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

Running title: PTPRs are hypermethylated in sporadic CRC

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Abstract

Introduction: 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.

Materials and methods: The analyses were carried out on 131 surgical specimens obtained from sporadic colorectal cancer patients. The methylation status of the four genes was examined using MSP.

Results: The analysis of promoter methylation using an Illumina 27K microarray revealed four protein tyrosine phosphatases *PTPRM*, *PTPRT*, *PTPRR* and *PTPRZ1* as being hypermethylated with beta-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 to 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 plays an important role in the etiology of colorectal cancer.

Key words: colorectal cancer, methylation, protein tyrosine phosphatase receptor-like, *PTPRM*, *PTPRT*, *PTPRR*, *PTPRZ1*

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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 play an important role in carcinogenesis acting as tumor suppressor genes (1,2,3).

Loss of the tumor suppressor function of PTPs, similarly 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 (5,6,7).

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: *PTPRF*, *PTPRG*, *PTPRT*, *PTPN3*, *PTPN13* and *PTPN14* in colorectal cancer. Epigenetic silencing was observed for *PTPRR* and *PTPRG* in colorectal cancer 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 colorectal cancer (5,8,9,10,11,12).

The purpose of our study was to analyze the methylation status of colorectal cancer cells. 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 protein tyrosine phosphatases, were selected as they were found to be alternatively methylated by a microarray experiment .The results were then validated employing 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.

Materials and methods

Patient samples

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The study was performed on 131 sporadic colorectal cancer samples compared to matched healthy colon tissues. The samples were obtained immediately after surgery from the First Department of Surgical Oncology, Lower Silesian Oncology Centre in Wroclaw. The study group consisted of 59 females and 72 males with mean age 64.5±10.54 diagnosed with primary sporadic colorectal cancer. 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 Wroclaw Medical University Ethical Committee.

Methods

The samples were obtained during surgery and were frozen at -20^{o}C, until DNA isolation. All tissues were examined histopathologically. DNA was isolated from ~~cancer tissue~~ tumor samples with at least 40% viable neoplastic cells using a QIAamp DNA mini kit (Qiagen) following the recommended protocol. ~~All tissues were examined histopathologically.~~ Methylation status was evaluated for 12 pairs of CRC and matched normal colon tissue using Illumina Infinium HumanMethylation27. The Benjamini-Hochberg procedure for controlling ~~to~~ 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 for the detailed results). Further analysis on an additional 131 samples considered four protein tyrosine phosphatase 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 (Methyl Specific PCR). Primers for methylated and unmethylated promoter sequences were designed using MethPrimer software (13) (Table 1).

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

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CpG Methylated HELa Genomic DNA (New England Biolabs) 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: 0.6µl modified DNA was mixed with 0.75µl of each primer (10mM) 0.75µl dNTPs (40mM, Fermentas), 1.5µl buffer (10x, Qiagen), 0.15µl HotStart DNA Polymerase (5µ/µl Qiagen) in a total volume of 15 µl.

The cycle parameters were as follows: 95ºC 15min, 95ºC 30sec, 56ºC 30 sec (methylated and unmethylated *PTPRM* and *PTPRZ1*) or 60ºC (methylated and unmethylated *PTPRT*, unmethylated *PTPRR*) or 63ºC (methylated *PTPRR*) for 35-38 cycles, 72ºC 30 sec and a 72ºC 5 min of 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 *PTPR*s with various clinical factors (e.g. location of cancer, mutations, 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 protein tyrosine phosphatase genes were found as follows: *PTPRM*, *PTPRT*, *PTPRR* and *PTPRZ1* (see Suppl. 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

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sequences) were observed for the examined phosphatases in cancer tissues (Fig.1). A few samples amplified neither of the two sets of primers. The results are presented in Table 3.
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 (see Table 3).

Our results revealed a significantly higher level of methylation in tumors compared to ~~normal colon~~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 protein tyrosine phosphatases: *PTPRM*, *PTPRT*, *PTPRR* and *PTPRZ1*, are hypermethylated in colon cancer compared to 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~~0.01988~~). However, using MSP only the presence of a methylated allele in the tissue

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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~~a possible role of~~ PTPs playing a role in the development of cancer ~~transformation~~. As ~~it has been~~ published recently, ~~In several previous studies the biological role of selected the~~an involvement of PTPs in cancerogenesis has been observed for ~~-~~a variety of tumors.- *PTPRT* (*PTPR-rho*) 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-zeta* or *PTPR-beta*) is a receptor of VacA, a cytotoxin of *Helicobacter pylori*, inducing gastric ulcers (15). PTPRZ1 has also been reported to play 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-mu*) is a member of the Ig superfamily of adhesion molecules and has been shown to be expressed in neurons, glia, epithelia and prostate (16,17,18). PTPRM plays a crucial role in EGFR signaling – reduced expression of *PTPRM* increases the EGFR specific phosphorylation of tyrosine residue, thus causing phospholipase C-gamma (PLC)γ_1 activation and cell migration (18). It is also known that EGFR 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 to 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. 2009 to be highly downregulated in the early stages of colorectal tumorigenesis. This gene encoding a classical PTP receptor plays a crucial role in homeostasis regulating signaling in proliferation and differentiation (9).

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. (2004)

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(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 has been published. PTPRM is known to play a crucial role in the EGFR 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.

~~Earlier~~Recent epigenomic microarray analyses ~~of aberrantly methylated genes~~ in CRC tissues have identified many ~~hypermethylated~~ gene promoters which were hypermethylated~~-~~. Thus it has been postulated that these genes~~that can~~ may ~~influenc~~influence be involvede in colorectal tumorigenesis. In the studies~~y~~ of Kim et al. (2011) and Hinoue et al. (2012) the Illumina platform has been used to~~for~~ analyze~~sis of~~ 22 and 125 tumor samples respectively (19,20). ~~Although~~Although these ~~autors~~authors have not mentioned ~~about~~ PTPs in the~~irtop~~ "top ranking lists" of genes with aberrant methylation, the available raw data shows differences in PTP~~s~~ methylation ~~according~~as compared to healthy tissue (19,20).

Our study, which seems to be the first analysis using MSP of the methylation status of ~~*PTPRS*~~ *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 plays a still unknown role in the colon.

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