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Down-regulation of matrix Gla protein messenger RNA in human colorectal adenocarcinomas

Chung-Wei Fan[a], Di-Li Sheu[b,c], Hong-Arh Fan[a], Kung-Cheng Hsu[a], C. Allen Chang[b], Err-Cheng Chan[c,*]

[a]*Division of Colon and Rectal Surgery, Chang Gung Memorial Hospital, Taipei, Taiwan*
[b]*Institute of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan*
[c]*School of Medical Technology, Chang Gung University, 259 Wen-Hua 1st Road, Kwei-Shan, Taoyuan 333, Taiwan*

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Abstract

Matrix Gla protein (MGP) is a vitamin K-dependent extracellular matrix protein commonly found in a variety of tissues. In this study, we describe the potential use of MGP gene expression as the tumor marker of colorectal cancer. A decrease in expression of the MGP gene was also discovered in colorectal cancer using differential screening of cDNA libraries. The MGP expression in 80 human colorectal adenocarcinomas was quantified by a Northern blot analysis to better define the expression pattern of MGP in colorectal cancer. The expression of MGP mRNA was reduced in 63 of 80 (79%) colorectal adenocarcinomas ($P < 0.001$) as compared to the mRNA in adjacent normal tissue, implying that a decrease in MGP expression is associated with colorectal cancer development. The proportion of tumors with downregulated expression of MGP was lower in Duke's A/B than Duke's C/D (34 of 47 versus 26 of 33, respectively) tumors and was lower in moderate differentiation than poor differentiation (44 of 64 versus 16 of 16, respectively). However, χ^2 analysis does not reveal any correlation between a loss of MGP expression and tumor progression or differentiation state. In conclusion, the downregulation of MGP mRNA generally occurs in colorectal adenocarcinomas. Although the role of MGP in cancer development is unknown, the reduced expression of MGP may be used to distinguish the normal colorectal cells from malignant cells.

Keywords: Vitamin K-dependent protein; Northern blot; In situ RNA hybridization; Gla protein; cDNA library

1. Introduction

Matrix Gla protein (MGP) is an extracellular matrix protein that requires vitamin-K for its γ-carboxylation [1], and it is initially isolated from bone and cartilage [2,3] and consists of 79 amino acid residues with five post-translationally modified γ-carboxyglutamic acids. The distribution for MGP mRNA was latterly found not only in connective tissues of the rat but also in most of the soft tissues such as lung, heart, and kidney [4]. MGP is thought to inhibit hydroxyapatite formation in bone [4]. Although the function of MGP in soft tissues is presently unknown, it has been suggested that MGP expression is related to cellular differentiation and tumor progression [5,6].

Elucidating the molecular basis of cancer cell transformation requires knowledge of the gene mutations

* Corresponding author. Tel.: +886-3-3283016, ext. 5220; fax: +886-3-3288741.
E-mail address: chanec@mail.cgu.edu.tw (E.-C. Chan).

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Table 1
MGP expression and clinical characteristics of colorectal tissues

Case	Age (years)	Sex	Differentiation	Location	Histology	Duke's stage	MGP (T/N) ratio
1	56	Male	Moderately	Rectum	Adenocarcinoma	B	0.13
2	64	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	B	1.11
3	80	Female	Moderately	Ascending colon	Adenocarcinoma	B	1.6
4	67	Male	Moderately	Rectum	Adenocarcinoma	B	1.33
5	41	Female	Moderately	Rectum	Adenocarcinoma	D	1.19
6	57	Female	Moderately	Rectum	Adenocarcinoma	B	0.614
7	77	Male	Moderately	Rectum	Adenocarcinoma	B	1.11
8	82	Male	Well	Rectum	Adenocarcinoma	B	0.19
9	61	Female	Moderately	Rectum	Adenocarcinoma	A	0.09
10	63	Male	Moderately	Rectum	Adenocarcinoma	B	0.17
11	40	Female	Moderately	Rectum	Adenocarcinoma	B	1.78
12	75	Female	Moderately	Sigmoid colon	Adenocarcinoma	B	0.5
13	58	Female	Moderately	Ascending colon	Adenocarcinoma	C	0.65
14	43	Male	Moderately	Cecum	Adenocarcinoma	B	0.5
15	61	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.38
16	58	Female	Moderately	Rectum	Adenocarcinoma	B	0.24
17	72	Female	Moderately	Transverse colon	Adenocarcinoma	B	0.34
18	64	Female	Moderately	Cecum	Adenocarcinoma	C	0.45
19	59	Female	Moderately	Transverse colon	Adenocarcinoma	B	0.95
20	68	Male	Moderately	Ascending colon	Adenocarcinoma	C	0.35
21	61	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.83
22	54	Male	Moderately	Rectum	Adenocarcinoma	B	0.61
23	69	Male	Well	Rectosigmoid colon	Adenocarcinoma	A	0.12
24	68	Male	Moderately	Sigmoid colon	Adenocarcinoma	B	0.04
25	65	Female	Moderately	Rectum	Adenocarcinoma	C	0.1
26	69	Male	Moderately	Ascending colon	Adenocarcinoma	B	0.157
27	30	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.06
28	59	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	D	0.03
29	80	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.169
30	53	Male	Moderately	Rectum	Adenocarcinoma	B	0.45
31	77	Female	Moderately	Ascending colon	Adenocarcinoma	B	0.06
32	65	Male	Moderately	Rectum	Adenocarcinoma	B	2.66
33	39	Male	Moderately	Sigmoid colon	Adenocarcinoma	B	2.03
34	57	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	C	0.01
35	41	Female	Moderately	Rectum	Adenocarcinoma	D	1.21
36	30	Female	Moderately	Rectum	Adenocarcinoma	A	1.71
37	70	Female	Moderately	Transverse colon	Adenocarcinoma	B	0.06
38	69	Male	Moderately	Rectum	Adenocarcinoma	D	0.51
39	70	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.28
40	82	Female	Poorly	Transverse colon	Adenocarcinoma	B	0.22
41	64	Female	Moderately	Ascending colon	Adenocarcinoma	C	0.14
42	67	Male	Well	Sigmoid colon	Adenocarcinoma	B	1.5
43	65	Female	Moderately	Rectum	Adenocarcinoma	C	0.95
44	81	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	D	1.36
45	69	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	C	0.05
46	63	Female	Moderately	Rectum	Adenocarcinoma	D	0.43
47	64	Male	Moderately	Ascending colon	Adenocarcinoma	C	0.05
48	72	Male	Moderately	Rectum	Adenocarcinoma	B	0.14
49	38	Male	Moderately	Rectum	Adenocarcinoma	B	0.92
50	76	Female	Well	Rectum	Adenocarcinoma	B	0.24
51	74	Female	Mucinous	Ascending colon	Adenocarcinoma	C	0.85
52	71	Male	Poorly	Rectum	Adenocarcinoma	C	0.37

Table 1 (*continued*)

Case	Age (years)	Sex	Differentiation	Location	Histology	Duke's stage	MGP (T/N) ratio
53	67	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	C	1.42
54	48	Male	Moderately	Transverse colon	Adenocarcinoma	B	0.45
55	45	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.71
56	57	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	C	1.02
57	81	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.14
58	54	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.41
59	66	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.24
60	61	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	D	0.46
61	59	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	C	0.85
62	63	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.12
63	66	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	0.36
64	66	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	A	0.41
65	73	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	1.31
66	60	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	B	2.28
67	65	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	C	0.34
68	59	Female	Moderately	Rectosigmoid colon	Adenocarcinoma	D	0.21
69	54	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	D	0.33
70	72	Male	Moderately	Sigmoid colon	Adenocarcinoma	C	0.48
71	47	Male	Moderately	Sigmoid colon	Adenocarcinoma	A	0.49
72	72	Female	Moderately	Cecum	Adenocarcinoma	D	0.87
73	63	Female	Moderately	Sigmoid colon	Adenocarcinoma	D	0.47
74	64	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	D	0.34
75	66	Male	Poorly	Rectosigmoid colon	Adenocarcinoma	D	0.31
76	71	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	D	0.24
77	50	Male	Mucinous	Rectosigmoid colon	Adenocarcinoma	D	0.49
78	69	Male	Moderately	Rectosigmoid colon	Adenocarcinoma	D	1.02
79	75	Female	Moderately	Rectum	Adenocarcinoma	A	0.534
80	36	Male	Poorly	Rectosigmoid colon	Adenocarcinoma	D	0.884

and other genetic changes that occur during this complicated multi-step process. However, the unmutated genes also play an essential role in cancer development [7]. Expression genetics at the RNA level is a different approach to the identification of cancer-related genes [8]. Malignant transformation of the cell is believed to arise from the accumulation of genetic changes, which usually result in the alteration of gene expression in the cancer cells. Identification and characterization of such differentially expressed genes in cancer cells versus their normal counterparts will shed light on the underlying pathogenic mechanisms and provide the opportunity to develop useful biomarkers for diagnosis and prognosis on cancers. Several methods such as subtractive hybridization [9], serial analysis of gene expression [10], expressed-sequence-tag analysis [11], and differential display [12] can be used to identify genes that are differentially expressed between normal and cancer cells. In our laboratory, we used mRNA differential display reverse transcription polymerase chain reaction (DDRT-PCR) to search for the genes that are differentially expressed in colorectal tumor and paired normal tissues, and evaluated whether they are useful as potential genetic markers for colorectal cancer [13]. Our differential screening of a colorectal tissue cDNA-tags library revealed that MGP mRNA was downregulated in colorectal tumors.

In malignant human breast and urogenital tumor tissue and cell lines, MGP mRNA was found to be overexpressed, but a downregulated expression was observed to be correlated with an unfavorable clinical status such as a poor differentiated state, a larger tumor size, and lymph node metastasis [14,15]. The regulation of MGP mRNA expression has not previously been systematically addressed in human colorectal tumors. In this report, we describe differential expression of the MGP gene in colorectal adenocarcinomas.

2. Materials and methods

2.1. Tissue collection and sample preparation

Colorectal tumor samples were collected from 80 patients after surgery at Chang Gang Memorial Hospital, Taiwan, between 1995 and 1998. Of the 80 paired tissues, 44 were from males, and 36 were from females (Table 1). In Table 1, the degree of differentiation, the age of the patient, the localization of the tumor, the clinical stage, and the histological evaluation are also described. All the tumors were adenocarcinomas, and range from poorly to well differentiated. Samples were dissected into two parts; one was for routine histopathological evaluation, and the other was frozen immediately in liquid nitrogen after surgical resection and maintained at −70°C until RNA extraction. After the addition of RNA extraction solution, 100 mg of each sample was crushed and homogenized with a pestle and mortar in a liquid nitrogen bath and transferred to a plastic tube on ice for Northern blot analysis. The paired normal tissue samples were dissected at locations 10 cm away from tumors for comparison. The tissue was examined and confirmed by an experienced pathologist to differentiate the normal cell type from the carcinoma cell type.

2.2. Preparation and labeling of cDNA probe

DNA-free RNA from colorectal carcinoma tissue was prepared by treatment with Rnase-free DnaseI (Gibco BRL, Grand Island, NY). Total RNA was isolated by TRIzol reagent-chloroform (Gibco BRL) extraction [16]. After isopropanol precipitation, DNA-free total RNA (0.1 μg) was reverse transcribed in 1× MMLV buffer which contained 2 μM T_{15} as the 3′-primer oligonucleotide, 10 mM DTT, and 250 μM each of dGTP, dATP, dTTP, and dCTP. After the solution was heated at 70°C for 10 min and cooled to 42°C, 200 units of MMLV Superscript-II (Gibco BRL) was added for 50 min and then at 70°C for 15 min. To prepare a specific MGP cDNA probe for Northern hybridization analysis, PCR of cDNA from colorectal carcinoma tissue was used to generate a 584 bp full-length MGP cDNA. A forward primer (5′-CTGAGACTGACCTGCAGGACGAAAC-3′) and a reverse primer (5′-GAAGTATCACACCACAAGTTTATTA-3′) based on the reported sequence for the human MGP gene were designed to amplify MGP cDNA [17,18]. PCR was performed by adding dNTPs, the specific primer set, and *Tag* DNA polymerase in a buffer according to the manufacturer's protocol (Promega, Madison, WI). PCR was run in a GeneAmp system 9600 (Perkin Elmer Applied Biosystems, Foster City, CA) with conditions of 94°C for 30 s, 40°C for 40 s, and 72°C for 30 s for 40 cycles. The PCR product of MGP was separated on a 3% agarose gel, visualized by ethidium bromide staining, and subeluted for subcloning into pCRII TA vector (Invitrogen, San Diego, CA). DNA sequencing was performed directly from the TA cloning vector using the Sequenase 2.0 system (Amersham Life Science, Cleveland, OH), and T7 and SP6 promoter primers were used to locate the cloned insert. The DNA sequence was analyzed by the FASTA search program of the GCG package and confirmed the DNA to be a full-length cDNA of human MGP. The probe of MGP cDNA was prepared by a random prime labeling system with a rediprime II kit (Amersham Pharmacia Biotech, Piscataway, NJ).

2.3. Northern hybridization analysis

For Northern blots, total RNA (10 μg) obtained from colorectal tissues was separated on formaldehyde-denaturing 1% agarose gels and transferred to nylon membranes (Schleicher and Schuell, Hahnestrasse, Germany). After UV cross-linking, blots were prehybridized at 40°C in 1× SSC, 1% SDS, 1× Denhardt's solution, 200 μg/ml salmon sperm DNA, and 20 mM Tris buffer (pH 7.1) for 4 h. Hybridization was continued at 40°C overnight in the presence of 1×10^6 cpm/ml of random prime ^{32}P-labeled probe. Following a wash at room temperature with 2× SSC/0.1% SDS, blots were washed at 65°C for 20 min with 1× SSC/0.1% SDS. The washed membrane was autoradiographed for 6 h. Paired carcinoma and normal colorectal tissues were evaluated by Northern hybridization analysis and compared quantitatively. For the normalization control, the blots were stripped and reprobed with a ^{32}P-labeled human actin gene probe.

2.4. Quantification

MGP RNA as well as control actin RNA bands were quantified using image optical scanning and

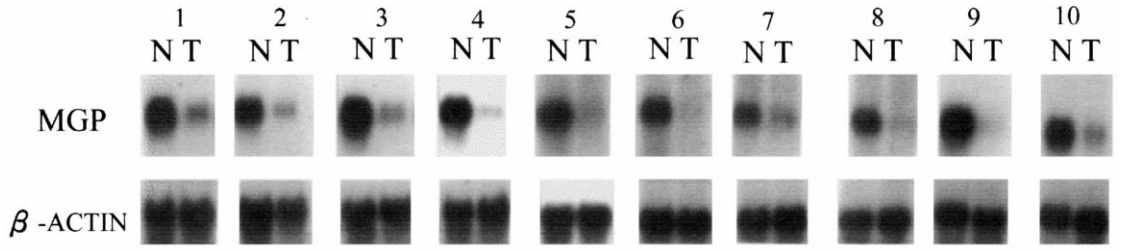

Fig. 1. Representative Northern blot analysis showing the expression of MGP mRNA in human colorectal adenocarcinomas of different histological types. Each lane contains approximate 10 μg of RNA from the human colorectal tissues. Lanes 1 and 2, Duke's A adenocarcinoma; lanes 3 and 4, Duke's B adenocarcinoma; lanes 5 and 6, adenocarcinoma with metastasis in a single or more than one regional lymph node; lanes 7 and 8, poorly differentiated adenocarcinoma; lanes 9 and 10, mucinous differentiated adenocarcinoma. As a control the same membrane strip was hybridized with β-actin. T, tumor; N, corresponding matched normal tissues.

ImageQuaNT software (Molecular Dynamics, Inc., Sunnyvale, CA). The ratio between the hybridization signals in tumor and matched normal tissue was calculated for each case and indicated as 'relative expression units' (REU). The REU between actin signals of tumor and normal tissue RNA was also calculated each time and used to correct for the amounts of RNA loaded in each lane.

2.5. Statistical analysis

The results of Northern blot analysis were analyzed by the unpaired *t*-test or χ^2-test to determine the significance of the difference between expression of MGP in paired samples of adjacent normal and tumor tissue. The relationship between the MGP expression and clinicopathological parameters was assessed by a χ^2-test (2×3 matrix) for validation of the *P* value.

3. Results

It has been reported that MGP mRNA was expressed in various rat tissues such as lung, heart, and kidney [11]. By Northern blot analysis, we also observed the expression of MGP mRNA in various soft tissues from humans. A further observation showed that MGP mRNA was expressed weakly in most colorectal adenocarcinoma cell lines (data not shown). We then proceeded to investigate MGP mRNA expression in colorectal adenocarcinomas to determine whether downregulation of this gene occurs in malignant transformation.

A total of 80 cases of paired sporadic adenocarcinoma and normal mucosa from surgical resections were evaluated (Table 1). All the RNA samples were examined as good quality by observing three clear 28S, 18S, and 5S ribosomal bands of an ethidium bromide-stained gel. Equal loading of RNA (15 μg) in each Northern blot was verified by an equivalent ratio of OD_{260}/OD_{280}, and the ratio between the MGP hybridization signals in tumor and paired normal tissue was corrected with actin hybridization signals of tumor and paired normal tissue. As shown in Fig. 1, mRNA of MGP exhibited downregulated expression in colorectal tumor tissues as compared with their paired normal tissues. The 80 pairs of RNA samples from the colorectal tissues showed REU (defined as a ratio of MGP mRNA of tumor/paired normal tissue) between 0.01 and 2.66 for the MGP probe.

Table 2 shows that the MGP mRNA was significantly reduced in 63 of 80 (79%) adenocarcinomas ($P < 0.001$), and the degree of downregulation of MGP mRNA as determined by densitometry ranged from 1.2- to 100-fold in the cancer compared with the paired normal tissue. Major differences in the amount of MGP mRNA were observed (REU from 0.01 to 0.5): 31 (50%) tumors gave an expression level decreased by two- to five-fold, 10 (16%) tumors gave an expression level decreased by five- to ten-fold, and nine (15%) tumors gave an expression level decreased by more than ten-fold relative to normal colorectal tissue RNA. In the colorectal tumor samples, MGP mRNA was expressed at highly variable levels, suggesting that the downregulation of MGP expression may be associated with clinical parameters. We then used χ^2-test analysis in colorectal adenocarcinomas. Although we found decreased MGP mRNA expression in most of the adenocarcino-

Table 2
Relationship between MGP mRNA downregulation and the standard clinical, pathological, and biological factors

	Total population (%)	Underexpressed MGP mRNA	
		No. of patients (%)	*P* value (χ^2-test)
Total	80 (100)	63 (79)	<0.01
Age (years)			
<60	30 (38)	16 (53.3)	> 0.05
>60	50 (62)	44 (88.0)	
Sex			
Male	44 (55)	34 (77.2)	>0.05
Female	36 (45)	26 (72.2)	
Differentiation[a]			
Well, Mod.	74 (92)	54 (72.9)	> 0.05
Poor., Muci.	6 (8)	6 (100)	
Duke's classification			
A, B	47 (59)	34 (72.3)	>0.05
C, D	33 (41)	26 (78.7)	
Sample location			
Colon	56 (70)	45 (80.3)	>0.05
Rectum	24 (30)	15 (62.5)	

[a] Well, well differentiated adenocarcinoma; Mod., moderately differentiated adenocarcinoma; Poor., poorly differentiated adenocarcinoma; Muci., mucinous differentiated adenocarcinoma.

mas studied, there was no statistically significant correlation between levels of MGP expression and Duke's stage, cell differentiation, tumor site, age, or patient's gender as reported in Table 2.

4. Discussion

Expression genetics is becoming an important approach in cancer research. Analysis of cancer tissue and its paired normal tissue RNAs by the arbitrarily primers is a novel approach to the detection of an alteration of gene expression linked to the tumorigenic process [2,6]. By using DDRT-PCR technology, we can identify several known and novel genes to show significant changes in expression in colorectal tumors [7]. In this study, we demonstrated a decreased expression of MGP mRNA in colorectal cancer by the same technique. MGP is reported to have the widest rat tissue distribution of any known vitamin K-dependent protein [11]. In addition, it is suggested that MGP functions as a mineralization inhibitor and is overproduced by the heart and kidney, which are susceptible to mineralization. This study is the first to report that MGP is expressed at a high level in colorectal tissue from humans. Recent reports suggest that alternation of MGP expression is associated with various pathological states. For example, MGP is expressed in proliferating vascular smooth muscle cells which are characteristic of atherosclerosis [19]. Upregulation of MGP mRNA was observed in malignant human breast cell lines and tissues [14]. An inverse correlation was found between MGP levels and urogenital tumor progression and metastasis [15]. In this study, we observed that mRNA of MGP was downregulated in colorectal cancer cells. However, the gene downregulation was not statistically correlated with histopathologic features such as tumor progression, size, and cell differentiation. At this moment, we do not have any explanation for this observation. However, at least the reduced MGP mRNA expression in malignant colorectal tumors (63 out of 80, 79%) can be used to distinguish them from the normal cells. It has been reported that although the MGP mRNA was expressed by normal and cancer cells in some cases, the MGP expression level of individual cancer cells varied even within a single cancer cell nest. The cause of such a difference is not clear [20,21]. It is also suggested that the variation of MGP expression may occur in the general population, which is due to

physiological determinants such as age, sex, exercise, diet, and disease state [22]. Those previous data may explain why it so difficult to study the association between MGP expression levels and colorectal histopathologic features.

The ongoing investigations are focused on a comprehensive analysis of MGP expression in colorectal adenomas and metastases. In particular, colorectal adenomas are being assessed to determine whether specific clinicopathologic or genotypic features are correlated with decreased MGP expression. Although the function of the MGP gene in colorectal tumorigenesis is not clear, it can begin to be assessed for possible use in clinical diagnosis. Further study is necessary to determine the putative role of this gene in colorectal carcinogenesis and whether the MGP gene is a possible target for gene therapy.

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