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Recurrent epigenetic silencing of the *PTPRD* tumor suppressor in laryngeal squamous cell carcinoma

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Abstract

Cellular processes like differentiation, mitotic cycle, and cell growth are regulated by tyrosine kinases with known oncogenic potential and tyrosine phosphatases that downmodulate the first. Therefore, tyrosine phosphatases are recurrent targets of gene alterations in human carcinomas. We and others suggested recently a tumor suppressor function of the *PTPRD* tyrosine phosphatase and reported homozygous deletions of the *PTPRD* locus in laryngeal squamous cell carcinoma. In this study, we investigated other gene-inactivating mechanisms potentially targeting *PTPRD*, including loss-of-function mutations and also epigenetic alterations like promoter DNA hypermethylation. We sequenced the *PTPRD* gene in eight laryngeal squamous cell carcinoma cell lines but did not identify any inactivating mutations. In contrast, by bisulfite pyrosequencing of the gene promoter region, we identified significantly higher levels of methylation ($p = 0.001$ and $p = 0.0002$, respectively) in 9/14 (64%) laryngeal squamous cell carcinoma cell lines and 37/79 (47%) of primary laryngeal squamous cell carcinoma tumors as compared to normal epithelium of the upper aerodigestive tract. There was also a strong correlation ($p = 0.0001$) between methylation and transcriptional silencing for the *PTPRD* gene observed in a cohort of 497 head and neck tumors from The Cancer Genome Atlas dataset suggesting that DNA methylation is the main mechanism of *PTPRD* silencing in these tumors. In summary, our data provide further evidence of the high incidence of *PTPRD* inactivation in laryngeal squamous cell carcinoma. We suggest that deletions and loss-of-function mutations are responsible for *PTPRD* loss only in a fraction of cases, whereas DNA methylation is the dominating mechanism of *PTPRD* inactivation.

Keywords

PTPRD, tumor suppressor gene, head and neck cancer, laryngeal cancer, epigenetics, bisulfite pyrosequencing, microRNA, mutation screen, cell lines

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Introduction

Deregulation of protein tyrosine phosphatases (PTPs) plays an important role in cancer development. These proteins are involved in signaling cascades that regulate a variety of cellular processes including cell growth, differentiation, and mitotic cycle. Therein, the inactivating PTPs function in a delicate balance with the activating protein tyrosine kinases.[1] Various alterations resulting in loss of function of the PTP genes observed in neoplasms emphasize their suppressive role in cancer formation.[2]

Recently, we reported homozygous deletions that target the *PTPRD* gene in laryngeal squamous cell carcinoma (LSCC) cell lines. Consequently, *PTPRD* was shown to be significantly downregulated in LSCC cell lines on messenger RNA (mRNA) and protein level.[3] Nevertheless, the highly recurrent transcriptional downregulation of *PTPRD* suggests also other inactivating mechanisms of this gene, beside the homozygous deletions in LSCC. Thus, we hypothesized that point mutations as well as epigenetic mechanisms such as promoter hypermethylation may lay behind its silencing in this tumor.

In this study, we analyzed these putative *PTPRD*-silencing mechanisms using Sanger sequencing and bisulfite pyrosequencing and showed their frequency and importance in the inactivation of this gene.

Materials and methods

LSCC cell lines, primary tumors, and the control samples

In total, 15 LSCC cell lines derived from patients diagnosed with LSCC at the Turku University Hospital in Finland were used (Supplementary Table S1). The cell lines were previously characterized.[4,5] Cells were grown in adhesive 25 cm^2 flasks in standard Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C under 5% CO_2 atmosphere. Cells were harvested at a confluence of 70%–90%. All cell lines were human papillomavirus (HPV) 16 and 18 negative.

Fresh frozen tumor samples were obtained from 79 patients diagnosed with LSCC who underwent surgical tumor resection followed by radiotherapy and chemotherapy in the Department of Otolaryngology and Laryngological Oncology, K. Marcinkowski University of Medical Sciences in Poznan, Poland. The local Institutional Ethical Board of K. Marcinkowski University of Medical Sciences has approved the collection and use of biological samples (904/06, 164/10, and 502/15).

Normal epithelial samples (n = 10; 5 men and 5 women), buccal swabs from the oral cavity lining, samples isolated from hypertrophies of vocal folds, and polyps of the vocal folds (n = 10) were collected as the control group. Histologically, these controls derived from the same tissue as the tumor are not neoplastic and do not turn malignant even during a follow-up for many years.

DNA from the cell lines primary tumor and non-cancerous tissue specimens was isolated using standard phenol/chloroform method described elsewhere.[6] DNA from buccal swabs was isolated with the Genomic Mini kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions.

Mutation screen of the PTPRD *gene in LSCC cell lines*

Coding exons of the *PTPRD* gene were Sanger sequenced in the eight LSCC cell lines (UT-SCC-6A, UT-SCC-11, UT-SCC-22, UT-SCC-29, UT-SCC-34, UT-SCC-57, UT-SCC-106A, and UT-SCC-107) using standard procedures. In detail, primer flanking coding exons, including the 5′ and 3′ splicing sites, were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/).[7,8] Primer sequences are listed in the supplementary data (Supplementary Table S2). Polymerase chain reactions (PCRs) were performed using the following conditions: at 95°C for 5′, 35 cycles: denaturation at 95°C for 30 s, annealing at 55°C or 60°C or 65°C for 30 s, elongation at 72°C for 30 s, and terminal elongation at 72°C for 5′. PCR products were visualized under ultraviolet (UV) light (BioDoc-it Imaging System, UVP, Upland, USA) on 1.8% agarose gels stained with ethidium bromide and purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's instructions. DNA sequencing reactions for DNA sequencing were performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, USA) under the following conditions: at 96°C for 2′, 26 cycles: denaturation at 96°C for 10 s, annealing at 55°C for 5 s, and elongation at 60°C for 4′. The products were purified by ethanol precipitation, separated using the ABI310 sequencer (Applied Biosystems), and analyzed using the Sequencing Analysis (ABI) software. Some of the PCR products were sequenced by the Genomed company (Warsaw, Poland). The .abi files were visualized using the Chromas Lite (Technelysium Pty Ltd, South Brisbane, Australia) and Sequencher Demo (Gene Codes) software and compared with the reference sequences downloaded from the Genome Browser hg19 assembly (http://genome.ucsc.edu/). Identified mutations were compared with the 1000 Genomes database (http://browser.1000genomes.org/) to distinguish between single-nucleotide polymorphism (SNP) and novel mutations. The SNPs were analyzed using the CADD tool (Combined Annotation Dependent Depletion; http://cadd.gs.washington.edu/home) to estimate their deleteriousness. We set the Phred-like scaled C-score at 15 as a cut-off score for identification of deleterious SNPs according to authors' recommendations.[9]

Multiplex reverse transcription polymerase chain reaction for the PTPRD *gene*

Primer sequences were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). The multiplex-included primer for amplification of the *PTPRD* complementary DNA (cDNA) sequence (exon 43–46 of *PTPRD* transcript variant 1; product size: 376 bp; forward: CATAGCTGGTCGTTGTGTTCT; and reverse: ACG GTCTGCAAGATACCAGT) and the reference primer for an arbitrarily chosen gene (cDNA product size: 598 bp; forward: CGGAGAGCACTAAGCCACTT; and reverse: GTCCATTGCGCAGACAAAAG).

RNA from the cell lines was isolated using standard phenol/chloroform method described elsewhere[6] and reverse transcribed using the Enhanced Avian First Strand Synthesis Kit (Sigma-Aldrich, Steinheim, Germany). PCR reactions were performed using the following conditions: at 95°C for 5′, 35 cycles: denaturation at 95°C for 30 s, annealing at 65°C for 30 s, elongation at 72°C for 30 s, and terminal elongation at 72°C for 5′.

The design of bisulfite pyrosequencing assay for PTPRD *promoter region DNA methylation analysis*

The primer was designed with PyroMark Assay Design Software 2.0 (Qiagen, Hilden, Germany). The assay was analyzed for potential SNP occurrence within the primer binding region. The primer sequences for the *PTPRD* promoter region were as follows: forward: 5′-GGGAATAGG AGGATATGTTAGGT-3′ (labeled with biotin at the 5′ end), reverse: 5′-ACACCCACTACCCCTTAAATATTA-3′, and sequencing: 5′-ACTACCCCTTAAATATTACC-3′. The primer amplifies the 175 bp sequence located in the *PTPRD* promoter region (chr9:10,613,333-10,613,507; hg19) that includes three CG dinucleotides. To test the assay sensitivity and its ability to reproduce low as well as high levels of methylation, a dilution series from 0% to 100% (every 10%) of fully methylated and unmethylated DNA was prepared and tested with the respective assay.

Bisulfite treatment of DNA, PCR reaction, and electrophoresis

Purified DNA (1 µg) was converted with bisulfite solution according to EpiTect DNA Modification Kit Protocol (Qiagen, Hilden, Germany). PCR was performed according to PyroMark PCR Kit (Qiagen, Hilden, Germany) program: at 95°C for 15 min (initial heating), followed by 45 cycles: denaturation for 30 s at 94°C, primer annealing in 60°C for 30 s and extension in 72°C for 30 s, and the final extension in 72°C for 10 min. PCR products were separated on 1.8% agarose gel stained with ethidium bromide and visualized under UV light (BioDoc-it Imaging System, UVP).

Pyrosequencing

Pyrosequencing was performed according to the standard protocol and as described previously.[10] PCR products were mixed with binding buffer (Qiagen, Hilden, Germany) and sepharose coated with streptavidin (GE Healthcare, Chicago, USA), shaked for 10 min and cleaned on vacuum pump station in the following buffers: 70% EtOH, 0.2% NaOH, and washing buffer (Qiagen, Hilden, Germany). Single-strand amplicons were then mixed with annealing buffer (Qiagen, Hilden, Germany), the sequencing primer (0.4 µM), and then heated for 2 min at 85°C and cooled for primer hybridization. Pyrosequencing was performed using the PyroMark Q24 (Qiagen, Hilden, Germany) and the results analyzed using the PyroMark Q24 (2.0.6 Qiagen software), which automatically calculates the G:A "–" strand (de facto mC:C) ratio at the CpG sites. The analyzed sequence was RACAACCTCCCTCRAAAAATAATA ATAATAATAATAACCRAAAA (hg19 chr9:10613464-10613481) and enclosed three CG repeats (R). Each pyrosequencing run was accompanied by fully methylated DNA sample (CpG Genome Universal Millipore, Darmstadt, Germany) and unmethylated whole genome amplified (WGA) sample prepared using the GenomePlex® Whole Genome Amplification Kit (Sigma-Aldrich, Steinheim, Germany) from pooled DNA isolated from peripheral blood of 10 healthy donors. The results for each analyzed sample were visualized as value bars of DNA methylation level in each CG repeat separately.

To calculate the normal methylation level for control samples (buccal swabs, hypertrophies of vocal folds, and polyps of the vocal folds), we added the maximal methylation level value observed in controls plus 3 × mean standard deviation of the whole control group. The obtained value was regarded as the "cut-off" level of methylation beyond which a sample was described as methylated.

The Cancer Genome Atlas data mining

We further sought to confirm our methylation data with the public online cancer database: The Cancer Genome Atlas (TCGA) at http://tcga-data.nci.nih.gov/. We downloaded the numerical data from two platforms: Illumina HiSeq mRNA expression RNA-seq V2 data and Illumina Human methylation 450K data for *PTPRD* gene (ID 5798) that included 479 head and neck squamous cell carcinoma (HNSCC) cases. The data were used to visualize the potential correlation between mRNA expression and methylation data for the *PTPRD*. The graph showing the outline of both datasets was constructed using Microsoft Excel and the correlation was performed by Pearson's and Spearman's test using GraphPad Prism 5. The RNA-seq by expectation

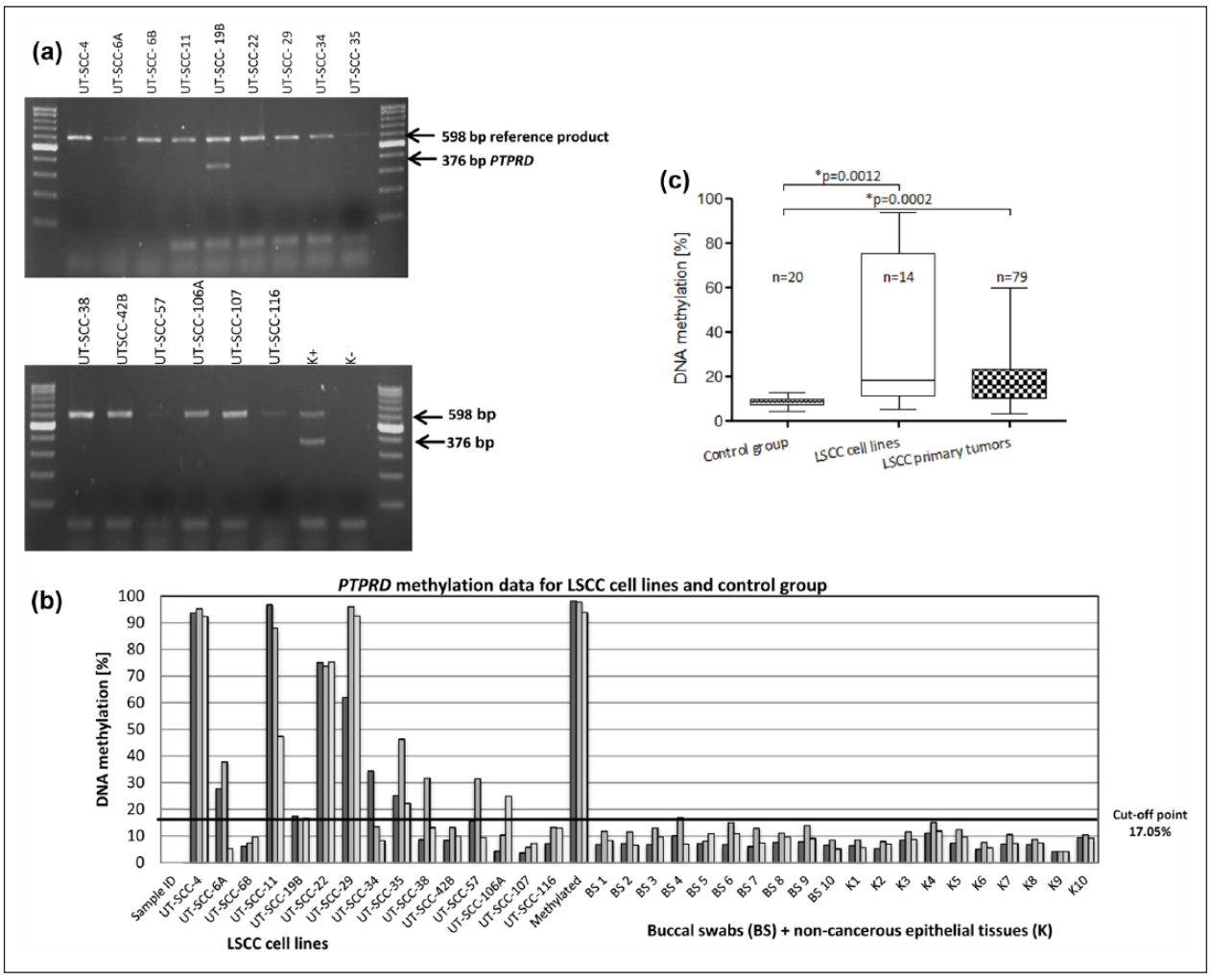

Figure 1. (a) Multiplex RT-PCR for the *PTPRD* gene (exons 43–46; *PTPRD* transcript variant 1). *PTPRD* expression is retained in one, the UT-SCC-19B cell line. K+: positive control, K−: negative control, (b) DNA methylation of *PTPRD* promoter region in LSCC cell lines and the control group (buccal swabs, hypertrophies of vocal folds, and polyps of the vocal folds). Each bar corresponds to a single CG repeat. Cut-off line (17.05%) indicates samples with increased DNA methylation, and (c) DNA methylation levels in LSCC cell lines, LSCC primary tumors, and the control group (buccal swabs, and samples isolated from hypertrophies of vocal folds and polyps of the vocal) shown as box plots with SD.

maximization (RSEM) expression values (Z score)<0 were considered as lack of expression.

Results

PTPRD *is recurrently transcriptionally silenced but inactivating mutations are rare in LSCC cell lines*

In our previous study, we showed recurrent downregulation of *PTPRD* on both mRNA and protein levels in LSCC cell lines.[3] Here, using multiplex reverse transcription polymerase chain reaction (RT-PCR), we further validate this finding and show complete transcriptional silencing of the *PTPRD* gene in 14/15 LSCC cell lines analyzed (Figure 1(a)). The mechanism behind the observed transcriptional loss may at least, in part, lie in the identified homozygous deletions targeting the gene. Nevertheless, the frequency of the homozygous deletions cannot accomplish for the frequency of *PTPRD* silencing. Therefore, in this study, we sequenced all coding exons of the gene in eight LSCC cell lines that showed an absent call for the *PTPRD* expression tag 214043_at (Affymetrix U133 plus 2.0 published elsewhere[3]) expecting to find recurrent loss-of-function mutations.

In the mutation screen, we identified the homozygous SNP rs35929428 (CADD score, 25.8) in the UT-SCC-11 and UT-SCC-57 cell lines and the heterozygous SNP rs370537821 (CADD score, 9.019) in the UT-SCC-22 cell line. The CADD score suggests that the SNP rs35929428 might possibly be deleterious, whereas the SNP rs370537821 is probably benign. Moreover, we found, in

the UT-SCC-6A cell line, a homozygous insertion of 25 nucleotides which due to a repetitive sequence in this locus can be located either in the position chr9:8,331,582 (hg19) in the exon 44 (assigned as rs3215098; NM_002839.3) or in the position chr9:8,331,574 (hg19) located in the intron 44/45 (assigned as rs146237556; NM_002839.3). Interestingly, the SNP rs3215098 leads to the shortened *PTPRD* protein caused by a premature stop codon, while the SNP rs146237556 is considered as a benign change. As there is no transcript of the *PTPRD* gene in these cell lines, it is not possible to clarify which of these SNPs is present on cDNA level.

In conclusion, we identified two SNPs (rs35929428 and rs3215098) which can putatively influence *PTPRD* functionality in 3/8 sequenced cell lines. Beside the SNPs, no novel alterations were identified suggesting that *PTPRD* loss-of-function mutations are rare in LSCC.

The promoter region of PTPRD *is recurrently hypermethylated in LSCC cell lines and primary tumors*

As the mutation screen did not identify frequent loss-of-function mutations, we performed a methylation analysis of the *PTPRD* promoter region in LSCC to identify hypermethylation as an alternative mechanism of gene inactivation. We first determined the level of methylation of normal epithelium of the upper aerodigestive tract in buccal swab samples (n = 10) and DNA samples isolated from hypertrophies of vocal folds and polyps of the vocal folds (n = 10). In these samples, the DNA methylation level ranged from 6.7% to 12.7% (standard deviation (SD) = 1.45) and the mean methylation level was 8.93%. By analyzing these results with respect to the control dilution series of methylated DNA, it demonstrates the lack of *PTPRD* methylation in these controls. It shows that methylation of the *PTPRD* gene is not a normal feature of human epithelial cells of the upper aerodigestive tract. We next used these results to calculate the cut-off point of methylation by adding the highest methylation value from the control group (12.7%) and three times the SD ($3 \times 1.45\% = 4.35\%$) that resulted in a cut-off methylation of 17.05%.

We then analyzed the 14 LSCC cell lines using the same pyrosequencing assay. Using the cut-off point for methylation (17.05%), we identified 9/14 (64%) LSCC cell lines to have elevated level of methylation, whereas 4 of them (UT-SCC-4, UT-SCC-11, UT-SCC-22, and UT-SCC-29) demonstrated hypermethylation defined as pyrosequencing-result showing methylation >60% (Figure 1(b)). By comparing methylation levels in controls (n = 20) and LSCC cell lines (n = 14) using the unpaired t-test, a significant difference of means ($p = 0.001$) was found (Figure 1(c)).

To exclude that the observed elevation of methylation is only a feature of cultured cells, we analyzed a cohort of 79 primary LSCC tumors. In line with the observation in cell lines, according to our criteria, we identified elevated methylation in 37/79 (47%) cases, and the mean methylation level of *PTPRD* promoter in LSCC tumors was significantly higher ($p = 0.0002$, t-test) from the control group (Figure 1(b)) (Supplementary Figure S1). Although, we did not observe hypermethylation in any of the analyzed LSCC primary tumors, which means that none of the samples' DNA methylation level was higher than 60%.

In conclusion, we found that in contrast to the normal human epithelial cells of the upper aerodigestive tract, LSCC tumors show recurrent elevated methylation of the *PTPRD* promoter region.

PTPRD *promoter methylation is significantly correlated with loss of mRNA expression*

In order to show that hypermethylation of the *PTPRD* promoter region results in transcriptional silencing, we mined available TCGA methylation and expression data for 497 HNSCC cases. Overall, there were 333/497 HNSCC cases (67%) showing loss of *PTPRD* expression. In detail, there were 410/497 (82.5%) samples with >60% DNA methylation level (hypermethylated), which included 100/410 (24.4%) samples showing expression (the highest RSEM Z score expression was only 3.9). In contrast, 87/497 (17.5%) samples demonstrated <60% methylation level, where 64/87 (73.6%) samples showed expression (the highest expression was 17.9) (Figure 2). Moreover, there was a significant ($p < 0.0001$, $r = -0.56$, Spearman's test) relation between loss of expression and hypermethylation, where methylation level >60% level resulted in silencing of *PTPRD* expression (Figure 2). Therefore, TCGA further corroborates that *PTPRD* is silenced primarily by epigenetic mechanism in head and neck tumors.

Discussion

The oncogenic potential of protein tyrosine kinases is well characterized and has led to the introduction of several novel therapeutics to daily clinical practice. Recently, increasing attention is focused on the PTPs that physiologically show a suppressive function towards the oncogenic kinases. The tumor suppressive functionality of PTPs, including the *PTPRD* gene, is further supported by homozygous deletions and loss-of-function mutations observed in various human neoplasms.[1,3,11,12] Nevertheless, considering the frequency of transcriptional silencing of *PTPRD*, additional mechanism must be co-responsible for its inactivation. In line with this hypothesis, in the eight LSCC cell lines sequenced in this study, which showed no expression of the *PTPRD* gene, beside the observed SNPs, none harbored a loss-of-function mutation. We therefore concluded that epigenetic mechanism might be responsible for the

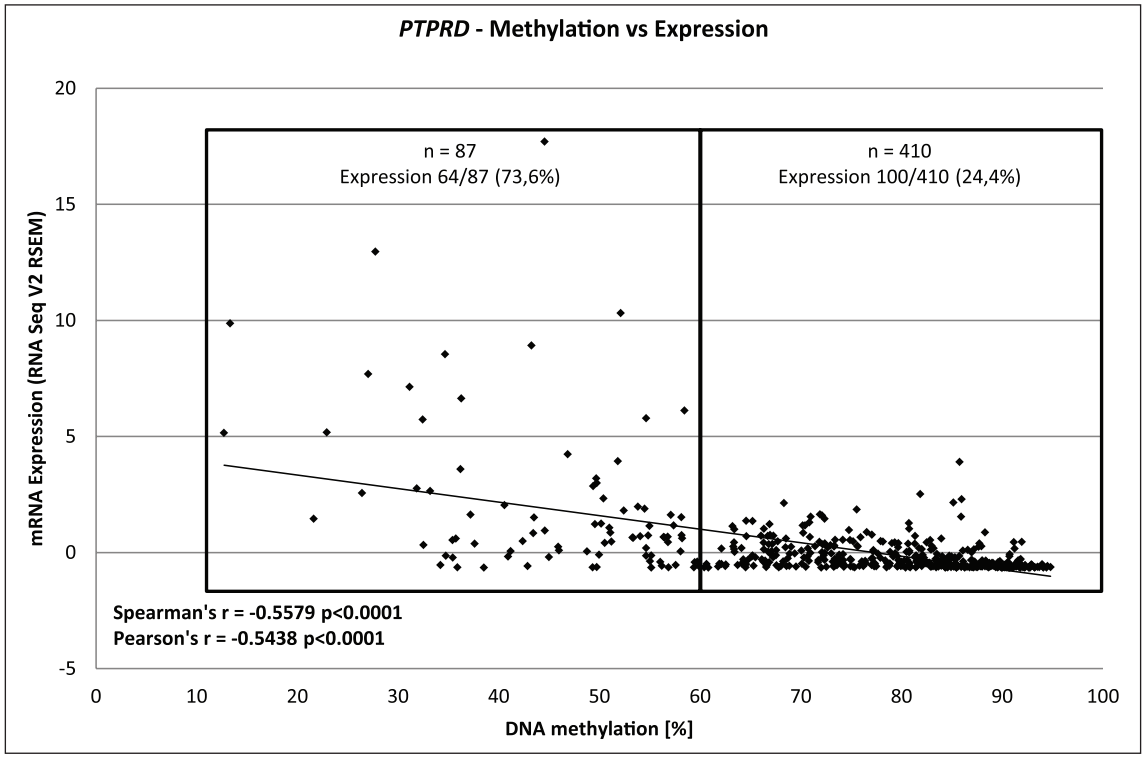

Figure 2. Correlation of *PTPRD* promoter methylation (Infinium HumanMethylation450 BeadChip) with *PTPRD* mRNA expression level (RNA Seq2 V2 RSEM) in head and neck tumors based on freely available TCGA data.
Source: http://tcga-data.nci.nih.gov/

observed downregulation and analyzed the methylation level of the gene promoter region by bisulfite pyrosequencing. The analysis showed elevated methylation of the promoter region in 10/14 (71%) LSCC cell lines ($p=0.024$) as well as 45/79 (60%) primary tumors ($p=0.01$) in contrast to controls ($n=20$) that were all unmethylated. The observation is supported by recent publication where *PTPRD* hypermethylation was reported in colorectal cancer[13] and the available TCGA data wherein hypermethylation of the *PTPRD* gene in head and neck tumors is frequent and correlates with downregulation of the gene's expression ($p<0.0001$, $r=-0.5579$). Therefore, these data suggest that methylation is the predominant mechanism of *PTPRD* inactivation in cancers derived from epithelial cells including head and neck cancer. In support to the epigenetic nature of *PTPRD* silencing, there are numerous microRNAs (miRNAs) listed in public available databases, including miR-20b, which are predicted in silico to regulate the *PTPRD* transcript. Interestingly, basing on our previously published miRNA expression profiles, we identified a 2.8-fold increased expression of miR-20b in LSCC as compared to no tumor controls.[14] It might suggest a potential involvement of this miRNA in the observed *PTPRD* silencing. However, this result requires further experimental validation.

The *PTPRD* gene is known for its property to regulate the *STAT3* oncogene, and its tumor suppressive function is mediated by its ability of downmodulate STAT3 through tyrosine dephosphorylation.[15] In light of this, *PTPRD* loss can result in constitutive oncogenic STAT3 signaling driving tumor formation.[16,17] Importantly, Ortiz et al. demonstrated that heterogeneous deletions of the *PTPRD* gene resulting in a retained wild-type allele are sufficient to deprive a cell of its suppressive function. The gene functions therefore in a haploinsufficient manner.[17] In this context, elevated methylation, but not hypermethylation, observed in a subset of the primary tumors studied here could reflect the same biological phenomenon.

Interestingly, it has been suggested recently that not only PTPRs losses result in the deregulation of STAT3 but also point mutations that modulate protein tyrosine phosphatase receptor (PTPR) proteins functionality.[18,19] This tempting possibility might be true for a subset of head and neck tumors and present an alternative mechanism for *PTPRD*-related STAT3 activation. Nevertheless, the results presented here strongly imply the conclusion that in the majority of LSCC cases, the *PTPRD* gene is transcriptionally inactive (compare Figure 2—TCGA data and our results). Thus, modulating point mutations can only rarely manifest their effects.

In conclusion, we provide here further evidence for the importance of *PTPRD* inactivation in the course of LSCC. We show, moreover, that epigenetic mechanisms, like

promoter hypermethylation, are the main mechanisms behind the frequent silencing of the *PTPRD* gene.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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