Colon Cancer and Protein Arginine Methyltransferase 1 Gene Expression

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Abstract. *Background: In this study, the possible relation of the expression pattern of arginine methyltransferase 1 and colon cancer progression is investigated. Materials and Methods: Colon cancer samples as well as normal colon samples were used to define the arginine methyltransferase 1 expression by RT-PCR. The results were associated with clinical and histological parameters of the tissues. Results: In colon cancer tissue, only PRMT1 variants v1 and v2 were often expressed. Statistical significance for the clinico-pathological parameters examined was found only for PRMT1 variant v2. PRMT1 v2 expression was associated with nodal status and tumour grade. PRMT1 v2 expression analysis in 25 pairs of cancerous/non-cancerous colon tissue showed higher or equal expression in cancer versus normal tissue. In 18 inflamed colon tissues examined for PRMT1 expression and compared with the expression of 90 colon cancer tissue samples, statistical significance was found only for variants v1 and v2. A higher percentage of PRMT1 v2 expression was observed in older patients. Conclusion: From the present preliminary results, it can be said that PRMT1 variant v2 can probably be regarded as a marker of unfavourable prognosis in colon cancer patients.*

Protein function is dependent on the covalent post-translational modifications of the 20 amino acid residues normally incorporated by ribosomes during protein synthesis. Some of these modifications are reversible, such as protein phosphorylation reactions, whereas others are apparently irreversible and can effectively create new types of amino acids to broaden the chemical diversity of polypeptides. In this latter group of modifications, a number of methylation reactions is included (1). Protein methylation involves transfer of a methyl group from *S*-adenosylmethionine to acceptor groups on substrate proteins. Proteins can be methylated on lysine, arginine, histidine or carboxyl residues (2).

Arginine methylation occurs on either or both of the two terminal guanidine nitrogen atoms, resulting in three possible products: monomethylarginine; N^G,N^G-dimethylarginine, in which both methyl groups are on the same nitrogen (asymmetric dimethylarginine); and N^G,N'^G-dimethylarginine, in which each nitrogen atom receives one methyl group (symmetric dimethylarginine) (2, 3). There are at least three distinct classes of protein arginine N-methyltransferases (PRMTs). The type I enzymes catalyse the formation of N^G-monomethylarginine and asymmetric N^G,N^G-dimethylarginine residues. The type II enzymes catalyse the formation of N^G-monomethylarginine and symmetric N^G,N'^G-dimethylarginine residues. The type III enzymes catalyse the monomethylation of the internal guanidine nitrogen atom to form ω-N^G-monomethylarginine (4). The specific importance of asymmetric or symmetric protein arginine methylation in terms of cellular function remains to be elucidated.

The PRMTs comprise a family of nine protein members so far elucidated (5-13). These enzymes interact with a variety of substrates including RNA-binding proteins (14), transcriptional factors (3) and cytokines (15). This variety of substrates reflects the interference of PRMTs in many diverse cellular processes, such as regulation of transcription (8) or signal transduction (16). Protein arginine *N*-methyltransferase 1 was the first type I PRMT in mammalian cells to be cloned and characterized (5). PRMT1 is the predominant type I enzyme in tissues and contributes most of the type I protein arginine methyltransferase

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activity in mammalian cells (15). The *PRMT1* gene is located on chromosome 19q13.3. The gene spans 11,163 bp of the genomic sequence, close to *RRAS* and *IRF3* genes (RRAS is the most telomeric) (17). The gene is comprised of 12, 11 or 10 exons (three PRMT1 isoforms in normal tissues) (6, 17).

PRMT1 prefers to methylate arginine residues in glycine-rich regions, which are found in many proteins that bind RNA and are involved in various aspects of RNA metabolism, such as hnRNPs (18), histone H4 (6), STAT protein (19) and Sam68 (20). Additionally, there is strong evidence that PRMT1 interacts with ILF3 (15) and IFNAR1 (16). Immunocytochemical localisation studies on RAT1 cells suggested that PRMT1 is predominantly nuclear (7), while in studies focusing on Ewing's sarcoma protein (EWS), there has been evidence of the presence of PRMT1 in the cytoplasm (21). According to experiments by Herrmann *et al*., PRMT1 is a highly dynamic enzyme with variable subcellular localisation and mobility (22).

Methylation events have been implicated in disease emergence and progression, including cancer (23-25). The action of PRMT1 seems to correlate with a variety of diseases that mainly concern the cell cycle and the appearance of a variety of malignancies. Additionally, the inhibition of methylation of proteins, such as STAT1 and $hnRNPA_2$, is considered responsible for the lack of interferon response observed in many malignancies (19) and also relates to the cellular localisation of these proteins and the appearance of malignancy (18). Finally, it has been reported that PRMT1 activity seems to be inversely correlated with cell growth and oncogenesis (5).

Colorectal adenocarcinoma is one of the most common malignancies and, if not diagnosed and treated early, the tumour spreads to the entire bowel wall, extends to adjacent organs, and eventually metastasizes to regional lymph nodes and distant sites. The majority of deaths from colorectal cancer occur in patients with late-stage tumours, which are usually incurable. Well-defined molecular alterations have been associated with cancer progression. In accordance with those findings and in an effort to find new markers for colon cancer, the expression of the *PRMT1* gene in colon cancer progression was examined.

Materials and Methods

Study group. The study group consisted of 25 pairs of colorectal carcinomas and their distal normal colonic mucosa in proximal surgical margin, 65 samples of colon cancer and 18 samples of inflammed colon tissues, collected at the St. Savvas Oncologic Hospital of Athens. Informed consent was obtained from all patients for the scientific analysis of tumour tissues. Patients' mean age±SE was 67.17±1.25 years with a range of 31-92 years (Table I). Clinical and pathological information documented at the time of surgery included stage and grade of the disease, histological type, size and nodal status of the tumours.

Table I. *Distribution of variants in 90 colon cancer patients*.

Numerical variable	No. of patients	Mean±SE	Median	Range
Age (years)	90	68.1±1.3	71.0	31-72
Tumor size (cm)	88	4.55±0.19	4.50	2.00-11.50
Dichotomous variable		**No. of patients**		**(%)**
PRMT1-v1				
Positive		88		97.8
Negative		2		2.2
PRMT1-v2				
Positive		43		47.8
Negative		47		52.2
PRMT1-v3				
Positive		3		3.3
Negative		87		96.7

RNA extraction and RT-PCR. Colon tissues (cancer/normal) were collected on surgery and kept in liquid nitrogen. The tissue samples were pulverized using a mirko dismembrator U (Sartorius, Germany) and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. The purity and concentration of the RNA were determined using spectrophotometry. Total RNA was reverse transcribed by RT-PCR using the Thermoscript®RT (Invitrogen, Carlsbad, USA). The integrity of the produced cDNA was examined by amplification of *β-actin* gene (housekeeping gene).

In order to optimize the PCR conditions, different quantities of cDNA from the HT-29 cell line were amplified under exponential, non-saturating conditions, for 30, 35, 37, 40 and 42 cycles to determine that amplification was in the linear range and the appropriate cycle number for PCR. The PCR conditions using different amounts of cDNA (0.4-2 μL) and different cycle numbers were examined and 0.8 μL cDNA and 40 cycles for *PRMT1*, and 0.6 μL and 35 cycles for *β-actin* were chosen as the optimum conditions.

PCR for *β-actin* was performed in a 20 μL reaction mixture containing 0.6 μL of cDNA, 2U of Platinum *Taq* DNA Polymerase (Invitrogen), 2 μL of 10x PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 0.8 μL of 50 mM $MgCl_2$, 0.4 μL of 10 mM dNTPs mix (Invitrogen) and 0.4 μL of each gene specific primer (0.1 μg/μL) (forward: 5'-ATCTCG CACCACACCTTCTA-3', reverse: 5'-CGTCATACTCCTGCTT GCTG-3'). The amplification protocol consisted of an initial incubation at 95˚C for 15 min, followed by 35 cycles of 95˚C for 30 s (denaturing step), 62˚C for 1 min (annealing step), 72˚C for 1 min (extension step) and a final extension step of 72˚C for 10 min. PCRs were performed on a PTC-200 thermal cycler (MJ Research, Inc, Waltham, Massachusetts, USA).

PCR for *PRMT1* was performed in a 20 μL reaction mixture containing 0.8 μL of cDNA, 2U of Platinum *Taq* DNA Polymerase (Invitrogen), 2 μL of 10x PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 0.8 μL of 50 mM $MgCl_2$, 0.4 μL of 10 mM dNTPs mix (Invitrogen) and 0.4 μL of each gene-specific primer (0.1 μg/μL) (forward: 5'-GAGGCCGCGAAC TGCATCAT-3', reverse: 5'-TGGCTTTGACGATCTTCACC-3').

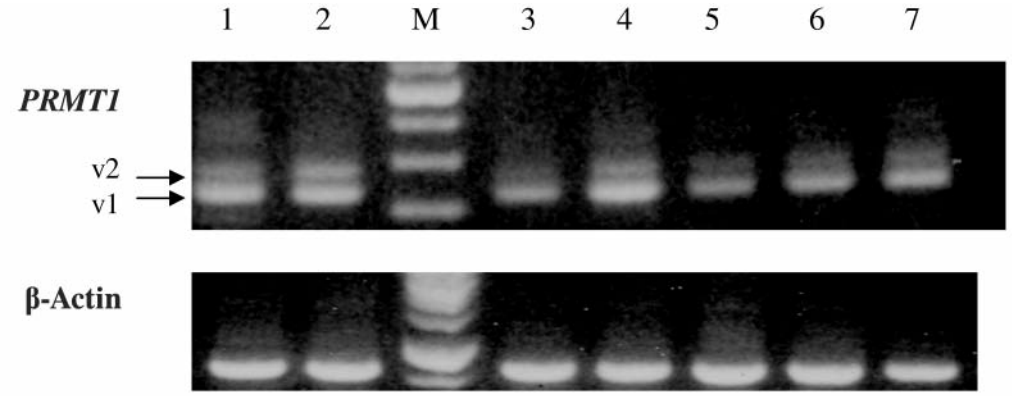

Figure 1. *Expression of PRMT1 gene splice variants in primary colon tumours (2, 4, 6) and normal colon tissues (1, 3, 5, 7). M: 100bp DNA ladder (molecular weight marker).*

The amplification protocol consisted of an initial incubation at 95˚C for 15 min, followed by 40 cycles of 95˚C for 30 s (denaturing step), 64.5˚C for 1 min (annealing step), 72˚C for 1 min (extension step) and a final extension step of 72˚C for 10 min. The identity of the 3 splice variants was verified by sequencing, with an automated DNA sequencer. Equal amounts of PCR products for *β-actin* and *PRMT1* were electrophoresed on a 1.5% agarose gel and visualization was based on ethidium bromide staining (Figure 1). Expression analysis was performed twice for each sample.

Statistical analysis. The expression of *PRMT1* splice variants was classified as positive or negative and was compared with the clinical and histopathological features of the patients. The associations between these variables and *PRMT1* status of each variant were analysed using several statistical models (Chi-square test (χ^2), Fisher's exact test, McNemar test, Mann-Whitney test) where appropriate.

Results

PRMT1 variant status and relation to clinical and histological variables. The *PRMT1* gene has three splice variants (6, 17), produced as a result of mRNA alternative splicing, named as v1, v2 and v3 (Figure 1). Additionally, it is a very low expression protein, so in order to determine the optimum PCR conditions, a variety of number of PCR cycles and template quantities were used. The ideal combination is that described in Materials and Methods.

During the study, it was revealed that *PRMT1* variants v1 and v2 were often expressed, whereas variant v3 only rarely (Table I). Additionally, *PRMT1* variant v3 did not seem to be expressed in any of the 25 normal colon tissues or the 18 inflamed colon tissues examined. *PRMT1* variant v2 was the only one out of the three variants produced that was associated in a statistically significant manner with the clinical and histological parameters examined. *PRMT1* v2 expression was associated with nodal status and tumour grade (p=0.018 and p=0.008, respectively). On the other hand, it did not appear to be associated with Dukes' stage (p=0.15) (Table II).

Table II. *Associations between PRMT1 v2 status and other clinicopathological variables.*

Variable	No. of patients (%)			
	Total	PRMT1 v2 -negative	PRMT1 v2 -positive	*P*-value
Nodal status				
Negative	27	18 (66.7)	9 (33.3)	**0.018**[a]
Positive	81	24 (39.3)	37 (60.7)	
x	2			
Dukes' stage				
A/B	34	21 (61.8)	13 (38.2)	
C	28	11 (39.3)	17 (60.7)	0.15[b]
D	26	11 (42.3)	15 (57.7)	
x	2			
Grade				
I	14	12 (85.7)	2 (14.3)	
II	31	14 (45.2)	17 (54.8)	**0.008**[b]
III	38	16 (42.1)	22 (57.9)	
IV	7	1 (14.3)	6 (85.7)	

[a]Fisher's exact test; [b]χ^2 test; x, status unknown.

Expression analysis of *PRMT1* variant v2 in 25 pairs of cancerous/non-cancerous colon tissues showed higher or equal expression in cancer *versus* normal tissue (p=0.008) (Table III). Additionally, the percentage of patients expressing *PRMT1* v2 was substantially higher in older patients compared to younger ones (p=0.004) (Figure 2).

Finally, after examining the expression of *PRMT1* variants in 18 inflamed colon tissues, the expression pattern was compared with that of the 90 colon cancer tissues; only the results concerning variants v1 and v2 were significant in a statistical manner (p=0.001 and p<0.001, respectively) (Table IV).

Table III. *PRMT1 v2 expression in pairs of cancerous and non-cancerous colon tissues.*

PRMT1 v2 expression	Number of patients (%)	*P*-value*
Higher in cancer *vs*. normal	8 (32.0)	
Lower in cancer *vs*. normal	0 (0.0)	**0.008**
Approx. equal in both tissues	17 (68.0)	

*Calculated by the McNemar Test.

Table IV. *Associations between PRMT1 variant expression and colon tissues examined.*

Type of tissue	No. of patients (%)			
	Total	PRMT1-v1 -negative	PRMT1-v1 -positive	*P*-value
Non-cancerous (inflammation)	18	5 (27.8)	13 (72.2)	0.001[a]
Cancerous	90	2 (2.2)	88 (97.8)	
	No. of patients (%)			
	Total	PRMT1-v2 -negative	PRMT1-v2 -positive	*P*-value
Non-cancerous (inflammation)	18	18 (100.0)	0 (0.0)	<0.001[a]
Cancerous	90	88 (97.8)	2 (2.2)	
	No. of patients (%)			
	Total	PRMT1-v3 -negative	PRMT1-v3 -positive	*P*-value
Non-cancerous (inflammation)	18	18 (100.0)	0 (0.0)	
Cancerous	90	88 (97.8)	2 (2.2)	0.69[a]

[a]Fisher's exact test.

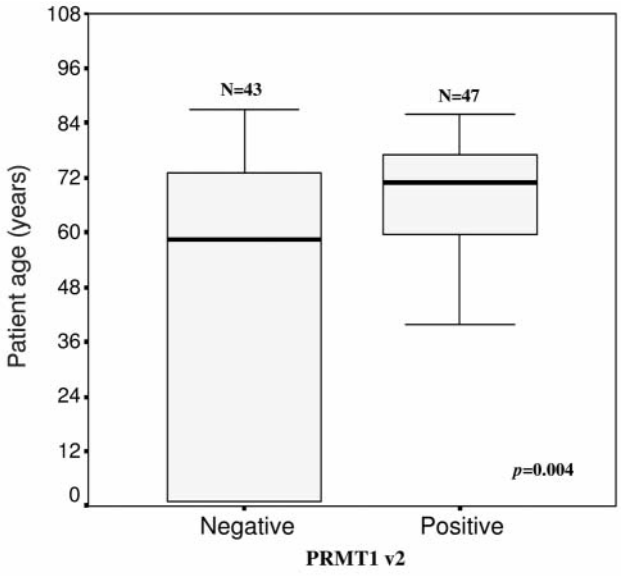

Figure 2. *Association of PRMT1 v2 expression with patient age. P-value was calculated by the Mann-Whitney test.*

Discussion

Arginine methyltransferases (PRMTs) are a large protein group responsible for one of the major post-translational modifications found in proteins. Their ability to transfer methyl groups to certain arginine residues in proteins gives them the advantage to contribute and control a number of cellular processes such as signal transduction, RNA metabolism, chromatin structure and protein-protein interactions. The member of the family most commonly found in human cells is PRMT1.

It is widely known in the scientific community that modifications in the expression pattern of certain genes are strongly correlated with cancer incidence. The majority of these genes are involved in developmental processes and regulate the cell cycle such as *MLH1* and *TP53* in colon. Though PRMT1 is known to methylate proteins such as histone H4 (26) and hnPNPs (18), its physiological role in the cell remains to be clarified. The central role that PRMT1 plays as a regulator of protein function is revealed by the disruption of this enzyme in mice, where *PRMT1*-knockout mice die shortly after implantation (27). Several hypotheses have been made for the importance of this enzyme including a main role in cancer progression.

Since only a few of the discovered biomarkers are well established and applied for routine use, the need for the discovery of new ones is quite urgent. These biomarkers must be able to predict in a credible manner the risk of recurrence in cancer patients. This study is an attempt to investigate the possible role of *PRMT1* as a new biomarker for colon cancer.

PRMT1 gene gives rise to three splice variants identified during this study. The statistical models used for the analysis of the experimental results revealed an association between *PRMT1* v2 splice variant and clinicopathological features of the tumours such as nodal status, grade of the tumour and patient age. The presence of splice variants is not unusual for eukaryotic genes. Approximately, 10-30% of alternatively spliced human genes have tissue-specific variants (28), while 316 genes have been shown to have cancer-specific variants (29). Moreover, there are many genes that present dramatic changes in alternative splicing patterns and when this happens it is associated with neoplasia and metastasis (30-31). It is very possible that alternative splicing regulation

plays a determinative role in the progression of some neoplasia and malignancies, an opinion that is in accordance with the presented findings.

Altered splicing patterns can serve as markers of the altered cellular state associated with disease even when they are not involved in the primary pathway of the disease mechanism. The participation of certain genes in secondary metabolic pathways does not deprive them of the potential to provide diagnostic and prognostic information. The results obtained from the present study suggest that *PRMT1* gene variant v2 expression may be used as a marker of unfavorable prognosis for colon cancer patients. Of course this is just a first attempt to elucidate the role of this enzyme in cancer. *PRMT1* combined with other markers could prove useful for physicians, but more extensive study including a larger study group is necessary.

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