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Expression and DNA methylation levels of prolyl hydroxylases PHD1, PHD2, PHD3 and asparaginyl hydroxylase FIH in colorectal cancer

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Abstract

Background: Colorectal cancer (CRC) is one of the most common and comprehensively studied malignancies. Hypoxic conditions during formation of CRC may support the development of more aggressive cancers. Hypoxia inducible factor (HIF), a major player in cancerous tissue adaptation to hypoxia, is negatively regulated by the family of prolyl hydroxylase enzymes (PHD1, PHD2, PHD3) and asparaginyl hydroxylase, called factor inhibiting HIF (FIH).

Methods: PHD1, PHD2, PHD3 and FIH gene expression was evaluated using quantitative RT-PCR and western blotting in primary colonic adenocarcinoma and adjacent histopathologically unchanged colonic mucosa from patients who underwent radical surgical resection of the colon (n = 90), and the same methods were used for assessment of PHD3 gene expression in HCT116 and DLD-1 CRC cell lines. DNA methylation levels of the CpG island in the promoter regulatory region of PHD1, PHD2, PHD3 and FIH were assessed using bisulfite DNA sequencing and high resolution melting analysis (HRM) for patients and HRM analysis for CRC cell lines.

Results: We found significantly lower levels of PHD1, PHD2 and PHD3 transcripts (p = 0.00026; p < 0.00001; p < 0.00001) and proteins (p = 0.004164; p = 0.0071; p < 0.00001) in primary cancerous than in histopathologically unchanged tissues. Despite this, we did not observe statistically significant differences in FIH transcript levels between cancerous and histopathologically unchanged colorectal tissue, but we found a significantly increased level of FIH protein in CRC (p = 0.0169). The reduced PHD3 expression was correlated with significantly increased DNA methylation in the CpG island of the PHD3 promoter regulatory region (p < 0.0001). We did not observe DNA methylation in the CpG island of the PHD1, PHD2 or FIH promoter in cancerous and histopathologically unchanged colorectal tissue. We also showed that 5-Aza-2′-deoxycytidine induced DNA demethylation leading to increased PHD3 transcript and protein level in HCT116 cells.

Conclusion: We demonstrated that reduced PHD3 expression in cancerous tissue was accompanied by methylation of the CpG rich region located within the first exon and intron of the PHD3 gene. The diminished expression of PHD1 and PHD2 and elevated level of FIH protein in cancerous tissue compared to histopathologically unchanged colonic mucosa was not associated with DNA methylation within the CpG islands of the PHD1, PHD2 and FIH genes.

Background

Colorectal cancer (CRC) belongs to one of the most extensively studied types of cancers due to its high mortality and severity. It is the third and second leading cause of death from malignant disease among adults in the US and Europe, respectively [1]. A decrease in oxygen concentration is widely seen during the formation

of many solid tumors, including CRC. Hypoxic regions may occur due to poorly formed vasculature, shunting of blood and vascular permeability [2]. Cancer cells can adjust to this microenvironment by altering gene transcription to enhance glucose uptake and angiogenesis [2]. The various adaptive responses involve multiple mechanisms, of which the best-characterized is mediated through transcriptional gene activation by the hypoxia inducible factor (HIF) [3]. HIF is a heterodimeric transcription factor assembled from an oxygen-regulated α subunit (HIF-α) and a constitutively expressed β subunit (HIF-β)

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[3,4]. Under hypoxic conditions, HIF-α translocates into the nucleus, where it forms a dimer with HIF-β to form an active transcriptional complex with a number of cofactors [3,4]. The HIF complex binds to the promoter hypoxia response elements (HREs) to induce the expression of target genes that regulate the cellular adaptive response to low oxygen tension [3,4].

HIF-α is constitutively expressed in the tissue; however, it has an extremely short half-life in normoxic conditions [3]. The level of HIF-α protein is regulated in several ways. The most well known is its degradation through post-translational hydroxylation. To date, two different oxygen-dependent hydroxylation mechanisms have been identified. The first pathway is initiated by three prolyl hydroxylase domain enzymes, PHD1, PHD2 and PHD3 [3]. The second pathway involves the factor inhibiting HIF (FIH) [5]. The PHD enzymes catalyze the hydroxylation of two conserved proline residues in the oxygen dependent degradation domain of the HIF-α protein. Hydroxylated proline residues are subsequently recognized by the E3 ligase complex containing von Hippel–Lindau tumour suppressor protein (pVHL), and targeted for degradation by the 26S proteasome [3]. Similarly, FIH hydroxylates the asparagine residue within the C-terminal transactivation domain of HIF-α [5,6]. This results in the prevention of HIF-α interaction with its coactivators. Hence, under normoxic conditions, there is a dual mechanism of HIF inhibition by its degradation or inactivation by PHDs and FIH enzymes, respectively.

Recently, various studies have demonstrated inconsistent data of FIH and PHD1, 2 and 3 expression changes during CRC development [7-10]. The mechanism by which these hydroxylases might be regulated is still not well elucidated. Interestingly, PHDs and FIH genes possess a CpG island within their promoter region. Similarly to genetic mutations, hyper- or hypomethylation of gene regulatory sequences have been shown to potentially change the expression of cancer related genes in different malignancies, including CRC [11]. To date, it has been demonstrated that the promoter region of the PHD3 gene is hypermethylated in plasma cell neoplasia, prostate, melanoma and mammary gland cancer cell lines [12,13]. The DNA methylation status of PHD1, PHD2 and FIH has also been investigated in breast, cervical and prostate cancer cell lines, but the results are inconsistent [12,14,15]. These reports prompted us to study whether altered PHD1, PHD2, PHD3 and FIH expression levels may be correlated with the DNA methylation status of their promoter regions in primary cancerous and histopathologically unchanged colorectal tissue from the same ninety patients. We also evaluated the effect of 5-Aza-2'-deoxycytidine (5-dAzaC), an inhibitor of DNA methyltransferases (DNMTs), on the DNA methylation level of the PHD3 gene and its effect on PHD3 transcript and protein levels in HCT116 and DLD-1 CRC cells under hypoxic and normoxic conditions.

Methods

Antibodies and reagents
Rabbit polyclonal (Rp) anti-PHD1 (NB100-310), -PHD2 (NB100-137), -PHD3 (NB100-139) and -FIH (NB100-428) antibodies (Ab) were provided by Novus Biologicals (Cambridge, UK). Rp anti-GAPDH Ab (FL-335) and goat anti-rabbit horseradish peroxidase (HRP)-conjugated Ab were provided by Santa Cruz Biotechnology (Santa Cruz, CA). 5-dAzaC was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Patient material
Primary colonic adenocarcinoma tissues were collected between June 2009 and July 2012 from ninety patients who underwent radical surgical resection of the colon at the Department of General and Colorectal Surgery, Poznań University of Medical Sciences, Poland (Table 1). Histopathologically unchanged colonic mucosa located at least 10–20 cm away from the cancerous lesions was obtained from the same patients. Since *ex vivo* stress may influence protein stability, one set of samples was

Table 1 Demographic and histopathological classification including stage, grade and tumour type of patients with CRC

Features	No. of patients
Total no. of patients	90
Gender (Female/Male)	41/49
Mean (± SD) age at radical surgical resection of colon (yrs)	68.60 ± 11.45
CRC localization	
Proximal colon (cecum to transverse)	32
Distal colon (splenic flexure to sigmoid)	18
Rectum	40
Histological grade	
G1	3
G2	65
G3	22
Dukes classification	
A	8
B	35
C	47
Tumour stage	
T1	4
T2	10
T3	65
T4	11

immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA/DNA/protein isolation [16]. Another set of samples was directed for histopathological examination. Histopathological classification including stage, grade and tumour type was performed by an experienced pathologist. No patients received preoperative chemo- or radiotherapy. Written informed consent was obtained from all participating individuals. The procedures of the study were approved by the Local Ethical Committee of Poznań University of Medical Sciences.

Cell culture
DLD-1 colon cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and HCT116 cells were kindly provided by the Department of Experimental and Clinical Radiobiology, Maria Skłodowska-Curie Cancer Center, Institute of Oncology Branch, Gliwice, Poland. These cells were cultured in DMEM GibcoBRL (Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine. To determine the effect of 5-dAzaC on DNA methylation, transcript and protein levels of selected genes, the HCT116 and DLD-1 cells were cultured for 24 hours in DMEM GibcoBRL (Grand Island, NY) supplemented with 10% FBS from Sigma-Aldrich Co. (St. Louis, MO). Cells were then cultured under normoxic or hypoxic (1% O_2) conditions either in the absence or in the presence of 5-dAzaC at a concentration of 1.00 or 5.00 μM for 6, 24 and 48 hours. Hypoxic conditions were achieved using a MCO-18 M multigas cell culture incubator, Sanyo (Wood Dale, IL), modified to permit flushing the chamber with a humidified mixture of 5% CO_2, 94% N_2. These cells were used for total DNA, RNA isolation, RQ-PCR, western blotting, and HRM analysis.

Reverse transcription and real-time quantitative polymerase chain reaction (RQ-PCR) analysis
Total RNA from primary tissues of patients with CRC and CRC cell lines was isolated according to the method of Chomczyński and Sacchi (1987) [17]. RNA samples were quantified and reverse-transcribed into cDNA. RQ-PCR was carried out in a Light Cycler®480 Real-Time PCR System, Roche Diagnostics GmbH (Mannheim, Germany) using SYBR® Green I as detection dye. The target cDNA was quantified by the relative quantification method using a calibrator for primary tissue or respective controls for HCT116 and DLD-1 cells. The calibrator was prepared as a cDNA mix from all of the patients' samples and successive dilutions were used to create a standard curve as described in Relative Quantification Manual Roche Diagnostics GmbH, (Mannheim, Germany). For amplification, 1 μl of total (20 μl) cDNA solution was added to 9 μl of IQ™ SYBR® Green Supermix, Bio-Rad Laboratories Inc. (Hercules, CA) with

primers (Additional file 1). To prevent amplification of sequences from genomic DNA contamination, primers and/or amplicons were designed at exon/exon boundaries and covered all gene splice variants (Additional file 1). The quantity of PHD1, PHD2, PHD3 and FIH transcript in each sample was standardized by the geometric mean of two internal controls. The internal control genes were *porphobilinogen deaminase* (PBGD) and *human mitochondrial ribosomal protein L19* (hMRPL19) (Additional file 1). They were selected from four candidate reference genes (*PBGD, hMRPL, peptidylprolyl isomerase A- PPIA, hypoxanthine phosphoribosyltransferase 1- HPRT*) based on the results achieved in geNorm VBA applet for Microsoft Excel (data not shown) [18,19]. The PHD1, PHD2, PHD3 and FIH transcript levels in the patients' tissues were expressed as multiplicity of cDNA concentrations in the calibrator. In HCT116 and DLD-1 cells, transcript levels were presented as multiplicity of the respective controls.

Western blotting analysis
Primary tissues from patients with CRC, HCT116 and DLD-1 cells were treated with lysis RIPA buffer and proteins were resuspended in sample buffer and separated on 10% Tris-glycine gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel proteins were transferred to a nitrocellulose membrane, which was blocked with 5% milk in Tris/HCl saline/Tween buffer. Immunodetection of bands was performed with Rp anti-PHD1, -PHD2, -PHD3 and -FIH Ab, followed by incubation with goat anti-rabbit HRP-conjugated Ab. To ensure equal protein loading of the lanes, the membrane was stripped and incubated with Rp anti-GAPDH Ab (FL-335), followed by incubation with goat anti-rabbit HRP-conjugated Ab. Bands were revealed using SuperSignal West Femto Chemiluminescent Substrate, Thermo Fisher Scientific (Rockford, IL) and Biospectrum® Imaging System 500, UVP Ltd. (Upland, CA). The amounts of analyzed proteins were presented as the protein-to-GAPDH band optical density ratio. For HCT116 and DLD-1 cells cultured in the absence of 5-dAzaC, the ratio of PHD3 to GAPDH was assumed to be 1.

DNA isolation and bisulfite modification
Genomic DNA was isolated using DNA Mammalian Genomic Purification Kit purchased from Sigma-Aldrich Co. (St. Louis, MO). 500 ng of genomic DNA was subjected to bisulfite conversion of cytosine to uracil according to the EZ DNA Methylation Kit™ procedure from Zymo Research Corporation (Orange, CA). The position of CpG islands and binding sites of transcription factors located in the regulatory region of the promoter was determined by online programs [20-22].

DNA methylation evaluation by bisulfite sequencing

DNA fragments containing CpG dinucleotides located in the promoter region of the *PHD1*, *PHD2*, *PHD3* and *FIH* genes were amplified from the bisulfite-modified DNA by the primer pairs (Additional file 1, Additional file 2) complementary to the bisulfite-DNA modified sequence. PCR amplification was performed by FastStart Taq DNA Polymerase from Roche Diagnostic GmbH (Mannheim, Germany). The PCR products were purified using Agarose Gel DNA Extraction Kit, Roche Diagnostic GmbH (Mannheim, Germany) with subsequent cloning into pGEM-T Easy Vector System I, Promega (Madison, WI) and transformation into TOPO10 *E. coli* strain cells. Plasmid DNA isolated from five positive bacterial clones was used for commercial sequencing of the cloned fragment of DNA. The results of bisulfite sequencing were assessed and presented using BiQ analyzer software and Bisulfite sequencing Data Presentation and Compilation (BDPC) web server, respectively [23,24].

DNA methylation assessment by high resolution melting (HRM) analysis

Methylation levels of DNA fragments located within the CpG island of the *PHD1*, *PHD2*, *PHD3* and *FIH* genes (Additional file 2) were determined by Real Time PCR amplification of bisulfite treated DNA followed by HRM profile analysis by Light Cycler®480 Real-Time PCR System, Roche Diagnostics GmbH (Mannheim, Germany). For PCR amplification, 1 μl of the bisulfite treated DNA from patients, HCT116, DLD-1 cells, or standards, and primers (Additional file 1, Additional file 2) was added to 19 μl of 5 X Hot FIREPol EvaGreen HRM Mix, Solis BioDyne Co. (Tartu, Estonia). Standardized solutions of DNA methylation percentage were prepared by mixing methylated and non-methylated bisulfite treated DNA from Human Methylated/Non-methylated DNA Set, Zymo Research Corp. (Orange, CA) in different ratios. To determine the percentage of methylation, the HRM profiles of patient DNA PCR products were compared with HRM profiles of standard DNA PCR product [25,26]. HRM methylation analysis was performed using Light Cycler®480 Gene Scanning software, Roche Diagnostics GmbH (Mannheim, Germany). Each PCR amplification and HRM profile analysis was performed in triplicate. Using HRM analysis we were able to detect heterogenous methylation with equal sensitivity (Additional file 3). The methylation for each patient was presented as a percentage of methylation in amplified fragments located in the CpG island of *PHD1*, *PHD2*, *PHD3* and *FIH*. Since low levels of methylation may not demonstrate significant biological effect and we are not able to quantify all CpG dinucleotides within the analyzed CpG island, the percentage results were divided into three groups: 0–1% methylation, 1–10% methylation and 10–100% methylation for statistical analysis [27-30].

Statistical analysis

The normality of the observed patient data distribution was assessed by Shapiro-Wilk test, and unpaired, two-tailed *t*-test or U Mann–Whitney test was used to compare the mean values. The chi-square test was used to examine significance in DNA methylation. To evaluate the association between different ranges of DNA methylation (0–1% methylation, 1–10% methylation and 10–100% methylation) and the ratio of cancerous tissue PHD3 mRNA level to histopathologically unchanged PHD3 mRNA level, the non-parametric Kruskal-Wallis test was employed. Data groups for cell lines were assessed by ANOVA to evaluate if there was significance ($p < 0.05$) between the groups. For all experimental groups, which fulfilled the initial criterion, individual comparisons were performed by post hoc Tukey test with the assumption of two-tailed distribution. Statistically significant results were indicated by $p < 0.05$. Statistical analysis was performed with STATISTICA 6.0 software.
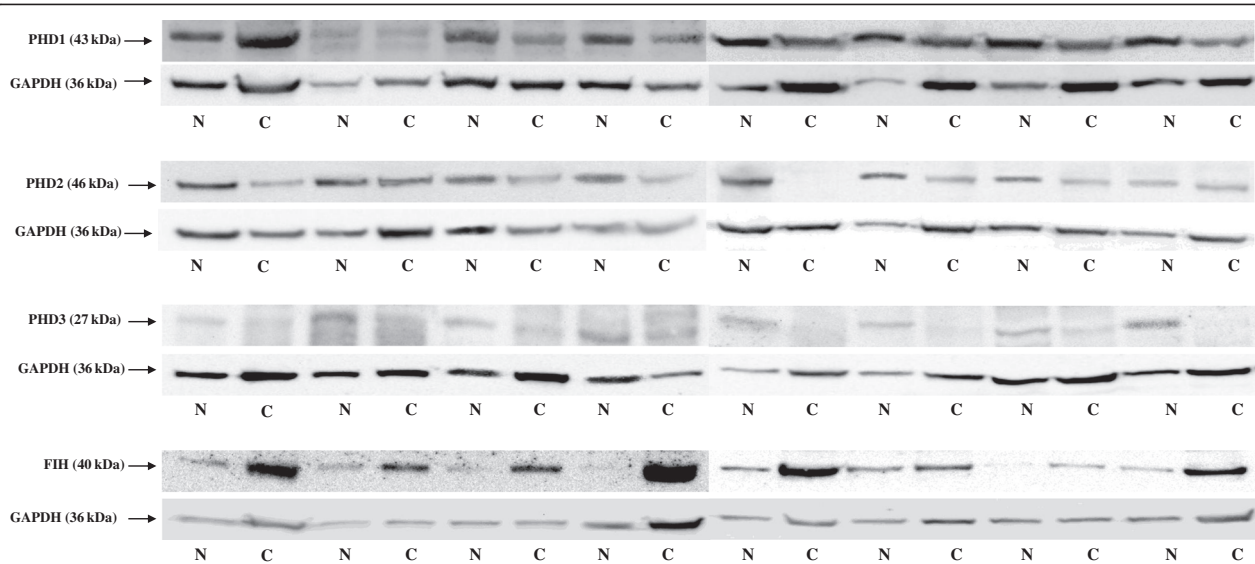
Results

PHD1, PHD2, PHD3 and FIH transcript and protein levels in primary cancerous and histopathologically unchanged tissues from patients with CRC

To compare PHD1, PHD2, PHD3, and FIH transcript and protein levels in cancerous and histopathologically unchanged tissues from ninety patients with CRC we used RQ-PCR and western blotting, respectively. We found significantly lower levels of PHD1, PHD2 and PHD3 transcript ($p = 0.00026$; $p < 0.00001$; $p < 0.00001$) and protein ($p = 0.004164$; $p = 0.0071$; $p < 0.00001$) in primary cancerous than in histopathologically unchanged tissues in ninety patients with CRC (Figure 1A, B; Figure 2). Moreover, we observed significantly lower levels of PHD1, PHD2, PHD3 transcript and protein in cancerous tissue in different age groups, among the genders, CRC localization, G2 and G3 histologic grade, levels of Dukes scale [31], and tumour stage (Additional file 4). There was no significant difference in the levels of FIH transcript between primary cancerous and histopathologically unchanged tissues in ninety patients with CRC ($p = 0.583$) (Figure 1A). However, we observed a statistically higher level of FIH protein in primary cancerous than in histopathologically unchanged tissue ($p = 0.0169$) (Figure 1B, Figure 2). We also found a significantly higher level of FIH protein in cancerous tissue in the male patient group ($p = 0.0210$), and in patients aged above 60 ($p = 0.0257$), with CRC localized in the rectum ($p = 0.031$) and G2 histologic grade ($p = 0.0226$) (Additional file 4).
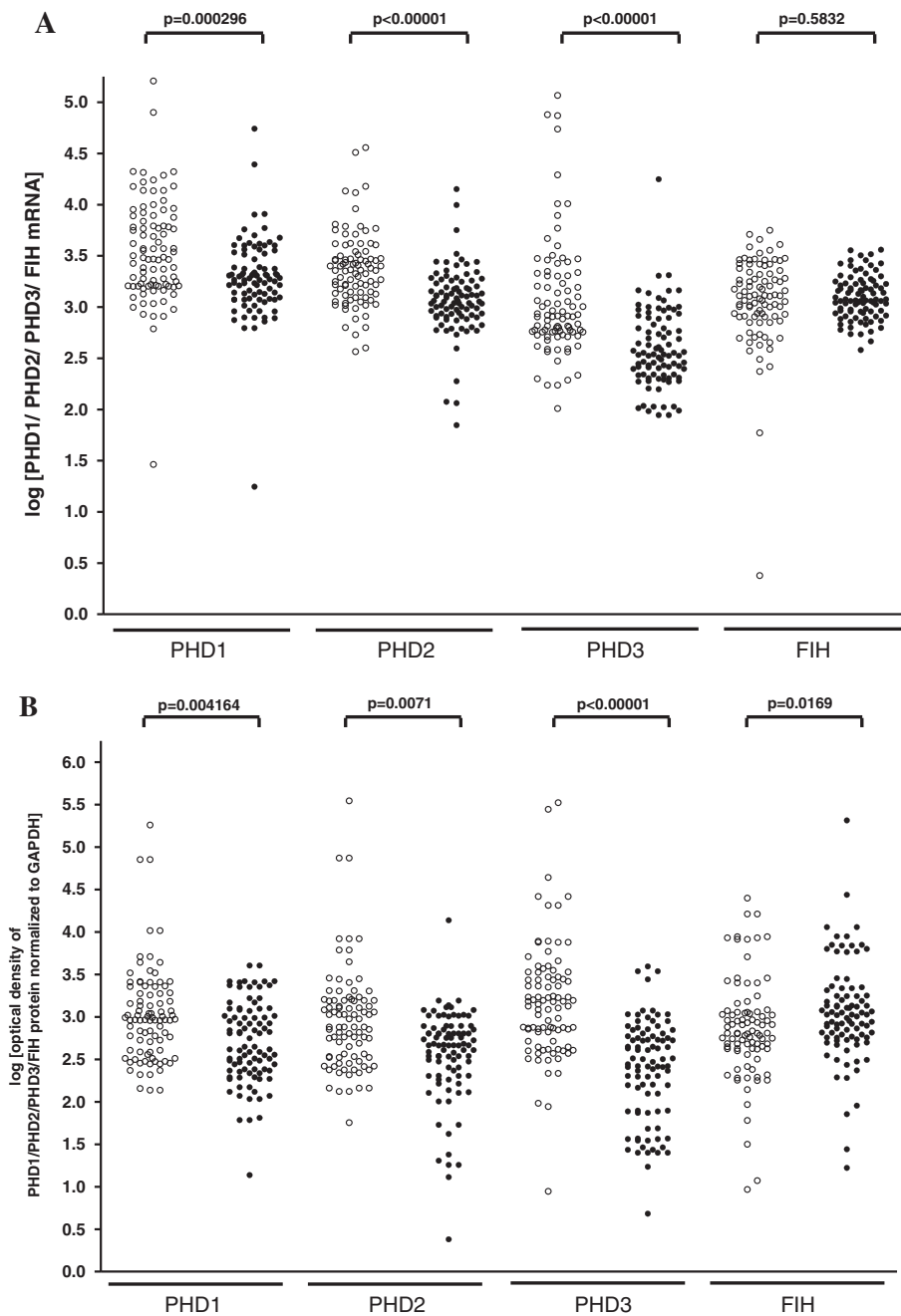
Figure 1 PHD1, PHD2, PHD3 and FIH transcript and protein levels in primary cancerous and histopathologically unchanged tissues from patients with CRC. A. The cancerous (●) and histopathologically unchanged tissues (○) from ninety patients with CRC were used for RNA and protein isolation. Total RNA was reverse-transcribed, and cDNAs were investigated by RQ-PCR relative quantification analysis. The PHD1, PHD2, PHD3 and FIH mRNA levels were corrected by the geometric mean of PBGD and hMRPL19 cDNA levels. The amounts of PHD1, PHD2, PHD3 and FIH mRNA were expressed as the decimal logarithm of multiples of these cDNA copies in the calibrator. **B.** Proteins were separated by 10% SDS-PAGE, and transferred to a membrane that was then immunoblotted with Rp anti- PHD1, - PHD2, - PHD3 and - FIH Ab and incubated with goat anti-rabbit HRP-conjugated Ab. The membrane was then stripped and blotted with Rp anti-GAPDH Ab, followed by incubation with goat anti-rabbit HRP-conjugated Ab. The amount of western blot-detected PHD1, PHD2, PHD3 and FIH proteins was presented as the decimal logarithm of PHD1, PHD2, PHD3 and FIH to GAPDH band optical density ratio. The p value was evaluated by unpaired, two-tailed t-test or U-Mann-Whitney test.

Figure 2 Representative picture of western blot in histopathologically unchanged tissue (N) and primary cancerous tissue (C) from patients with CRC. Immunodetection of bands was performed with Rp anti- PHD1, - PHD2, - PHD3 and - FIH Ab, followed by incubation with goat anti-rabbit HRP-conjugated Ab. The membrane was stripped and incubated with Rp anti-GAPDH Ab, followed by incubation with goat anti-rabbit HRP-conjugated Ab. Bands were revealed using SuperSignal West Femto Chemiluminescent Substrate, Thermo Fisher Scientific (Rockford, IL) and Biospectrum® Imaging System 500, UVP Ltd. (Upland, CA).
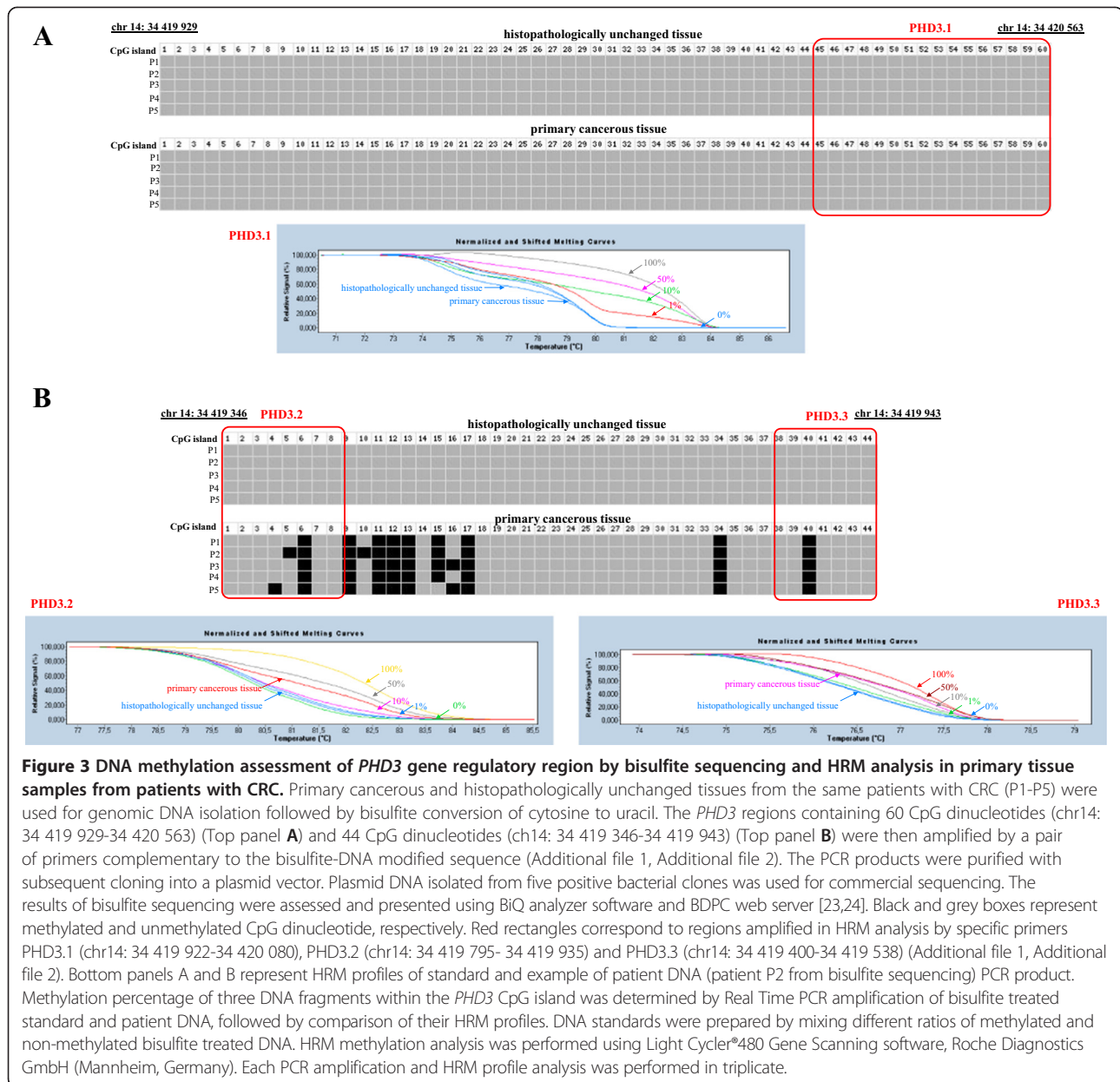
DNA methylation levels in primary cancerous and histopathologically unchanged tissues from patients with CRC

To compare DNA methylation levels in the promoter region of the *PHD1*, *PHD2*, *PHD3*, and *FIH* genes between DNA samples from cancerous and histopathologically unchanged tissues, we performed sodium bisulfite DNA sequencing and HRM analysis (Additional file 1, Additional file 2). Bisulfite sequencing was used for preliminary evaluation of DNA methylation in large regions of selected CpG islands in randomly selected patients. We detected a similar pattern of DNA methylation within all individual clones of each patient. The DNA methylation level evaluation for *PHD3* revealed significant differences between cancerous and histopathologically unchanged tissue in region chr14: 34 419 346–34 419 943 (Figure 3B, Additional file 1, Additional file 2). However, we observed no changes of DNA methylation within the promoter of *PHD3* in region chr14: 34 419 929–34 420 563 (Figure 3A, Additional file 1, Additional file 2). Moreover, we did not detect DNA methylation in the regulatory region of the *PHD1*, *PHD2* and *FIH* genes in cancerous and histopathologically unchanged tissue in selected patients with CRC (Additional file 1, Additional file 2, Additional file 5). To extend DNA methylation studies and to confirm bisulfite sequencing data for all analyzed genes, we employed HRM analysis of PCR amplified bisulfite treated DNA for patients. Depending on the length of the CpG island and the amplification possibilities of bisulfite treated DNA, one to three primer pairs was used in HRM analysis (Additional file 1,

Additional file 2). In keeping with the bisulfite sequencing data, we observed no DNA methylation within the promoter region of the *PHD1*, *PHD2* and *FIH* genes in cancerous and histopathologically unchanged tissue from ninety patients with CRC (Additional file 1, Additional file 2, Additional file 6). We also detected no DNA methylation for *PHD3* in region chr14: 34 419 922–34 420 080 in cancerous and histopathologically unchanged tissue using HRM analysis (Figure 3A, Additional file 1, Additional file 2). However, HRM evaluation showed a significant increase in the average DNA methylation level in cancerous compared to histopathologically unchanged tissue from ninety patients with CRC in the CpG island of the *PHD3* gene in regions chr14: 34 419 795–34 419 935 and chr14: 34 419 400–34 419 538 ($p < 0.00001$) (Figure 3B; Table 2, Additional file 1, Additional file 2). HRM results were compared with those obtained in bisulfite sequencing for all analyzed genes in reconstituted samples. A similar pattern of DNA methylation was observed between these two methods (Figure 3). Moreover, we observed that an increase in the average DNA methylation level of *PHD3* in regions chr14: 34 419 795–34 419 935 and chr14: 34 419 400–34 419 538 correlated to a decrease in the ratio of cancerous-to-histopathologically-unchanged tissue PHD3 mRNA level ($p < 0.0001$) (Figure 4).

DNA methylation level of the *PHD1*, *PHD2* and *FIH* genes in HCT116 and DLD-1 CRC cells

To assess DNA methylation levels in the promoter region of the *PHD1*, *PHD2*, and *FIH* genes in DLD-1 and

Figure 3 DNA methylation assessment of *PHD3* gene regulatory region by bisulfite sequencing and HRM analysis in primary tissue samples from patients with CRC. Primary cancerous and histopathologically unchanged tissues from the same patients with CRC (P1-P5) were used for genomic DNA isolation followed by bisulfite conversion of cytosine to uracil. The *PHD3* regions containing 60 CpG dinucleotides (chr14: 34 419 929-34 420 563) (Top panel **A**) and 44 CpG dinucleotides (ch14: 34 419 346-34 419 943) (Top panel **B**) were then amplified by a pair of primers complementary to the bisulfite-DNA modified sequence (Additional file 1, Additional file 2). The PCR products were purified with subsequent cloning into a plasmid vector. Plasmid DNA isolated from five positive bacterial clones was used for commercial sequencing. The results of bisulfite sequencing were assessed and presented using BiQ analyzer software and BDPC web server [23,24]. Black and grey boxes represent methylated and unmethylated CpG dinucleotide, respectively. Red rectangles correspond to regions amplified in HRM analysis by specific primers PHD3.1 (chr14: 34 419 922-34 420 080), PHD3.2 (chr14: 34 419 795- 34 419 935) and PHD3.3 (chr14: 34 419 400-34 419 538) (Additional file 1, Additional file 2). Bottom panels A and B represent HRM profiles of standard and example of patient DNA (patient P2 from bisulfite sequencing) PCR product. Methylation percentage of three DNA fragments within the *PHD3* CpG island was determined by Real Time PCR amplification of bisulfite treated standard and patient DNA, followed by comparison of their HRM profiles. DNA standards were prepared by mixing different ratios of methylated and non-methylated bisulfite treated DNA. HRM methylation analysis was performed using Light Cycler®480 Gene Scanning software, Roche Diagnostics GmbH (Mannheim, Germany). Each PCR amplification and HRM profile analysis was performed in triplicate.

HCT116 cells, we performed HRM analysis (Additional file 1, Additional file 2). We observed no DNA methylation of the promoter region of *PHD1*, *PHD2* and *FIH* gene in the analyzed regions using HRM analysis under hypoxic and normoxic conditions (Additional file 6).

The hypermethylated *PHD3* gene in HCT116 is not induced upon hypoxia conditions

To evaluate the association between DNA methylation of the *PHD3* gene and its expression in HCT116 and DLD-1 CRC cell lines we performed HRM analysis, RQ-PCR, and western blotting. We observed a high level of DNA methylation in HCT116 and no DNA methylation in

DLD-1 cells in the chr14: 34 419 922–34 420 080, chr14: 34 419 795–34 419 935 and chr14: 34 419 400–34 419 538 regions of *PHD3* gene CpG island using HRM analysis in both hypoxic and normoxic conditions (Figure 5A). We detected a lower level of PHD3 transcript and protein in HCT116 cells compared to DLD-1 cells in both hypoxic and normoxic conditions (Figure 5B, C). However, statistical significance in these differences occurred only under hypoxic conditions (Figure 5B, C). Moreover, we observed a statistically significant induction of *PHD3* transcript and protein level upon hypoxia in DLD-1 cells, with no changes in HCT116 cells under the same conditions (Figure 5B, C).

Table 2 Methylation level of the regulatory region of the *PHD3* gene in primary cancerous tissue and histopathologically unchanged tissue sample from patients with CRC
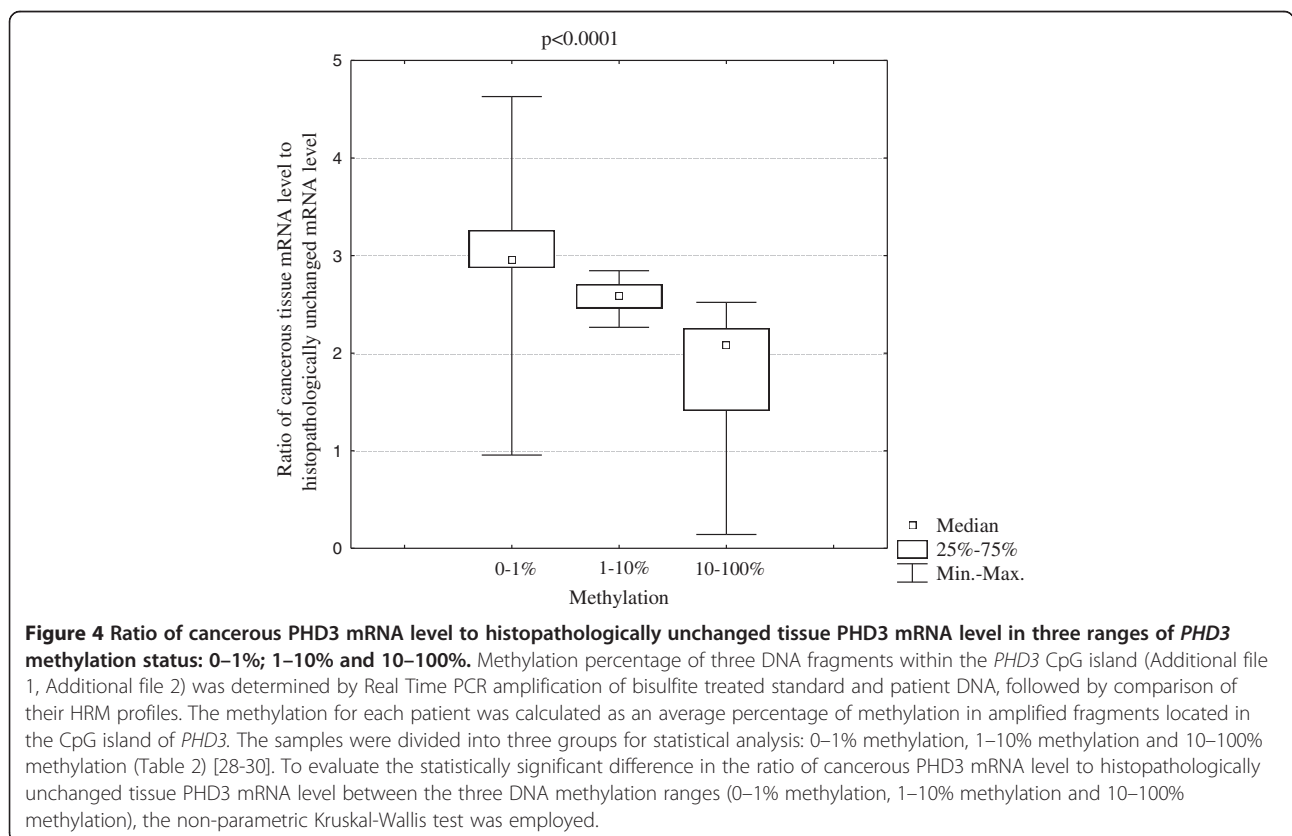
	Total no. of patients	< 1% methylation	1–10% methylation	10–100% methylation	p[a]
Histopathologically unchanged tissue	90	63	27	0	p < 0.00001
Primary cancerous tissue		37	31	22	

The primary cancerous and histopathologically unchanged tissue samples from the same patients were used for genomic DNA isolation, followed by bisulfite conversion of cytosine to uracil. The DNA fragments of the CpG island were then amplified by three pairs of primers complementary to the bisulfite-DNA modified sequence (Additional file 1, Additional file 2). To determine the percentage of methylation, the HRM profiles of the patients' DNA PCR products were compared to HRM profiles of the prepared standard PCR products (Figure 3). DNA methylation of the *PHD3* regulatory region for each patient was calculated as mean of the percentage of methylation in two DNA amplified fragments where the differences between primary cancerous and histopathologically unchanged tissue where observed. [a] Chi square test.

5-dAzaC induced DNA demethylation of *PHD3* promoter region, PHD3 transcript and protein contents in HCT116 cells, and did not affect *PHD3* DNA methylation or expression levels in DLD-1 cells under hypoxic and normoxic conditions
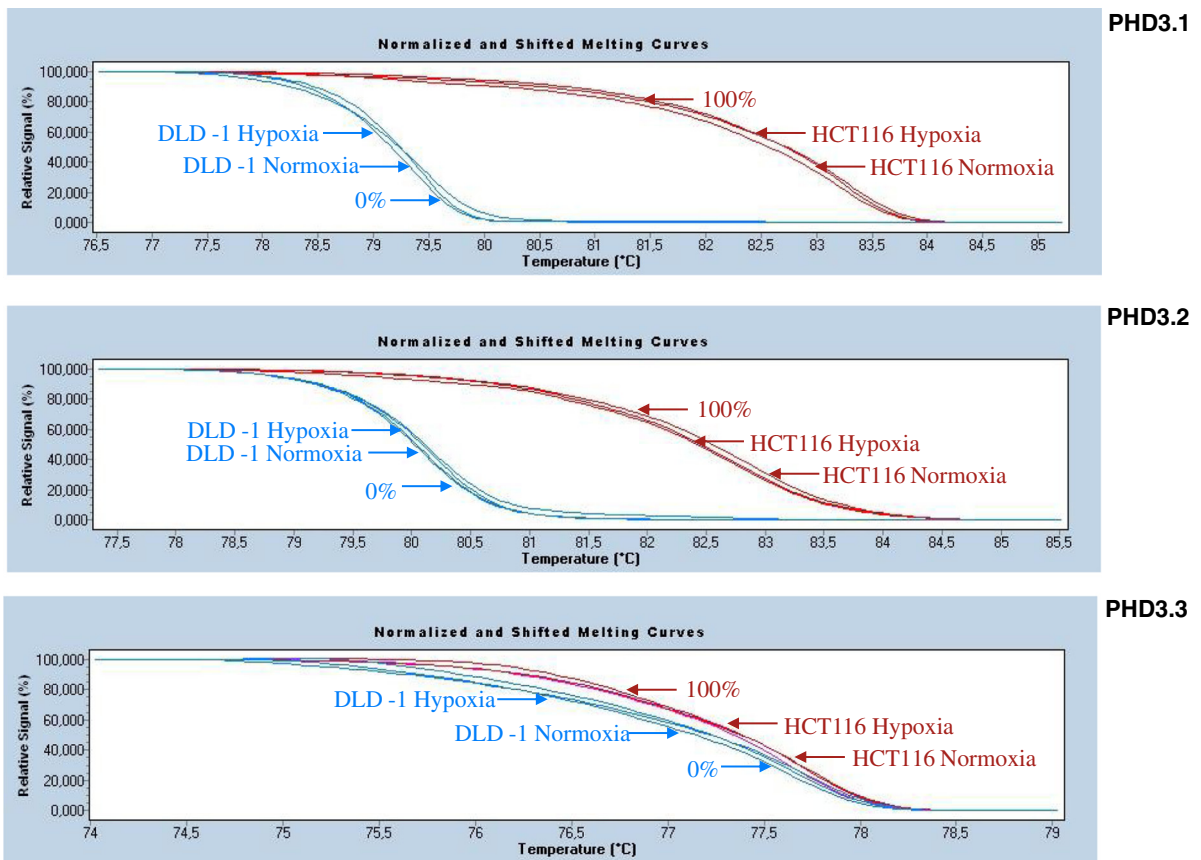
In order to assess the effect of 5-dAzaC on DNA methylation and *PHD3* gene expression levels we used HRM analysis, RQ-PCR, and western blotting. We observed no effect of 5-dAzaC treatment on the DNA methylation status in the analyzed regions of the *PHD3* promoter region in DLD-1 cells upon hypoxic and normoxic conditions (Figure 6A, B, C). On the contrary, using HRM analysis we noticed significant DNA demethylation in chr14: 34 419 922–34 420 080, chr14: 34 419 795–34 419 935 and chr14: 34 419 400–34 419 538 regions of the CpG island of the *PHD3* gene in HCT116 cells cultured for 48 hrs in the presence of 5.00 µM 5-dAzaC in both hypoxic and normoxic conditions (Figure 6A, B, C). The changes in DNA methylation level were accompanied by 5-dAzaC induced expression of *PHD3* in HCT116 cells. We observed that 5-dAzaC resulted in a progressive increase in PHD3 transcript levels in HCT116 cells and no significant changes for DLD-1 cells (Figure 7A). For HCT116 we found approximately a 2.45- and 2.59-fold significant increase in PHD3 transcript levels at 48 hrs of incubation under normoxic and hypoxic conditions, respectively (Figure 7A). Alterations in PHD3 transcript levels in HCT116 cells were associated with increased PHD3 protein
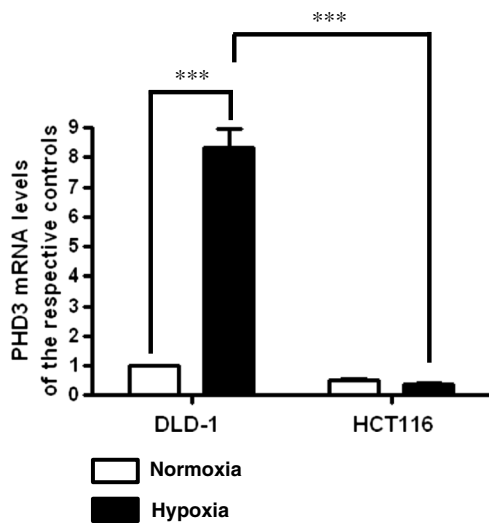

Figure 4 Ratio of cancerous PHD3 mRNA level to histopathologically unchanged tissue PHD3 mRNA level in three ranges of *PHD3* methylation status: 0–1%; 1–10% and 10–100%. Methylation percentage of three DNA fragments within the *PHD3* CpG island (Additional file 1, Additional file 2) was determined by Real Time PCR amplification of bisulfite treated standard and patient DNA, followed by comparison of their HRM profiles. The methylation for each patient was calculated as an average percentage of methylation in amplified fragments located in the CpG island of *PHD3*. The samples were divided into three groups for statistical analysis: 0–1% methylation, 1–10% methylation and 10–100% methylation (Table 2) [28-30]. To evaluate the statistically significant difference in the ratio of cancerous PHD3 mRNA level to histopathologically unchanged tissue PHD3 mRNA level between the three DNA methylation ranges (0–1% methylation, 1–10% methylation and 10–100% methylation), the non-parametric Kruskal-Wallis test was employed.
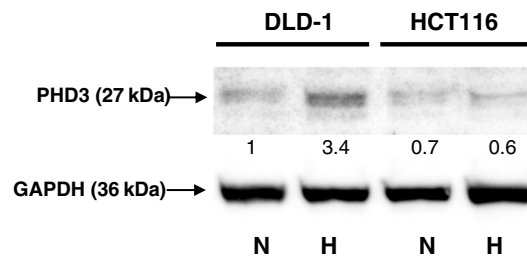
Figure 5 (See legend on next page.)

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Figure 5 DNA methylation and expression level of the *PHD3* gene in HCT116 and DLD-1 CRC cells. A. HCT116 and DLD-1 cells were cultured under normoxic or hypoxic (1% O$_2$) conditions for 48 hrs. Cells were then used for DNA isolation followed by bisulfite modification. Methylation percentage of three DNA fragments within the *PHD3* CpG island (Additional file 1, Additional file 2) in HCT116 and DLD-1 cells under hypoxic and normoxic conditions was determined by Real Time PCR amplification of bisulfite treated standard and cell line DNA, followed by comparison of their HRM profiles. **B.** Cells were cultured in DMEM either in hypoxic (1%O$_2$) or normoxic conditions for 48 hrs. After incubation, the cells were used for total RNA isolation and reverse transcription. The PHD3 cDNA levels were determined by RQ-PCR relative quantification analysis. RQ-PCR results were standardized by the geometric mean of PBGD and hMRPL19 cDNA levels. PHD3 cDNA levels are expressed as a multiplicity of these cDNA copies in the cell line's calibrator. **C.** Cells were cultured in DMEM either in hypoxic (1%O$_2$) (**H**) or normoxic (**N**) conditions for 48 hrs. Cells were then used for protein isolation. Proteins were separated by 10% SDS-PAGE, and transferred to a membrane that was then immunoblotted with Rp anti - PHD3 Ab and incubated with goat anti-rabbit HRP-conjugated Ab. The membrane was then stripped and reblotted with Rp anti-GAPDH Ab, followed by incubation with goat anti-rabbit HRP-conjugated Ab. The band densitometry readings were normalized to GAPDH loading control. The ratio of PHD3 to GAPDH for DLD-1 in normoxic conditions was assumed to be 1.
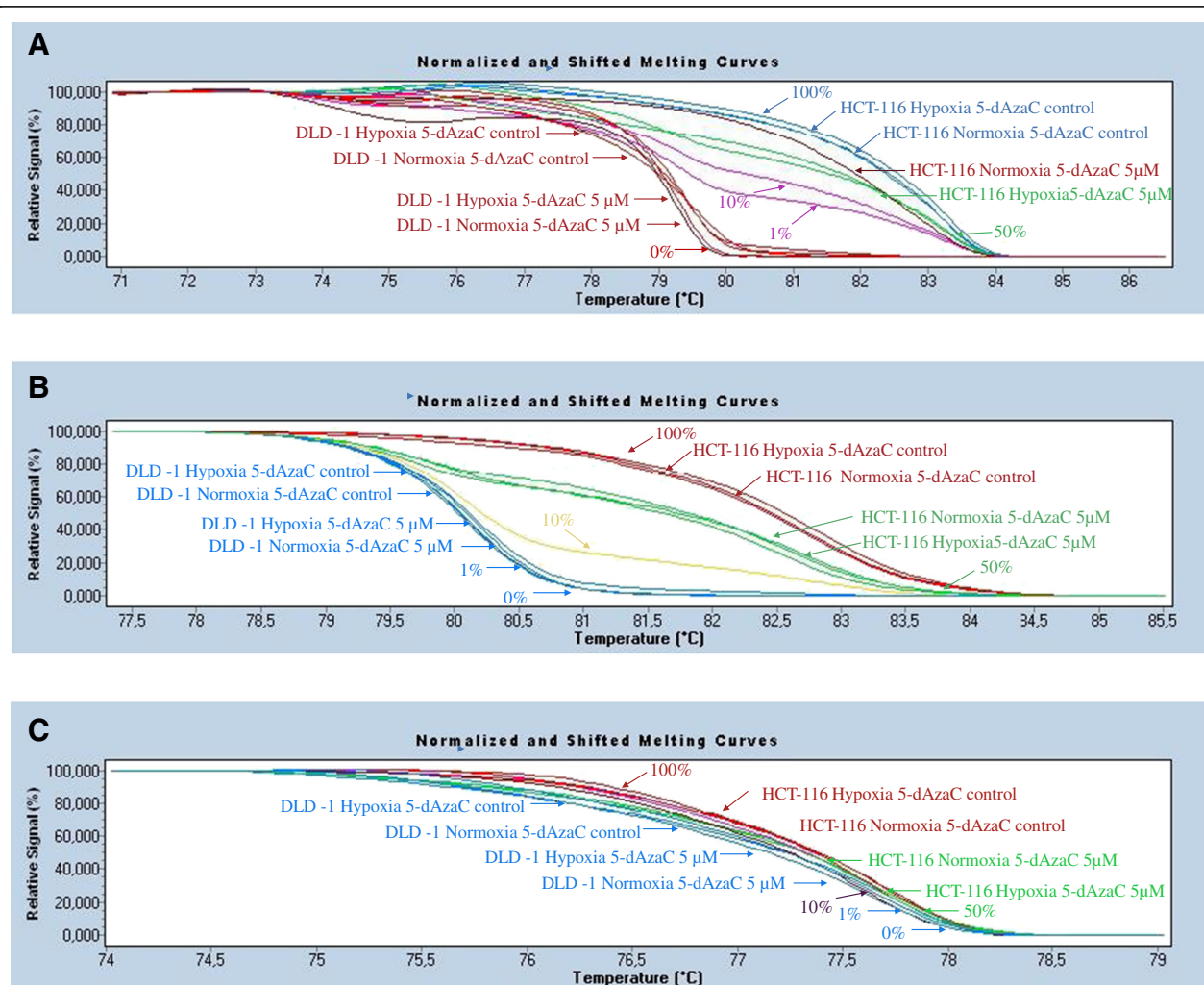
Figure 6 Effect of 5-dAzaC on *PHD3* gene DNA methylation in HCT116 and DLD-1 CRC cells. HCT116 and DLD-1 cells were cultured under normoxic or hypoxic (1% O$_2$) conditions either in the absence or in the presence of 5-dAzaC at a concentration of 5.00 μM for 48 hrs. Cells were then used for DNA isolation followed by bisulfite modification. Methylation percentage of three DNA fragments within the *PHD3* CpG island: **A** (chr14: 34 419 922–34 420 080), **B** (chr14: 34 419 795–34 419 935) and **C** (chr14: 34 419 400–34 419 538) (Additional file 1, Additional file 2) in HCT116 and DLD-1 cells under hypoxic and normoxic conditions was determined by Real Time PCR amplification of bisulfite treated standard and cell line DNA, followed by comparison of their HRM profiles.
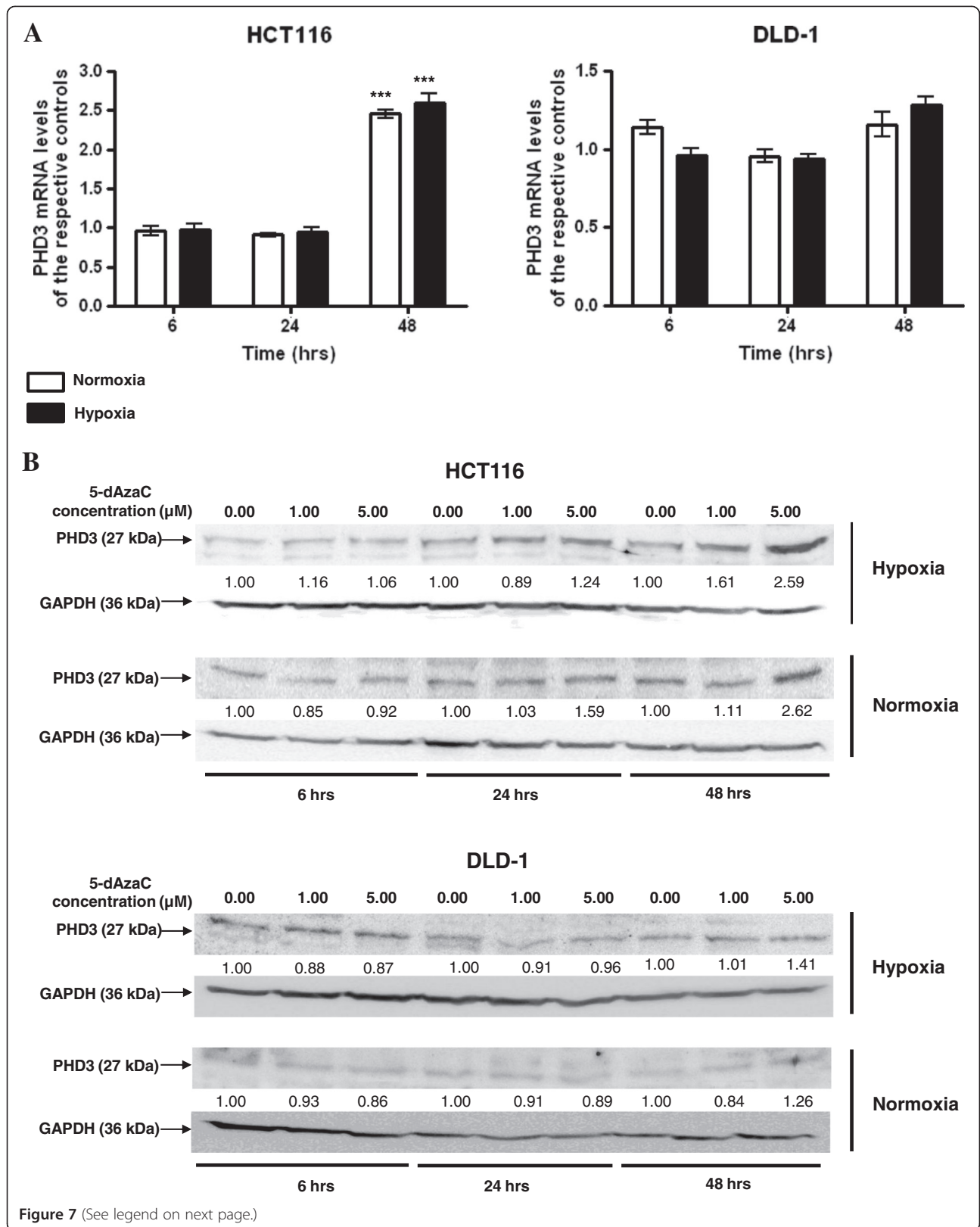
A

HCT116

DLD-1

Normoxia

Hypoxia

B

HCT116

DLD-1

Figure 7 (See legend on next page.)

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Figure 7 5-dAzaC effect on PHD3 transcript (A) and protein (B) levels in HCT116 and DLD-1 CRC cells. HCT116 and DLD-1 cells were cultured in DMEM for 6, 24 and 48 h either in the absence or in the presence of 5-dAzaC at a concentration of 1.00 or 5.00 μM under hypoxic or normoxic conditions. After incubation the cells were used for total RNA isolation and protein isolation. Total RNA was reverse-transcribed, and PHD3 cDNA levels were determined by RQ-PCR relative quantification analysis. RQ-PCR results were standardized by the geometric mean of PBGD and hMRPL19 cDNA levels. PHD3 cDNA levels are expressed as a multiplicity of the respective controls. Each sample was determined in triplicate and results are presented as the mean ± SE from three experiments *P < 0.05. The cell protein was separated by 10% SDS-PAGE, and transferred to a membrane that was then immunoblotted with Rp anti - PHD3 Ab and incubated with goat anti-rabbit HRP-conjugated Ab. The membrane was then stripped and reblotted with Rp anti-GAPDH Ab, followed by incubation with goat anti-rabbit HRP-conjugated Ab. The band densitometry readings were normalized to GAPDH loading control. The ratio PHD3 to GAPDH for control was assumed to be 1.

levels in both hypoxic and normoxic conditions (Figure 7B). Densitometric analysis of western blotting bands indicated an approximately 2.59- and 2.62- fold increase in PHD3 protein level in HCT116 cells incubated with 5.00 μM 5-dAzaC for 48 hrs as compared to the respective controls under hypoxic and normoxic conditions, respectively. Incubation of DLD-1 cells with 5-dAzaC at various concentrations for different time periods did not significantly increase PHD3 protein contents under either hypoxic or normoxic conditions (Figure 7B).

Discussion

The maintenance of oxygen homeostasis is a crucial physiological process [32]. Hypoxia is a common feature of human cancers, associated with higher aggressiveness and resistance to chemotherapy [33,34]. The cellular environment response to hypoxia is mediated by HIF, a heterodimeric transcriptional complex, which is post-translationally regulated by prolyl and asparaginyl hydroxylases. The first group, PHD enzymes, govern the ubiquitin- mediated mechanism of HIF-α degradation under normoxic conditions [3]. Since hydroxylation reactions require the presence of oxygen, dioxygenases are unable to function in hypoxic conditions. Thus, the HIF is not directed to proteasomal degradation and may exert its effect on genes during CRC progression. However, oxygen concentration is not the only cause of altered PHD function during tumorigenesis. The contribution of PHD to cell behaviour depends on several conditions, including the relative abundance of PHD proteins in a specific tissue [3]. Since changes in the amount of available PHD enzymes will alter the rate of hydroxylation of HIF-α, research has focused on the regulation of the quantity of PHDs. Aberrant expression of PHDs has been observed in a variety of human cancers. Detailed analysis of the expression patterns of all three PHD isoforms has shown differences depending on cancer cell type [7,35-37].

PHD1 protein levels were elevated in non-small cell type lung cancer (NSCLC) and pancreatic endocrine tumors [36,38]. Moreover, PHD1 inactivation resulted in lower levels of Cyclin D1 and impaired breast tumour formation [39]. However, it has been demonstrated that colon cancer cells expressing the *PHD1* gene cause inhibition of tumour growth and angiogenesis under hypoxic conditions when injected into nude mice [40].

PHD2 was also previously studied in various cancer types. Low levels of *PHD2* expression were correlated with poor survival rate in CRC and breast cancer [9,41]. Nonetheless, studies in head and neck squamous cell carcinoma (HNSCC) demonstrated an association between increased levels of nuclear PHD2 protein with strongly proliferative and malignant tumour phenotypes [42,43]. Furthermore, immunohistochemical studies have found that high *PHD2* and *PHD3* expression was significantly associated with worse 5-year survival rate in pancreaticobiliary cancer [44]. PHD3 transcript levels were shown to be increased in HNSCC, and *in vitro* analysis revealed inhibition of cell cycle progression in cancerous cells in the absence of PHD3 activity [45]. Elevated levels of PHD3 protein in cancerous tissue were also observed in immunohistochemical studies of NSCLC and gastric cancer [38,46]. However, PHD3 catalytic activity has demonstrated an ability to induce apoptosis independent of HIF in different cancer cell lines [12,47-49]. Additionally, Peurala et al. presented that increased PHD3 expression and was associated with an increased survival rate in breast cancer patients [41].

Discrepancies between *PHD1*, *PHD2* and *PHD3* experimental results might be partly explained by the demonstration that HIF and PHDs can form a feedback loop that limits hypoxic signaling in reoxygenated cells [50]. Moreover, these discrepancies imply a dual function of PHDs in the control of tumour progression that depends on cell type, other PHDs- interacting factors, and function independent of HIF. The HIF- independent functions of PHDs include PHD3 directed inhibition of phosphorylation of the inhibitor of κβ kinase β and activation of NF κβ in CRC, neuronal apoptosis and myoblast differentiation by PHD3, or control of myocardial development by PHD2 [8,51,52].

We found a statistically significant decrease in the expression levels of the *PHD1*, *PHD2* and *PHD3* genes in cancerous tissue compared to histopathologically unchanged colorectal tissue. Although we did not observe statistically significant differences in FIH transcript levels between cancerous and histopathologically unchanged

colorectal tissue, we found a significant increase of FIH protein in CRC. FIH, in addition to PHDs, hydroxylates HIF-α at a conserved asparagine residue. Through this modification, FIH prevents interaction of HIF with its transcriptional coactivators [5]. More importantly, FIH is able to suppress HIF activity under severe hypoxic conditions, where PHDs are inactive [53,54]. Additionally, FIH exerts HIF-independent functions by interaction with pVHL, histone deacetylases, p105, Notch 1, and SOCS box proteins [55-58]. FIH is widely expressed; however, its expression varies between tissue and cancer types [7]. To date, FIH protein overexpression was correlated with a more malignant phenotype and poor prognosis in pancreatic endocrine tumours and NSCLC, respectively [36,38]. Moreover, it has been established that FIH gene silencing reduced colon cancer cell proliferation in vitro and in vivo through the p53-p21 mediated pathway [54]. We observed a significant increase in the protein level of FIH in CRC tissue. This observation may result from E3 ligase activity of a member of seven in absentia homolog 1 (SIAH-1). Therefore, SIAH-1 facilitates ubiquitination and proteasomal degradation of FIH [59,60]. To date, SIAH-1 has been found to be widely distributed in human cell lines and tissues, including CRC, with a decreased expression in breast and hepatocellular cancer [61-63].

Since the prolyl and asparaginyl hydroxylases have so wide and profound an effect on tumorigenesis, studies on the regulation of the expression of these enzymes may help in our understanding of CRC progression. There are many factors involved in the development and occurrence of CRC, and they are classified as genetic, epigenetic and environmental [64-67]. One of the epigenetic mechanisms, namely altered DNA methylation in a gene's regulatory region, is observed from the early stages of lesions in aberrant crypt foci and hyperplastic polyps [68,69]. It has been shown that DNA hypermethylation of MLH1, MGMT, CDKN2A and many others is associated with CRC progression [67,70-72]. Our study is the first to investigate the methylation status of the promoter regulatory regions of PHD1, PHD2, PHD3 and FIH in primary cancerous tissue from patients with CRC, and HCT116, DLD-1 CRC cell lines. We did not observe DNA methylation within the CpG island of the PHD1, PHD2 and FIH gene promoter in either patients or cell lines. To date, the DNA methylation status of the promoter region of PHD1, PHD2, PHD3 and FIH was studied in a few cancers, including breast, prostate, cervical, melanoma, kidney and plasma cell neoplasia [12-15]. In cervical cancer cell lines the hypomethylation of the CpG island of the PHD2 gene promoter was associated with an increase in PHD2 expression [15]. Similarly to our results, no DNA methylation within the CpG island of PHD1, PHD2 and FIH has been observed

in breast and plasma cell neoplasia [13,14]. There was also no DNA methylation in the promoter region of the PHD3 gene in clinical samples from breast and prostate cancer [12,14]. We also observed no DNA methylation within the PHD3 gene using bisulfite sequencing in region chr14: 34 419 929–34 420 563 and HRM analysis in region chr14: 34 419 922–34 420 080 in a group of patients, which is consistent with the results of Huang et al. and Place et al. [12,14]. However, we found a significantly higher level of DNA methylation within the first exon and intron of the PHD3 gene (chr14: 34 419 346–34 419 943 using bisulfite sequencing; chr14: 34 419 795–34 419 935 and chr14: 34 419 400–34 419 538 using HRM analysis) in cancerous tissue compared to histopathologically unchanged tissue. To the best of our knowledge, DNA methylation within the chr14: 34 419 346–34 419 943, chr14: 34 419 795–34 419 935 and chr14: 34 419 400–34 419 538 regions was not previously analyzed in other studies. Data from Encyclopedia of DNA elements project showed that these regions are DNase I hypersensitive and able to bind various transcription factors, which suggests a promoter or enhancer activity [22]. Moreover, Pescador et al. (2005) identified a functional HRE within the first intron of the PHD3 gene and proposed a model of HIF-mediated hypoxic induction of PHD3 [73].

Since hypoxic conditions may induce global DNA hypomethylation in cancer cells, we investigated DNA methylation and expression levels of the PHD3 gene in HCT116 and DLD-1 cells under hypoxic and normoxic conditions [74]. We reported a high level of DNA methylation and no transcript and protein level changes under hypoxic and normoxic conditions in HCT116 cells. In the PHD3 gene promoter region in DLD-1 cells, we did not detect DNA methylation under either experimental condition, but we observed a significant induction of PHD3 transcript and protein level upon hypoxia. The PHD3 gene possesses in its promoter region a putative HRE and can be induced by HIF transcription factor complex under hypoxic conditions [35]. A lack of increase in PHD3 expression in HCT116 cells might be the result of DNA methylation of its promoter region in hypoxic conditions. To date, a decreased expression of PHD3 mRNA was correlated with high CpG island methylation status in plasma cell neoplasia and selected melanoma, prostate and mammary gland cancer cell lines [12,13].

In order to verify the role of DNA methylation within the CpG island of the PHD3 gene, we treated HCT116 and DLD-1 cells with 5-dAzaC under normoxic and hypoxic conditions. 5-dAzaC was previously shown to induce the expression of many genes in different types of cancer and inhibit the growth of CRC cells [75,76]. We observed significant DNA demethylation in the chr14: 34 419 922–34 420 080, chr14: 34 419 795–34

419 935 and chr14: 34 419 400–34 419 538 regions of the CpG island of the *PHD3* gene in HCT116 cells incubated with 5-dAzaC, which was correlated with an increase in PHD3 transcript and protein levels. The same regions were unmethylated in DLD-1 cells at different experimental conditions and 5-dAzaC did not affect *PHD3* gene expression.

The presence of DNA hypermethylation of the *PHD3* promoter region in a broad range of human cancers suggests its role in tumour survival. In glioblastoma cell lines, accumulation of PHD3 protected tumour cells against hypoxia-induced cell death via control of HIF [77]. However, reduction of *PHD3* expression by DNA methylation may allow for stable HIF directed cellular response during hypoxia [12]. Moreover, in a subset of breast, prostate, melanoma and renal carcinoma cell lines, HIF-1α accumulation during hypoxia was independent of DNA hypermethylation of the *PHD3* promoter region, which suggests its role in other pathways and hydroxylase independent function [12].

Conclusion

Our results showed increased DNA methylation levels in the CpG island of *PHD3* in CRC as compared to normal colonic epithelium from the same patients. These epigenetic alterations were associated with a significant decrease of PHD3 expression levels in patients with CRC. However, the reduction in *PHD1* and *PHD2* expression in cancerous tissue was not due to altered methylation within the CpG island in the promoter region of *PHD1* and *PHD2*, respectively. Therefore, other mechanisms might be responsible for the observed decreased expression levels of *PHD1* and *PHD2* in CRC patients. Furthermore, we observed an increased level of FIH protein in CRC, with no changes in the FIH transcript level between cancerous and histopathologically unchanged colonic mucosa. Additionally, we observed different statistical values of transcript and protein levels of the analyzed genes for subgroups of patients classified according to different features. However, a larger group of patient and deeper molecular investigation of these observations should be studied to determine whether the results within subgroups may be clinically important.

We also found that an inhibitor of DNMTs, 5-dAzaC, induced DNA demethylation of the *PHD3* promoter region, which was associated with increased transcript and protein levels in HCT116 cells under hypoxic and normoxic conditions.

Although we presented epigenetic transcriptional downregulation of *PHD3* gene expression in CRC patients and HCT116 CRC cell line for the first time, further studies are required to verify and determine the role of CpG island methylation in *PHD3* expression in CRC to a greater extent. Moreover, DNA methylation is often associated with other alterations in chromatin structure, namely histone acetylation. High levels of DNA methylation accompanied with a low degree of histone acetylation may decrease the transcription of many genes [78]. In consideration of this, we also need to determine the possible role of histone modifications in *PHD3* gene expression.

Additional files

Additional file 1: Primer sequences.

Additional file 2: Schematic representation of the CpG distribution within the 5' regulatory region of the *PHD1* (A), *PHD2* (B), *PHD3* (C) and *FIH* (D) genes.

Additional file 3: Detection of heterogeneous methylation with HRM analysis.

Additional file 4: PHD1 (A), PHD2 (B), PHD3 (C), FIH (D) transcript and protein levels in primary cancerous and histopathologically unchanged tissue samples from patients with CRC.

Additional file 5: Bisulfite sequencing of DNA CpG rich region of the *PHD1*, *PHD2* and *FIH* genes in primary tissue samples from patients with CRC.

Additional file 6: DNA methylation assessment of the *PHD1*, *PHD2* and *FIH* gene regulatory region by HRM analysis.

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