

ORIGINAL ARTICLE

Protein tyrosine phosphatase receptor-like genes are frequently hypermethylated in sporadic colorectal cancer

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The activity of phosphatases could be influenced by genetic, as well as epigenetic alterations. In our study, we have investigated the methylation status of four PTPRs: *PTPRM*, *PTPRT*, *PTPRR* and *PTPRZ1*, which were pre-selected using microarray techniques as being alternatively methylated in sporadic colorectal cancer (CRC). The analyses were carried out on 131 surgical specimens obtained from sporadic CRC patients. The methylation status of the four genes was examined using methyl specific PCR (MSP). The analysis of promoter methylation using an Illumina 27K microarray revealed four protein tyrosine phosphatases *PTPRM*, *PTPRT*, *PTPRR* and *PTPRZ1* as being hypermethylated with β -value ≥ 0.2 and $P \leq 0.05$. Subsequent analysis using MSP confirmed these observations—the frequency of promoter methylation was significantly higher in tumor cells compared with matched normal tissue for each of the analyzed genes. There was no association observed between the methylation status of PTPRs and either *CIMP*, *K-ras* (codon 12) and *BRAF* (exon 15, V600E) mutations or tumor localization (proximal/distal). The results of our study show a statistically significant difference between promoter methylation in cancerous and healthy tissue. This result supports the hypothesis that the PTPR family has an important role in the etiology of CRC.

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INTRODUCTION

Via dephosphorylation of multiple enzymes, protein tyrosine phosphatases (PTPs) regulate many processes, such as cell differentiation and proliferation, apoptosis, gene transcription, mRNA processing, molecule transport out of and into cells, as well as cell signaling.^{1,2} The PTP Cys-based superfamily is composed of three classes: Class I (classical PTPs) consisting of transmembrane receptor PTPs, non-receptor PTPs, as well as dual-specific PTPs; Classes II and III consisting of Cdc25 family members and low molecular weight phosphatases, respectively.¹

PTPs have an important role in carcinogenesis acting as tumor suppressor genes.^{1–3}

Loss of the tumor suppressor function of PTPs, similar to that of other tumor suppressors, is a consequence of gene inactivation resulting from a biallelic inactivation event, such as gene deletion, mutation or promoter hypermethylation.⁴ Methylation of CpG islands located in a gene promoter is associated with the downregulation of gene transcription through the alteration of chromatin conformation influencing DNA availability to transcription factors.^{5–7}

Until now, several phosphatases have been examined in human cancers, enabling the assessment of their contribution to the development of cancer. Somatic mutations have been found in the following six phosphatases: *PTPRE*, *PTPRG*, *PTPRT*, *PTPN3*, *PTPN13* and *PTPN14* in colorectal cancer (CRC). Epigenetic silencing was observed for *PTPRR* and *PTPRG* in CRC and cutaneous T-cell lymphoma, for *PTPRD* in glioblastoma, head and neck squamous cell carcinoma and lung cancer, as well as for *PTPRO* in hepatocellular carcinoma. Moreover, loss of heterozygosity was identified for *PTPRJ* in CRC.^{5,8–12}

The purpose of our study was to analyze the methylation status of CRC tissues. Using a genome-wide approach (Illumina Infinium HumanMethylation27), we found 422 differentially methylated probes including four members of the tyrosine phosphatase family (PTPRs): *PTPRT*, *PTPRR*, *PTPRM* and *PTPRZ1*.

These four, but no other PTPs, were selected as they were found to be alternatively methylated by a microarray experiment. The results were then validated using methyl specific PCR (MSP). The tumor suppressor function of PTPs allowed us to hypothesize that epigenetic silencing is a possible mechanism adding to the loss of functioning of these genes in colon cancer cells.

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MATERIALS AND METHODS

Patient samples

The study was performed on 131 sporadic CRC samples compared with matched healthy colon tissues. The samples were obtained immediately after surgery from the First Department of Surgical Oncology, Lower Silesian Oncology Centre in Wrocław. The study group consisted of 59 females and 72 males with mean age 64.5 ± 10.54 diagnosed with primary sporadic CRC. None of them were treated with preoperative therapy. The molecular characteristics of the cancer samples are as follows: the *BRAF* V600E mutation (exon 15) was observed in 8, while the *K-ras* (codon 12) mutation was found in 31 colon cancer samples. No *K-ras* mutation in codon 13 was revealed in our material. CIMP-high and CIMP-low/0 status were observed for 7 and 124 tumors, respectively.

The study was accepted by Wrocław Medical University Ethical Committee.

Methods

The samples were obtained during surgery and were frozen at -20°C , until DNA isolation. All tissues were examined histopathologically. DNA was isolated from tumor samples with at least 40% viable neoplastic cells using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the recommended protocol. Methylation status was evaluated for 12 pairs of CRC and matched normal colon tissue using Illumina Infinium HumanMethylation27 (Illumina, San Diego, CA, USA). The Benjamini–Hochberg procedure for controlling the false discovery rate was used to correct *P*-values for multiple testing. All probes with β -values ≥ 0.20 and $P \leq 0.05$ were retained (see Supplementary Table 1 for the detailed results). Further analysis on an additional 131 samples considered four PTP receptor-like genes: *PTPRM* (probeID: cg01946574), *PTPRT* (probeID: cg13168820), *PTPRR* (probeID: cg23694248) and *PTPRZ1* (probeID: cg25167643), as they showed differential methylation characterized by β -values ≥ 0.20 and $P \leq 0.05$. The methylation status of these genes was confirmed using MSP. Primers for methylated and unmethylated promoter sequences were designed using MethPrimer software¹³ (Table 1).

DNA modification was performed using the EpiTect Bisulfite Kit (Qiagen) according to the standard procedure. One microgram of DNA isolated from both CRC and healthy colon tissue was modified for each patient.

CpG Methylated HELa Genomic DNA (New England Biolabs, Pickering, ON, Canada) and DNA from the peripheral blood of a healthy person were used as control DNA for methylated and unmethylated sequences, respectively.

The PCR mixture was prepared separately for methylated and unmethylated sequences for all the genes examined. Briefly: 0.6 μl modified DNA was mixed with 0.75 μl of each primer (10 mM) 0.75 μl dNTPs (40 mM, Fermentas, Burlington, ON, Canada), 1.5 μl buffer ($10 \times$, Qiagen), 0.15 μl HotStart DNA Polymerase ($5 \text{ u} \mu\text{l}^{-1}$ Qiagen) in a total volume of 15 μl .

The cycle parameters were as follows: 95°C 15 min, 95°C 30 s, 56°C 30 s (methylated and unmethylated *PTPRM* and *PTPRZ1*), or 60°C (methylated and unmethylated *PTPRT*, unmethylated *PTPRR*) or 63°C (methylated *PTPRR*) for 35–38 cycles, 72°C 30 s and a 72°C 5 min final extension in an MJ thermocycler. The products were evaluated using 2.5% agarose gel.

Statistical analysis

The association between the methylation of *PTPRM*, *PTPRZ1*, *PTPRT* and *PTPRR* and the type of colon cell (cancerous or normal) was analyzed using Fisher's exact test of association. This test was also used to analyze the association of the methylation status of these four *PTPRs* with various clinical factors (for example, location of cancer, mutations and CIMP status).

RESULTS

In this study, we analyzed the methylation status of four *PTPs* in 131 sporadic colon cancers (30 proximal/101 distal) in comparison to matched healthy colon tissues (95 pairs tumor/healthy tissue and 36 cancer tissues without paired healthy tissue).

Microarray results

Using genome-wide microarray analysis, we found over 400 differentially methylated genes in tumor tissues, among which four *PTP* genes were found as follows: *PTPRM*, *PTPRT*, *PTPRR* and *PTPRZ1* (see Table 2 and Supplementary Table 1).

MSP results

Partial methylation (the presence of both methylated and unmethylated sequences), lack of methylation (only unmethylated sequences), as well as complete methylation (only methylated sequences) were observed for the examined phosphatases in cancer tissues (Figure 1). A few samples amplified neither of the two sets of primers. The results are presented in Table 3.

Table 1 Primer sequences for the analyzed genes

Gene, localization and no. of nucleotides from ATG for forward primer with reference sequence	%GC	'C's	Primer sequences FM, RM –forward/reverse, methylated FU, RU – forward/ reverse, unmethylated	Product size
<i>PTPRR</i> 12q21.2–440 (NM_002849.3)	64.00	9	FM 5'TGGAGTTTATTTGTTTTAAGTCGT	197
	64.00	6	RM 5'GACAAATACCTAACCTTCTAAACGC	
	64.00	9	FU 5'TGGAGTTTATTTGTTTTAAGTTGT	199
	66.67	7	RU 5'ACAACAATACCTAACCTTCTAAACAC	
<i>PTPRM</i> 18p11.2–1230 (NM_002845.3)	75.00	6	FM 5'TCGGGTTTATACGGGATATC	220
	65.22	6	RM 5'GAAAACTACGAACTTCTCTACG	
	73.91	7	FU 5'GTTTTGGGTTTATATGGGATATT	225
	68.00	6	RU 5'CCAAAAACTACAAACTTCTCTACA	
<i>PTPRT</i> 20q12–q13.1–723 (NM_133170.3)	75.00	9	FM 5'TGTTTCGAGATTTTGGTTAGTAGTC	231
	73.91	8	RM 5'ACATCGTCTACCCTATAAAAACG	
	76.00	9	FU 5'GTGTTTTCGAGATTTTGGTTAGTAGTT	233
	72.00	8	RU 5'AACATCATCTACCCTATAAAAACAC	
<i>PTPRZ1</i> 7q31.1–454 (NM_001206839.1)	75.00	5	FM 5'AGGTACGTTTTAGCGGAGC	225
	50.00	5	RM 5'ATAATCTAAAAAAACCCGACGAT	
	78.26	5	FU 5'GAGGTATGTTTTAGTGGAGTGG	228
	50.00	6	RU 5'AAATAATCTAAAAAAACCCACACAAT	

In healthy colon tissues, no complete methylation occurred, but cases of partial methylation and lack of methylation were common in the following genes: *PTPRM*, *PTPRT* and *PTPRZ1* with the exception of *PTPRR*, for which complete methylation was found in 2 normal samples (Table 3).

Our results revealed a significantly higher level of methylation in tumors compared with normal colon tissue (see Table 4).

There was no significant association of the methylation status of *PTPRM*, *PTPRT*, *PTPRR* or *PTPRZ1* with *CIMP*, the *K-ras* (codon 12) and *BRAF* (exon 15, V600E) mutations, or tumor localization (proximal/distal) (data not shown).

A clear association between the methylation of *PTPRZ1* and *PTPRM* in healthy cells ($P < 0.001$) was also found (Table 5).

DISCUSSION

An abnormal methylation pattern is a widely described biological phenomenon in cancer cells. Global hypomethylation, promoting genomic instability and regional hypermethylation of CpG islands at

gene promoters associated with transcriptional silencing of the tumor suppressor gene, has been reported in many types of cancers.⁶ In particular, the hypermethylation of tumor suppressor gene promoters leading to loss of their function is an important event in the molecular pathogenesis of cancer.¹⁴

Our results revealed that the promoter sequences of four PTPs: *PTPRM*, *PTPRT*, *PTPRR* and *PTPRZ1*, are hypermethylated in colon cancer compared with healthy colon tissue. These data were obtained using a methylation microarray and then confirmed by methylation-specific PCR. Methylated and unmethylated sequences were both often observed in healthy colon tissues in our study, although the frequency of methylated sequences was significantly lower than in cancer tissues (for *PTPRM*, *PTPRT*, *PTPRZ1* $P = 0$ and for *PTPRR* $P = 0.02835$). However, using MSP only the presence of a methylated allele in the tissue examined could be shown without the possibility of distinguishing between uni- and biallelic methylation.

These observations should be analyzed in the context of the possibility of PTPs having a role in the development of cancer. As published recently, the involvement of PTPs in cancerogenesis has been observed for a variety of tumors. *PTPRT* (*PTPR-ρ*) is a known tumor suppressor, mutations of which are associated with melanoma, colon, stomach and lung cancer.³ As reported previously, *PTPRT* mutations may cause loss of its function (nonsense or frameshift mutations), a decrease in phosphatase activity or defective cell adhesion (missense mutations in phosphatase and extracellular domains, respectively).^{3,8} Moreover, an increase in *PTPRT* activity in a tumor cell culture inhibited cell growth.⁸

PTPRZ1 (*PTPR-ζ* or *PTPR-β*) is a receptor of VacA, a cytotoxin of *Helicobacter pylori*, inducing gastric ulcers.¹⁵ *PTPRZ1* has also been

Table 2 Results obtained from an Illumina27K microarray for selected PTPs

Gene	OMIM and localization	P-value	Diff. Beta
<i>PTPRM</i>	176888/18p11.2	0.00271947	0.28216614
<i>PTPRT</i>	608712/20q12-q13.1	0.00093595	0.29058374
<i>PTPRR</i>	602853/12q21.2	0.04606745	0.21622813
<i>PTPRZ1</i>	176891/7q31.1	0.0485365	0.26768926

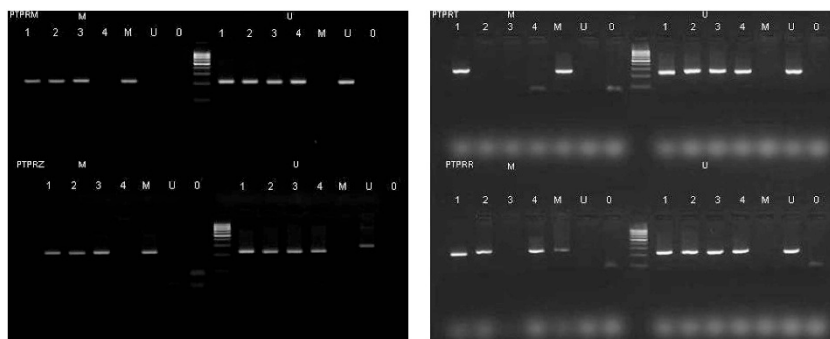


Figure 1 Methylation-specific polymerase chain reaction electrophoresis for cancerous and normal tissues (M—primers for methylated sequence, U—primers for unmethylated sequence) for four analyzed genes: *PTPRM* (220 and 225 bp), *PTPRZ1* (225 and 228 bp), *PTPRT* (231 and 233 bp) and *PTPRR* (197 and 199 bp). Lanes 1 and 2—cancer and matched normal DNA, lanes 3 and 4—cancer and matched normal DNA, M—positive control for methylation, U—positive control for lack of methylation, O—negative control. Size standard—O'GeneRuler 100 bp DNA Ladder (Thermo Scientific Fermentas, Burlington, ON, Canada).

Table 3 Methylation status in the analyzed tissues

Gene	Cancer tissue 131				Healthy tissue 95			
	Partial methylation	Complete methylation	Lack of methylation	Lack of both sequences	Partial methylation	Complete methylation	Lack of methylation	Lack of both sequences
<i>PTPRM</i>	122 (93.1%)	1 (0.8%)	7 (5.3%)	1 (0.8%)	54 (56.8%)	0	41 (43.2%)	0
<i>PTPRT</i>	104 (79.4%)	4 (3.1%)	21 (16.0%)	2 (1.5%)	0	0	95 (100%)	0
<i>PTPRR</i>	101 (77.1%)	5 (3.8%)	22 (16.8%)	3 (2.3%)	62 (65.3%)	2 (2.1%)	31 (32.6%)	0
<i>PTPRZ1</i>	123 (93.9%)	7 (5.3%)	0	1 (0.8%)	49 (51.6%)	0	46 (48.4%)	0

Table 4 Number of samples with methylation in the promoter sequence for the PTPs examined

Gene	Cancer tissue 131	Healthy tissue 95	P-value
<i>PTPRM</i>	123 (93.9%)	54 (56.8%)	0
<i>PTPRT</i>	108 (82.4%)	0 (100%)	0
<i>PTPRR</i>	106 (80.9%)	64 (67.4%)	0.02835
<i>PTPRZ1</i>	130 (99.23%)	49 (51.6%)	0

Abbreviation: PTP, protein tyrosine phosphatase.

Table 5 Association between methylation of *PTPRZ1* and *PTPRM* in healthy cells $P < 0.001$

	Methylation not observed at <i>PTPRM</i>	Methylation observed at <i>PTPRM</i>
Methylation not observed at <i>PTPRZ1</i>	29	17
Methylation observed at <i>PTPRZ1</i>	12	39

reported to have an important role in the functioning of neurons.¹⁵ There are no reports on its role as a tumor suppressor or its significance for cell homeostasis.

PTPRM (*PTPR-μ*) is a member of the Ig superfamily of adhesion molecules and has been shown to be expressed in neurons, glia, epithelia and prostate.^{16–18} *PTPRM* has a crucial role in epidermal growth factor receptor signaling—reduced expression of *PTPRM* increases the epidermal growth factor receptor specific phosphorylation of tyrosine residue, thus causing phospholipase C-gamma ($PLC\gamma_1$) activation and cell migration.¹⁸ It is also known that epidermal growth factor receptor activation *via* phosphorylation can itself increase cell proliferation, differentiation and survival.¹⁸ The increased expression of *PTPRM* in normal cell and low-grade astrocytomas compared with high-grade glioblastomas, along with the results of *in vivo* experiments, also confirmed that loss of this protein promotes tumor cell migration and propagation.¹⁷ The lack of expression of *PTPRM* in prostate cancer cells connected with the lack of *PTPRM*-mediated adhesion also suggests a role of *PTPRM* as a tumor suppressor gene.¹⁶

PTPRR was firstly described by Menigatti *et al.*,⁹ to be highly downregulated in the early stages of colorectal tumorigenesis. This gene encoding a classical PTP receptor has a crucial role in homeostasis regulating signaling in proliferation and differentiation.

PTPRT was the only gene for which there was no methylated sequence observed in the control samples. The importance of *PTPRT* in colon cancer has been postulated by Wang *et al.*⁸ Our results support this hypothesis, showing that the most probable role of *PTPRT* is the suppression of carcinogenesis. However, further studies are needed to confirm this hypothesis.

We also found a clear association between the methylation of *PTPRZ1* and *PTPRM* in healthy cells ($P < 0.001$). Up to now, no data suggesting any biological interaction between these genes have been published. *PTPRM* is known to have a crucial role in the epidermal growth factor receptor signaling pathway and thus in cell migration, while the role of *PTPRZ1* is still poorly described. Up to now, the expression of *PTPRZ1* has only been shown in neurons. Our results are the first revealing a potential role of this gene in colon cells.

Recent epigenomic microarray analyses in CRC tissues have identified many gene promoters, which were hypermethylated. Thus,

it has been postulated that these genes may be involved in colorectal tumorigenesis. In the studies of Kim *et al.*¹⁹ and Hinoue *et al.*,²⁰ the Illumina platform has been used to analyze 22 and 125 tumor samples, respectively. Although these authors have not mentioned *PTPs* in the ‘top ranking lists’ of genes with aberrant methylation, the available raw data show differences in PTP methylation compared with healthy tissue.^{19,20}

Our study, which seems to be the first analysis using MSP of the methylation status of *PTPRs* in sporadic colon cancer, has enabled us to conclude that the methylation of *PTPRs* is significantly different in cancerous and healthy tissue. This suggests a potential role of these genes as suppressors in colorectal carcinogenesis. Moreover, our results revealing alterations in *PTPRZ1* methylation in normal colon samples led us to hypothesize that this gene has a still unknown role in the colon.

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