prognostic factor for poor survival in this cancer. Using staining techniques PD/ECGF is often over-expressed in tumor infiltrating cells, mainly macrophages, and colon cancer cells themselves. Accordingly, expression of this marker by infiltrating cells provides an additional alternate mechanism for tumor neovascularization (32).

Cathepsin-B was significantly higher in homogenates of gastric cancer tissue than in those of cancer-free samples obtained from the same stomach (33), clearly indicating that this enzyme is involved in development and growth of this disease. In addition, Russo et al. (34) found that, cathepsin-B significantly correlated with TNM stage, nodal status, histological grade and presence of metastasis and patients with poorly differentiated tumors, indicating that the synthesis and release of Cathepsin-B is related to bad prognosis. In agreement of our findings, Cathepsin-B protein and its activity levels have been found to be higher in both gastric and colorectal cancer (34) and higher activities of Cathepsin-B have been observed in the initial stages of cancer development and higher levels of Cathepsin-B have been detected in metastatic versus non-metastatic cancer tissue homogenates. They studied the involvement of Cathepsin in the invasion, metastasis and proliferation of cancer cells. Their results indicated that cancer cells orchestrate various Cathepsins to progress malignant disease and it may be a potential target for cancer therapy (35). In addition, Miyamoto et al. (8) concluded that, this markers is highly expressed in GIT tumors compared to its expression in other cancerous lesions, this identifies Cathepsins as new diagnostic marker for GIT tumors.

Gastric specimens have been found to contain increased IL-8 protein levels and many gastric cancer cell lines express high levels of IL-8 mRNA and protein (36). In addition, they found that gastric cancer cells express not only IL-8, but also its receptors increases the invasive capacity of gastric cancer cells. The may have autocrine/paracrine roles in the progressive growth of human gastric cancer

(36). Moreover, over-expression of this marker promotes the adhesion, migration, invasion and chemo-resistance of gastric cancer cells (13,14), indicating that it is an important therapeutic target in gastric cancer. The relationship of IL-8 in colorectal carcinoma and its levels were significantly higher than those normal tissues (37).The level was significantly associated with tumor size, depth of infiltration, stages and liver metastases so it was responsible for tumor progression.

Also, a correlation between expression and the development of liver metastasis in colorectal cancer (38) suggesting that monitoring the expression may potentially help to assess the course of cancerous conditions and its prognosis. Moreover, IL-8 is a valuable panel of biomarkers, whose detection can be used in early detection and prognosis of colon cancer (39). Also, Bünger et al. (4) reported that, the screening value of additional blood markers, as IL-8, and the potential advantage of combining serum biochip testing with fecal occult blood needs to offers an innovative approach to colorectal cancer screening (4).

The present findings are in agreement with previous studies which showed that, NO activity and NO synthesis were high in GIT tumor tissue and in plasma (40). Measuring serum nitrate/nitrite concentration has been suggested to have potential in estimating the gastric juice nitrate/ nitrite concentration which could be useful as a marker for mutagenesis (41). Also, inducible nitric oxide synthase expression correlates with lymph node metastasis, vascular invasion, distant metastasis (3), TNM stage and poor survival rate in gastric cancer. They propose that synthesized inducible nitric oxide synthase increases angiogenesis, and lymphangiogenesis thus promotes tumor progression.
Inducible NO synthase expression may be a good biomarker for poor prognosis in gastric cancer (3). The levels of NO critically modulate the recognition of tumor cells by dendritic cells, and proposes a new potential therapeutic approach against chemo-immunoresistant tumors (17). On the other hand, NO not only inhibit cell growth and proliferation, but also induces apoptosis in GIT cancer and such effects of NO showed significant dose-dependant activity (16).

The findings of the present study were in agreement with previous reports who found significantly elevated levels of hyaluronic acid in GIT cancer patients and its serum levels showed fairly good sensitivity for discrimination of gastric cancer patients from controls (41). Additionally, two different patterns have been suggested for expression of HA in tumors arising from simple and stratified epithelia (42). With the first group, such as cancers of the colon and stomach, the expression correlates with tumor grade and invasiveness. With poorly differentiated tumors and local or distant invasion, more cancer cells will be hyalyronan-positive or have more intense hyaluronan staining. On the other hand, early stage cancer arising from stratified epithelia may have increased hyaluronan expression compared to normal epithelium, but high-grade aggressive squamous cancers are associated with decrease in HA expression (41). Sumiya et al. (43) reported that, the intracellular HA binding protein related to the invasion and metastasis through promotion of cancer cell motility through a number of pathways including focal adhesion kinase and MAP kinase. HA derived from malignant cells educates neutrophils to adopt an activated phenotype, and in that way stimulates the metastasis of malignant cells, which represents a positive regulatory loop between tumor and their stroma during neoplastic progression (43).

In the present study, there were positive correlations between different studied indices. These results were in agreement with Brown et al. (44) who suggested that thymidine catabolism by PD-ECGF stimulate carcinoma cell secretion of IL-8 and VEGF. Also, Sandhu et al. (45), reported the role of inflammatory cells infiltrating tumor which explain the presence of chemotactic factor as IL-8 that identified in human gastric and colorectal cancer. Hypoxic vascular endothelial cells may be another source of IL-8 which explain the positive correlation between NO and IL-8. Bünger et al. (4) reported that, the assessment of serum levels of IL-8, VEGF, and other angiogenic factors could be useful for GIT cancer screening with the potential of also detecting early stage tumor. Also they found that, the usage of additional markers on a multiplex array formats, utilizing serum samples, offers an innovative approach for cancer screening.

In conclusion, our study reveals a gradation in the angiogenic biomarkers as VEGF, PD-ECGF, Cathepsin-B, IL-8, HA and NO markedly increased in patients with gastric and colorectal cancers. The increased levels were observed in late stage of cancer and in patients with high tumor burden. Accordingly, estimation of these biomarkers may be used in early tumor detection and targeted tumor therapy by using anti-angiogenic therapy which thought to be a promising approach to cure cancer.

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