Chromatin, Gene, and RNA Regulation

Prognostic Potential of DNA Methylation and Transcript Levels of HIF1A and EPAS1 in Colorectal Cancer

Agnieszka Anna Rawluszko-Wieczorek1, Karolina Horbacka2, Piotr Krokowicz2, Matthew Misztal1, and Paweł Piotr Jagodziński1

Abstract

Hypoxic conditions during the formation of colorectal cancer may support the development of more aggressive tumors. Hypoxia-inducible factor (HIF) is a heterodimeric complex, composed of oxygen-induced HIFα and constitutively expressed HIFβ subunits, which mediates the primary transcriptional response to hypoxic stress. Among HIFα isoforms, HIF1α (HIF1A) and endothelial PAS domain–containing protein 1 (EPAS1) are able to robustly activate hypoxia-responsive gene signatures. Although posttranslational regulation of HIFα subunits is well described, less is known about their transcriptional regulation. Here, molecular analysis determined that EPAS1 mRNA was significantly reduced in primary colonic adenocarcinoma specimens compared with histopathologically nonneoplastic tissue from 120 patients. In contrast, no difference in HIF1A mRNA levels was observed between cancerous and noncancerous tissue. Bisulfite DNA sequencing and high-resolution melting analysis identified significant DNA hypermethylation in the EPAS1 regulatory region from cancerous tissue compared with nonneoplastic tissue. Importantly, multivariate Cox regression analysis revealed a high HR for patients with cancer with low EPAS1 transcript levels (HR, 4.91; 95% confidence interval, CI, 0.42–56.15; $P = 0.047$) and hypermethylated EPAS1 DNA (HR, 33.94; 95% CI, 2.84–405.95; $P = 0.0054$). Treatment with a DNA methyltransferase inhibitor, 5-Aza-2′-deoxycytidine (5-aza-dC/Decitabine), upregulated EPAS1 expression in hypoxic colorectal cancer cells that were associated with DNA demethylation of the EPAS1 regulatory region. In summary, EPAS1 is transcriptionally regulated by DNA methylation in colorectal cancer.

Implications: DNA methylation and mRNA status of EPAS1 have novel prognostic potential for colorectal cancer.

MoL Cancer Res; 12(8); 1112–27. ©2014 AACR

Introduction

Immense proliferation of tumor cells and their inadequate perfusion results in hypoxia, which is a hallmark of many solid tumors, including colorectal cancer (1). Mechanisms by which tumor cells alter their expression profile to adjust to low oxygen tension involve hypoxia-inducible factor (HIF; ref. 1). HIF is a heterodimeric transcription factor assembled from α and β subunits. It recognizes the hypoxia response element (HRE) and promotes expression of many genes involved in glucose metabolism, angiogenesis, or metastasis (1). The β subunit of HIF is constitutively expressed, whereas HIFα is mainly controlled by a posttranslational mechanism (1, 2). In normoxic conditions, HIFα is hydroxylated at specific residues, which results in proteasomal degradation (2). There are three α isoforms referred to as: HIF1α, HIF2α (officially designated as endothelial PAS domain–containing protein 1—EPAS1) and HIF3α, which are encoded by the HIF1A, EPAS1, and HIF3A genes, respectively (2). Among them, HIF1α or EPAS1 may bind together with β and other coactivators to HRE and activate HIF-dependent gene transcription (2). Many articles describe aberrant HIF1α or EPAS1 protein levels and their association with colorectal cancer prognosis (3–10). However, there are only few articles about the same issues on the transcriptional regulation of HIF1A and EPAS1. It should be noted that both of them possess a CpG island in their promoter region. DNA methylation within the CpG island associates the gene transcriptional repression, and aberrant DNA methylation patterns are observed during colorectal tumorigenesis (13). To date, only one article indicates DNA methylation of HIF1A in colorectal cancer (14), and there is no scientific reports about this type of epigenetic regulation of EPAS1 expression. Therefore, we aimed to examine DNA methylation and mRNA levels of the HIF1A and EPAS1 genes in...
primary cancerous and histopathologically unchanged colorectal tissues from the same 120 patients. Moreover, we evaluated the impact of EPAS1 DNA methylation and transcript level in colorectal cancer tissue with respect to patient survival. We also assessed the effect of 5-Aza-2'-deoxycytidine (5-dAzaC), an inhibitor of DNA methyltransferases, on DNA methylation level of the EPAS1 gene and on the EPAS1 transcript, as well as protein level in HCT116 and DLD-1 colorectal cancer cells under hypoxic and normoxic conditions.

Materials and Methods

Antibodies and reagents
Rabbit polyclonal (Rp) anti-HIF1α (NB100-449) and anti-EPAS1 (NB100-122) Abs were provided by Novus Biologicals. Rp anti-GAPDH Ab (FL-335) and goat anti-rabbit horseradish peroxidase (HRP)–conjugated Ab were provided by Santa Cruz Biotechnology. 5-dAzaC was purchased from Sigma-Aldrich Co.

Patient material
Primary colonic adenocarcinoma tissues were collected between June 2009 and March 2013 from 120 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). The histopathologically unchanged colonic mucosa located at least 10 to 20 cm away from the cancerous lesions was obtained from the same patients. One set of samples was immediately snap-frozen in liquid nitrogen and stored at −80°C until DNA/RNA isolation. The other set of samples was directed for histopathologic examination. Histopathologic classification was performed by an experienced pathologist. No patients received preoperative chemo- or radiotherapy. An 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.

Measurement of overall and disease-free survival
Follow-up data were available for 80 patients, who were observed from August 11, 2009, until death or October 15, 2013, whichever came first. Nine patients were excluded from further analysis because they did not fulfill criteria given below. Disease-free survival (DFS) is defined as the time elapsed from surgery to the first occurrence of any of the following events: recurrence or distant metastasis of colorectal cancer, development of a second noncolorectal malignancy. In overall survival (OS) analysis, deaths from any cause without clinical documentation of cancer related event were excluded from the study.

Cell culture
DLD-1 colon cancer cells were obtained from the ATCC, 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 containing 10% heat-inactivated FBS and 2 mmol/L glutamine. To determine the effect of 5-dAzaC on DNA methylation, transcript and protein level of the HIF1A and EPAS1 genes, the HCT116 and DLD-1 cells were cultured for 24 hours in DMEM GibcoBRL supplemented with 10% FBS from Sigma-Aldrich Co. Cells were then cultured under normoxic or hypoxic (1% O2) conditions, either in the absence or in the presence of 5-dAzaC. 5-dAzaC was at concentrations of 1.00 and 5.00 m mol/L for 6, 24, and 48-hour time frames. Hypoxic conditions were achieved using a MCO-18M multi-gas cell culture incubator, Sanyo, modified to permit flushing the chamber with a humidified mixture of 5% CO2, 94% N2. These cells were used for DNA and RNA isolation, quantitative real-time PCR (qRT-PCR), Western blotting, and high-resolution melting (HRM) analysis.

DNA isolation and bisulfite modification
Genomic DNA from tissues of patients with colorectal cancer and cell lines were isolated using the DNA Mammalian Genomic Purification Kit purchased from Sigma-Aldrich Co. A total of 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. The position of the CpG islands and binding sites of transcription factors located in the HIF1A and EPAS1 promoter were determined by online programs (15–17).

Table 1. Demographic and histopathologic classification of patients with colorectal cancer

<table>
<thead>
<tr>
<th>Features</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>120</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>54/66</td>
</tr>
<tr>
<td>Mean (±SD) age at radical surgical resection of colon (y)</td>
<td>67.94 ± 12.45</td>
</tr>
<tr>
<td>Colorectal cancer localization</td>
<td></td>
</tr>
<tr>
<td>Proximal colon (cecum to transverse)</td>
<td>45</td>
</tr>
<tr>
<td>Distal colon (splenic flexure to sigmoid)</td>
<td>22</td>
</tr>
<tr>
<td>Rectum</td>
<td>53</td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>7</td>
</tr>
<tr>
<td>G2</td>
<td>77</td>
</tr>
<tr>
<td>G3</td>
<td>36</td>
</tr>
<tr>
<td>TNM classification</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>17</td>
</tr>
<tr>
<td>IIA</td>
<td>47</td>
</tr>
<tr>
<td>IIC</td>
<td>6</td>
</tr>
<tr>
<td>IIIA</td>
<td>3</td>
</tr>
<tr>
<td>IIIB</td>
<td>34</td>
</tr>
<tr>
<td>IIIC</td>
<td>13</td>
</tr>
</tbody>
</table>
DNA methylation evaluation by bisulfite sequencing

The DNA fragments containing CpG dinucleotides located in the promoter region of the HIF1A and EPAS1 genes were amplified from the bisulfite-modified DNA by the primer pairs (Supplementary Table S1A) complementary to the bisulfite DNA-modified sequences in 5 patients. Clinicalopathologic parameters for these patients are given in Supplementary Table S1B. PCR amplification was performed by FastStart Taq DNA Polymerase from Roche Diagnostic GmbH. The PCR products were purified using the Agarose Gel DNA Extraction Kit, Roche Diagnostic GmbH with subsequent cloning into pGEM-T Easy Vector System I, Promega and transformation into TOP10 E. coli strain cells. The 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 (18, 19).

DNA methylation assessment by HRM analysis

Methylation level of DNA fragments located within the CpG island of the HIF1A and EPAS1 genes was determined by RT-PCR amplification of bisulfite-treated DNA, followed by HRM profile analysis by Light Cycler480 Real-Time PCR System, Roche Diagnostics GmbH. For PCR amplification, 1 μL of the bisulfite-treated DNA from patients, HCT116, DLD-1 cells, or standards, and primers (Supplementary Table S1A) was added to 19 μL of 5X Hot FIREPol EvaGreen HRM Mix, Solis BioDyne Co. Standardized solutions of DNA methylation percentage were prepared by mixing methylated and nonmethylated bisulfite-treated DNA from Human Methylated/Non-methylated DNA Set, Zymo Research Corp. in different ratios. To determine the percentage of methylation, the HRM profiles of patients DNA PCR products were compared with HRM profiles of standard DNA PCR products (20, 21). HRM methylation analysis was performed using Light Cycler480 Gene Scanning software, Roche Diagnostics GmbH. Each PCR amplification and HRM profile analysis was performed in triplicate. The HRM results were compared with those obtained from bisulfite sequencing for analyzed genes in reconstituted samples. A similar pattern of DNA methylation was observed between these two methods. The methylation for each patient was presented as a percentage of methylation in amplified fragments located in the CpG island of HIF1A and EPAS1. Because low level of methylation may not demonstrate significant biologic effects and we were not able to quantify all the CpG dinucleotides within the analyzed CpG island, the percentage results were divided into three groups: 0% to 1% methylation, 1% to 10% methylation, and 10% to 100% methylation for statistical analysis (22–25).

Reverse transcription and quantitative real-time PCR analysis

Total RNA from tissues of patients with colorectal cancer and cell lines were isolated according to the method of Chomczynski and Sacchi (26). The RNA samples were quantified and reverse-transcribed into cDNA, qRT-PCR was carried out in the Light Cycler480 Real-Time PCR System, Roche Diagnostics GmbH using SYBR Green I as the detection dye. The target CDNA was quantified by the relative quantification method using a calibrator for the primary tissues. 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. For amplification, 1 μL of (total 20 μL) CDNA solution was added to 9 μL of IQ SYBR Green Supermix, Bio-Rad Laboratories Inc. with primers (Supplementary Table S1A). 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. The quantity of HIF1A and EPAS1 transcripts in each sample was standardized by the geometric mean of two internal controls: porphobilinogen deaminase (PGBD) and human mitochondrial ribosomal protein L19 (hMRPL19; Supplementary Table S1A). The selection of internal control genes was made as previously (27). The HIF1A and EPAS1 transcript levels in the patients’ tissues were expressed as multiplicity of the cDNA concentrations in the calibrator. In HCT116 and DLD-1 cells, transcript levels were presented as multiplicity of the respective controls.

Western blotting analysis

HCT116 and DLD-1 cells were treated with lysis RIPA buffer, and proteins were resuspended in the sample buffer and separated on 10% Tris-glycine gel using 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 Rpi anti-HIF1α and -EPAS1 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 Rpi anti-GAPDH Ab (FL-335), followed by incubation with goat anti-rabbit HRP-conjugated Ab. The bands were revealed using SuperSignal West Femto Chemiluminescent Substrate, Thermo Fisher Scientific, and Biospectrum Imaging System 500, UVP Ltd. The amount of analyzed proteins was 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 EPAS1 to GAPDH was assumed to be 1.

Statistical analysis

The normality of the observed patient data distribution was assessed by the Shapiro–Wilk test, and the Mann–Whitney U test was used to compare the median values. The χ² test was used to examine the significance in DNA methylation. Fisher exact probability test was used for data that do not fulfilled criteria for Cochran theorem. To evaluate the association between different ranges of DNA methylation the χ² test was used for data that do not fulfilled criteria for Cochran theorem. To evaluate the association between different ranges of DNA methylation, the analysis was performed by the inverse variance weighting method.
DNA Methylation and mRNA Level of HIF1A and EPAS1 in Colorectal Cancer

EPAS1 mRNA level, the nonparametric Kruskal–Wallis test was used. Survival curves were plotted using the Kaplan–Meier method, and survival differences were achieved using the log-rank test. Multivariate Cox proportional hazard model was used to estimate the adjusted HR. Data groups for cell lines were assessed by ANOVA to evaluate if there were significant differences (P < 0.05) between the groups. For all experimental groups that fulfilled the initial criteria, 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 10.0 software.

Results
DNA hypermethylation of EPAS1 regulatory region is associated with a decrease in EPAS1 mRNA level in primary cancerous tissue compared with histopathologically unchanged tissue from patients with colorectal cancer, whereas there is neither DNA methylation nor transcript changes of HIF1A.

To compare the HIF1A and EPAS1 transcript and DNA methylation levels in the HIF1A and EPAS1 promoter regions in cancerous and histopathologically unchanged tissues from 120 patients with colorectal cancer, we used RQ-PCR and bisulfite DNA sequencing followed by HRM analysis, respectively. We found significantly lower levels of the EPAS1 transcript (P = 0.000011) in primary cancerous than in the histopathologically unchanged tissues in patients with colorectal cancer (Fig. 1A). Moreover, we observed significantly lower levels of the EPAS1 transcript in cancerous tissues in different age groups, genders, colorectal cancer localizations, histologic grades, and tumor–node–metastasis (TNM) stages (Supplementary Table S2). There was no significant difference in the level of the HIF1A transcript between primary cancerous and histopathologically unchanged tissues in 120 patients with colorectal cancer (P = 0.87; Fig. 1A). We also undetected DNA methylation within the HIF1A promoter in the analyzed regions (chr14: 62 161 804–62 162 333 and chr14: 62 162 250–62 163 074 using bisulfite sequencing; chr14: 62 161 655–62 161 825 and chr14: 62 162 301–62 162 427 using HRM analysis; Fig. 1B and C). Moreover, we did not observe DNA methylation in the regulatory region of the EPAS1 gene in cancerous and histopathologically unchanged tissues in regions chr2: 46 524 336–46 524 767 and chr2: 46 524 751–46 525 189 using bisulfite sequencing; chr2: 46 524 636–46 524 769 and chr2: 46 524 969–46 525 075 using HRM analysis (Fig. 1D and E). However, study of EPAS1 gene regulatory region chr2: 46 526 521–46 527 161 revealed significant DNA hypermethylation in cancerous tissue compared with histopathologically unchanged tissue, using bisulfite sequencing in 5 patients (Fig. 1F). In keeping with the bisulfite sequencing data, we observed significantly higher DNA methylation within EPAS1 regulatory region chr2: 46 526 762–46 526 905 in cancerous compared with histopathologically unchanged tissue from 120 patients with colorectal cancer (P < 0.00001; Fig. 1F and Table 2). Patients were also stratified by gender, age, histologic grades, and TNM stages for DNA methylation analysis. We observed higher DNA methylation within the analyzed region of the EPAS1 CpG island in primary cancerous tissue for a majority of subgroups and no distinctive subgroup biased DNA methylation (Supplementary Table S3). Moreover, we observed that an increase in DNA methylation level of EPAS1 in region chr2: 46 526 762–46 526 905 correlated to a decrease in the ratio of cancerous to histopathologically unchanged tissue EPAS1 mRNA level (P = 0.0036; Fig. 2).

DNA hypermethylation and low mRNA level of the EPAS1 gene are prognostic factors for patients’ OS with colorectal cancer

To investigate the effect of transcript and DNA methylation level of EPAS1 on patients’ survival, we carried out retrospective clinical analysis of 71 patients. The median survival was 36 months (range, 9–51 months). On the basis of RQ-PCR data, the EPAS1 mRNA level in histopathologically unchanged and cancerous tissue was subdivided into three groups: low, intermediate, and high EPAS1 transcript levels. Univariate analysis of OS revealed that patients with low mRNA expression level of EPAS1 in histopathologically unchanged tissue had a significant increase in risk of death compared with patients with an intermediate and/or high expression level (Fig. 3A). This related to survival: 33 months in low EPAS1 mRNA subgroup versus 36 in intermediate and high EPAS1 mRNA subgroups (Fig. 3A). Moreover, the Kaplan–Meier analysis revealed benefit of a high EPAS1 transcript level in histopathologically unchanged tissue of a 7-month median increase in survival compared with the intermediate and 14-month increase compared with the low EPAS1 mRNA subgroup in patients not treated with postoperative chemotherapy (Fig. 3B). Analysis of cancerous tissue disclosed lack of impact of EPAS1 mRNA level on OS (Fig. 3A and B). Furthermore, there was no evidence of impact of EPAS1 mRNA level on DFS in both the histopathologically unchanged and cancerous tissues (Supplementary Fig. S1). Impact of DNA methylation of EPAS1 regulatory region was done by comparison of two groups: absent DNA methylation and present DNA methylation in the EPAS1 gene regulatory region. Of note, 1% to 10% and 10% to 100% subgroups were merged into one because of limited number of patients in 10% to 100% subgroup (n = 4). We found that patients with DNA hypermethylation of EPAS1 in cancerous tissue compared with histopathologically unchanged had shorter OS rate compared with patients with no changes in DNA methylation status (Fig. 3C). Although, result was statistically insignificant, it suggests that there may have been a reduction in the risk of death for patients with the hypomethylated EPAS1 gene regulatory region. The analysis in the group of patients without postoperative chemotherapy and analysis of impact of EPAS1 DNA methylation on DFS did not reveal statistically significant data (Fig. 3C and D; Supplementary Fig. S1). However, multivariate Cox regression analysis with...
Figure 1. DNA methylation of promoter region and transcript levels of HIF1A and EPAS1 in primary cancerous and histopathologically unchanged tissues from patients with colorectal cancer. The cancerous and histopathologically unchanged tissues from 120 patients with colorectal cancer were used for RNA and DNA isolation. A, total RNA was reverse-transcribed, and cDNAs were investigated by RQ-PCR relative quantification analysis. The HIF1A and EPAS1 mRNA levels were corrected by the geometric mean of PBGD and hMRPL19 cDNA levels. The amounts of mRNA were expressed as the decimal logarithm of multiples of these cDNA copies in the calibrator. The P value was evaluated by the Mann–Whitney U test. (Continued on the following page.)
Figure 1. (Continued.) B to F, primary cancerous and histopathologically unchanged tissues from the same patients with colorectal cancer (P1–P5; Supplementary Table S1B) were used for genomic DNA isolation followed by bisulfite conversion of cytosine to uracil. The **HIF1A** regions containing 49 CpG dinucleotides (chr14: 62,161,804-62,162,333; B) and 70 CpG dinucleotides (chr14: 61,162,250-62,163,074; C) as well **EPAS1** regions containing 49 CpG dinucleotides (chr2: 46,524,336-46,524,767; D), 44 CpG dinucleotides (chr2: 46,524,751-46,525,189; E), and 37 CpG dinucleotides (chr2: 46,526,521-46,527,161; F) were then amplified by a pair of primers complementary to the bisulfite DNA-modified sequence (Supplementary Table S1A). (Continued on the following page.)
respect to age, gender, and postoperative chemotherapy status revealed that mRNA level and DNA methylation are both independent prognostic factors for patient’s survival (Table 3). Low EPAS1 mRNA level in histopathologically unchanged tissue and DNA hypermethylation in cancerous tissue compared with histopathologically unchanged have significant HR equal to 4.91 and 33.94, respectively (Table 3). Neither EPAS1 mRNA status nor DNA methylation were associated with DFS in multivariate analysis (Table 3).

EPAS1 gene regulatory region is hypermethylated in HCT116 colorectal cancer cells in normoxic and hypoxic conditions

To evaluate DNA methylation and expression level of the HIF1A and EPAS1 genes in HCT116 and DLD-1 colorectal cancer cells, we performed HRM analysis, RQ-PCR, and Western blotting. We observed no DNA methylation of the HIF1A promoter region in the analyzed regions using HRM analysis under hypoxic and normoxic conditions in HCT116 and DLD-1 cells (Fig. 4A). Moreover, we detected DNA hypomethylation in the EPAS1 CpG island in DLD-1 cells (Fig. 4A). Nonetheless, we detected a high level of DNA methylation in HCT116 in the chr2: 46 526 762- 46 526 905 and no DNA methylation in chr2: 46 524 636-46 524 769 and chr2: 46 524 969-46 525 075 (Fig. 4A). We revealed a lower level of the HIF1A and EPAS1 transcript in HCT116 cells compared with DLD-1 cells in both hypoxic and normoxic conditions (Fig. 4B). The HIF1A transcript level was not induced upon hypoxia in both cell lines (Fig. 4B). However, we observed a significant induction of the EPAS1 transcript level upon hypoxia in DLD-1 cells, with no changes in HCT116 cells under the same conditions (Fig. 4B). In both analyzed cell lines, hypoxic conditions induced HIF1α and EPAS1 protein level (Fig. 4B).

5-dAzaC induced DNA demethylation of EPAS1 gene regulatory region, EPAS1 transcript, and protein contents in HCT116 cells; did not affect EPAS1 DNA methylation or expression level in DLD-1 cells under hypoxic conditions

To assess the effect of 5-dAzaC on DNA methylation and the EPAS1 gene expression level, we used HRM analysis, RQ-PCR, and Western blotting. We observed no effect of 5-dAzaC treatment on DNA methylation status in the analyzed region of the EPAS1 promoter in DLD-1 cells under hypoxic and normoxic conditions (Fig. 4C). On the
studies reported a correlation between the HIF1 metabolism and pH homeostasis, thereby reinforcing tumor glioma cancers (28 in breast, head and neck, cervix, gastric, hepatocellular, and expression of oncogenes and genes involved in angiogenesis protein and patient survival, response to chemotherapy, results are inconclusive. HIF1 protein was also determined in colorectal cancer, but the

### Discussion

HIFα initiates adaptive responses that maintain proper metabolism and pH homeostasis, thereby reinforcing tumor growth and metastasis (1). Many immunohistochemical studies reported a correlation between the HIF1α or EPAS1 protein and patient survival, response to chemotherapy, expression of oncogenes and genes involved in angiogenesis in breast, head and neck, cervix, gastric, hepatocellular, and glioma cancers (28–32). The level of HIF1α and EPAS1 protein was also determined in colorectal cancer, but the results are inconclusive. HIF1α was correlated with poor patient prognosis by three independent studies, but three consecutive articles showed lack of such association in colorectal cancer (3–9). Moreover, Yoshimura and colleagues demonstrated strong positive immunohistochemical staining of EPAS1 in advanced colorectal cancer compared with low-grade tumors (6). Nonetheless, a study conducted by two other research teams described the opposite results (9, 10). The HIF1A and EPAS1 transcript level was not analyzed extensively. In the esophageal squamous cell carcinoma, pancreatic, gastric, cervical and colon cancers,
Histopathologically unchanged tissue

A

Event Censored

Cancerous tissue

Event Censored

Histopathologically unchanged tissue

N Events Median OS

Low EPAS1 mRNA 15 4 36
Int EPAS1 mRNA 39 2 36
High EPAS1 mRNA 17 1 33

Cancerous tissue

N Events Median OS

Low EPAS1 mRNA 18 2 36
Int EPAS1 mRNA 36 5 34.5
High EPAS1 mRNA 17 0 37

B

Histopathologically unchanged tissue

N Events Median OS

Low EPAS1 mRNA 9 3 24
Int EPAS1 mRNA 22 4 33
High EPAS1 mRNA 11 1 38

Cancerous tissue

N Events Median OS

Low EPAS1 mRNA 11 1 35
Int EPAS1 mRNA 21 4 21
High EPAS1 mRNA 10 0 36.5

C

DNA methylation of EPAS1 present
DNA methylation of EPAS1 absent

N Events Median OS

DNA methylation of EPAS1 present 47 3 36
DNA methylation of EPAS1 absent 24 4 33

DNA methylation of EPAS1 present
DNA methylation of EPAS1 absent

N Events Median OS

DNA methylation of EPAS1 present 25 3 33
DNA methylation of EPAS1 absent 17 2 31
HIF1α mRNA levels were increased in cancerous compared with noncancerous tissue (8, 11, 12, 33, 34). However, other studies reported a constant HIF1A mRNA level in tumor cells and suggested mainly posttranslational regulation of HIF1A expression (35–37), which is consistent with our observations. We have not detected significant HIF1A transcript changes between cancerous and histopathologically unchanged tissues isolated from 120 patients. Only one publication described a correlation of elevated EPAS1 gene expression (35) in colorectal cancer with low EPAS1 mRNA level in histopathologically unchanged tissues, which supports the idea of EPAS1 as having a tumor-protective role. Obviously, large multicenter studies on various patient populations with extended follow-up need to confirm these results.

Data suggest that the importance of HIF1α and EPAS1 in response to hypoxia may differ among tumor types and different stages of tumor progression. Moreover, recent articles indicate that these two HIFα subunits exhibit distinct roles in hypoxic conditions (31). HIF1α and EPAS1 may regulate the expression of many of the same target genes, but each has unique responsive genes as well (40). The mechanism responsible for the activation of HIF1α- or EPAS1-specific target genes seems to be the interaction of N-transactivation domain of HIF protein with different coactivators (41, 42). In renal cell carcinoma, tumor progression and metastasis were predominantly dependent on EPAS1 (43). However, an in vitro study in colorectal cancer cells revealed the induction number of genes associated with glycolysis and angiogenesis by HIF1α and tumor-suppressor genes such as cyclin G2 or angiopoietin-like 4 by EPAS1 (10).

Xenograft studies support the hypothesis of a protective function of EPAS1 in colorectal cancer. Silencing of EPAS1 expression in a mouse model is associated with a more intensive development of colorectal cancer (10). In a KRAS-driven non–small cell lung carcinoma mouse model, the loss of EPAS1 expression resulted in increased tumor growth and progression (39). Moreover, siRNA knockdown of EPAS1 reduced apoptosis in glioblastoma cells (44). Our observation illustrated an increased risk of death in patients with low EPAS1 mRNA level in histopathologically unchanged tissues, which supports the idea of EPAS1 as having a tumor-protective role. Obviously, large multicenter studies on various patient populations with extended follow-up need to confirm these results.

For the first time, we examined a relationship between epigenetic silencing of EPAS1 and clinical prognosis of patients with colorectal cancer. We found that a reduced EPAS1 mRNA level was associated with DNA methylation and mRNA level of HIF1A and EPAS1 in Colorectal Cancer.
hypermethylation in cancerous tissues, and EPAS1 DNA methylation was a prognostic factor for patient survival in multivariate Cox regression analysis. An investigated fragment of the EPAS1 CpG island is the region of a transcription factor binding and different types of epigenetic modifications such as histone acetylation (17). Hence, it may be

Figure 4. DNA methylation, expression level of the HIF1A and EPAS1 genes as well effect of 5-dAzaC on DNA methylation and expression of EPAS1 in HCT116 and DLD-1 colorectal cancer cells. HCT116 and DLD-1 cells were cultured under normoxic or hypoxic (1% O2) conditions for 48 hours. Cells were then used for DNA isolation followed by bisulfite modification, RNA and protein isolation. A, methylation percentage of DNA fragments within the HIF1A and EPAS1 CpG island (Supplementary Table S1A) in HCT116 and DLD-1 cells under hypoxic and normoxic conditions was determined by RT-PCR amplification of bisulfite-treated standard and cell line DNA, followed by comparison of their HRM profiles. (Continued on the following page.)
Figure 4. (Continued.) B, cells were cultured in DMEM either in hypoxic (1% O2; H) or normoxic (N) conditions for 48 hours. After incubation, the cells were used for total RNA isolation followed by reverse transcription and protein isolation. The HIF1A and EPAS1 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. HIF1A and EPAS1 cDNA levels are expressed as a multiplicity of these cDNA copies in the cell line’s calibrator. Proteins were separated by 10% SDS-PAGE, and transferred to a membrane that was then immunoblotted with specific primary and secondary Ab. The band densitometry readings were normalized to GAPDH loading control. The ratio of HIF1α or EPAS1 to GAPDH for DLD-1 in normoxic conditions was assumed to be 1. C, HCT116 and DLD-1 cells were cultured under normoxic or hypoxic (1% O2) conditions either in the absence or in the presence of 5-dAzaC at a concentration of 5.00 μmol/L for 48 hours. Cells were then used for DNA isolation followed by bisulfite modification. Methylation percentage of DNA fragment within the EPAS1 CpG island (chr2: 46,526,762-46,526,905) in HCT116 and DLD-1 cells under hypoxic and normoxic conditions was determined by RT-PCR amplification of bisulfite-treated standard and cell line DNA, followed by comparison of their HRM profiles. (Continued on the following page.)
Figure 4. (Continued.) D, HCT116 and DLD-1 cells were cultured in DMEM for 6, 24, and 48 hours either in the absence or in the presence of 5-dAzaC at a concentration of 1.00 or 5.00 μmol/L under hypoxic or normoxic conditions. After incubation, the cells were used for total RNA isolation and protein isolation. Total RNA was reverse-transcribed, and EPAS1 cDNA levels were determined by RQ-PCR relative quantification analysis. EPAS1 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 < 0.01; *** P < 0.001. The cell protein was separated by 10% SDS-PAGE, and transferred to a membrane that was then immunoblotted with specific primary and secondary Ab. The band densitometry readings were normalized to GAPDH loading control. The ratio of EPAS1 to GAPDH for control was assumed to be 1.
recognized as a putative enhancer or a promoter region. Data about DNA methylation of the HIF1A promoter region are ambiguous. The absence of DNA methylation of the HIF1A promoter was observed in advanced uterine cervical carcinoma and datasets available at ENCODE project, whereas DNA hypermethylation was observed in an immature hematopoietic cell line HMC-1 and normal colon tissues isolated from 20 patients with colorectal cancer (14, 34, 45). In our studies, DNA methylation of the HIF1A promoter was undetected in a group of 120 patients. The difference to previous studies in colorectal cancer may result from the used methods for determining DNA methylation, and may suggest complexity in epigenetic regulation of HIF1A. Moreover, many environmental factors like tobacco smoking, diet, and physical activity may affect DNA methylation status in colorectal cancer (46). Further experiments need to verify the potential impact of these factors on EPAS1 DNA methylation. In addition, even though we have not detected subgroup-biased DNA methylation in patients, we cannot exclude potential impact of gender, age, and other clinical-pathologic features in different patient populations.

The main limitation of our studies is the lack of association of DNA methylation status and mRNA level with protein expression of analyzed genes. However, because the amount of samples was limited, we preferred to evaluate HIF1A, EPAS1 DNA methylation, and transcript level as the prognostic significance of transcript, and DNA methylation has not been investigated previously. We assessed DNA methylation and expression level of the HIF1A and EPAS1 genes under hypoxic and normoxic conditions in DLD-1 and HCT116 colorectal cancer cells. We undetected changes in DNA methylation of the HIF1A promoter region and the HIF1A mRNA level under normoxic and hypoxic conditions in both analyzed cell lines. However, an increase of HIF1α protein in hypoxia was observed when compared with normoxic conditions, which indicates that the main regulator of HIF1A expression in colorectal cancer is oxygen-dependent posttranslational modification. The EPAS1 transcript level remained stable in normoxic and hypoxic conditions in HCT116 cells, whereas EPAS1 protein level was higher in hypoxic conditions. On the other hand, a significant increase in both the amount of EPAS1 transcript and protein in hypoxic conditions was observed in DLD-1 cells. EPAS1 mRNA changes in DLD-1 were associated with DNA hypomethylation of EPAS1 CpG island. The same region (chr2: 46 526 762-46 526 905) was hypermethylated in HCT116 cells, which may explain the lack of mRNA induction under hypoxic conditions. HCT116 and DLD-1 cells were also incubated for different time periods with 5-dAzA-C under hypoxic and normoxic conditions. 5-dAzA-C may induce expression of many genes and inhibit growth of colorectal cancer cell lines (47, 48). We observed 5-dAzA-C-induced DNA demethylation of the EPAS1 CpG island in HCT116 cells, regardless of oxygen concentration. An increase in the EPAS1 transcript and protein levels under hypoxic conditions suggests the role of EPAS1 DNA methylation in HCT116 cells. In normoxic conditions, the increase of EPAS1 protein was not observed in both cell lines, probably due to oxygen-dependent degradation. However, an increase of EPAS1 mRNA in DLD-1 cells was observed under normoxic conditions after 5-dAzA-C treatment, despite the lack of DNA methylation. The absence of mRNA and protein upregulation in DLD-1 cells upon hypoxia suggests that other factors must be involved in the induction of EPAS-1 gene expression besides DNA methylation. Moreover, discrepancies of the results obtained from the two cell lines may be a result of their different genetic background.

In conclusion, our findings present epigenetic transcriptional downregulation of EPAS1 in patients with colorectal cancer and the HCT116 cell line. In addition, low EPAS1 mRNA level in histopathologically unchanged tissues and DNA hypermethylation in cancerous tissues compared with the histopathologically unchanged might be an independent prognostic factor and potentially useful for selecting patients with a higher risk of death after resection. The clinical value of changes in EPAS1 mRNA and DNA methylation levels needs to be confirmed by large longitudinal studies as well as verified in other cancer types.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.A. Rawiszko-Wieczorek
Development of methodology: A.A. Rawiszko-Wieczorek
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.A. Rawiszko-Wieczorek, K. Horbacka, P. Krokowicz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.A. Rawiszko-Wieczorek
Writing, review, and/or revision of the manuscript: A.A. Rawiszko-Wieczorek, M. Mierzal, P.P. Jagodziński
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Horbacka
Study supervision: P.P. Jagodziński

Acknowledgments
The authors thank the Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, for access to the MCO-18M multi-gas cell culture incubator, Sanyo.

Grant Support
This work is supported by grant 2012/05/N/NZ5/00844 from the National Science Center, Poland.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 29, 2014; revised April 7, 2014; accepted April 25, 2014; published OnlineFirst May 13, 2014.

References


31. Carroll VA, Ashcroft M. Role of hypoxia-inducible factor (HIF)1-alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau


Prognostic Potential of DNA Methylation and Transcript Levels of HIF1A and EPAS1 in Colorectal Cancer

Agnieszka Anna Rawluszko-Wieczorek, Karolina Horbacka, Piotr Krokowicz, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-14-0054

Supplementary Material
Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2014/05/13/1541-7786.MCR-14-0054.DC1

Cited articles
This article cites 45 articles, 9 of which you can access for free at:
http://mcr.aacrjournals.org/content/12/8/1112.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://mcr.aacrjournals.org/content/12/8/1112.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mcr.aacrjournals.org/content/12/8/1112.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.