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Original article

Reduced expression of *PHD2 prolyl hydroxylase* gene in primary advanced uterine cervical carcinoma

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ABSTRACT

Decreased *PHD2* expression in human carcinomas has been considered a critical factor in supporting tumor angiogenesis and growth. We studied the levels of *PHD2* transcript and protein in advanced cervical cancer specimens (n = 27) and normal uterine cervical tissue samples ($n = 27$). Real-time quantitative PCR and Western blotting analysis showed significantly lower levels of *PHD2* transcript ($P = 0.0088$) and protein ($P = 0.0095$) in cancerous tissues as compared to corresponding normal tissue. Using DNA sequencing analysis, we also found an accumulation of mutations in promoter regions of *PHD2* in advanced cervical cancer specimens. Moreover, computer analysis of these mutations showed a loss of binding sites for many transcription factors. Our results suggest *PHD2* as a possible target in anti-angiogenic therapies in advanced uterine cervical carcinoma.

1. Introduction

Primary uterine cervical cancer mainly occurs in women of relatively young age, when many of them are active in their careers or looking after their families. In the European Union, 34,000 new cases and more than 16,000 deaths resulted from cervical cancer in 2004 [1]. Most cervical carcinomas are etiologically related to oncogenic subtypes of the human papilloma virus (HPV), which is considered a primary etiological factor of cervical cancerogenesis [2,3].

Despite the promise of the HPV16/18 vaccine and screening programs, a large cohort of currently HPV infected women may develop the advanced cervical malignancies over the next four decades. An outstanding property of clinically advanced cervical cancers is hypoxia at the tumor site [4,5]. It is considered a prognostic and therapeutic factor linked to radio- and apoptotic-resistance, changes in malignant cell proliferation, cell signaling, and genomic stability [5–7].

A major regulator of cellular response to hypoxia is hypoxia-inducible factor-1 (HIF-1), which is a transcription factor that increases the expression of genes encoding proteins involved in angiogenesis, glucose transport, glycolysis, tissue invasion/metastasis, and cell proliferation [8]. HIF-1 is a heterodimer consisting of an oxygen-labile HIF-1α subunit and a constitutively expressed HIF-1β subunit [8]. During normoxia, HIF-1α is degraded by a ubiquitin-mediated mechanism, which is mainly regulated by hydroxylation of Pro402 and Pro564 situated in its oxygen-dependent degradation (ODD) domain [9,10]. These hydroxylated prolines are recognized by Hippel-Lindau (VHL) protein, a component of an E3 ubiquitin ligase complex, and recognition is followed by proteosomal degradation [11,12]. Under hypoxia conditions, HIF-1α prolyl hydroxylases are inhibited and VHL and HIF-1α interaction is impaired leading to HIF-1α stabilization, its dimerization with HIF-1β, and formation of active HIF-1 transcription factor [8,11,12].

Hydroxylation of prolines in HIF-1α ODD domain is carried out by the three HIF prolyl 4-hydroxylases designated PHD1, PHD2, and PHD3, also named EGLN1, EGLN2 and EGLN3, respectively [13,14]. In humans, HIF-1α levels are mainly under the control of the PHD2 isoform, which exploits oxygen and α-ketoglutarate to hydroxylate Pro^{402} and Pro^{564} of HIF-1α [14].

Therefore, we studied the PHD2 transcript and protein levels in cancerous tissue from women with advanced cervical cancer ($n = 27$) and normal uterine cervical tissue from women with ovarian cysts ($n = 27$) from a Polish cohort. We also compared the sequence of *PHD2* promoter regions having binding sites for transcription factors in cancerous and corresponding normal tissues.

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2. Materials and methods

2.1. Patient material

Twenty-seven primary squamous cell cervical carcinoma tissue samples were collected between December 2009 and June 2010 from 27 patients treated at the Department of Radiotherapy, Greater Poland Cancer Center in Poznań, Poland (Table 1). Normal uterine cervical tissues were obtained from women with ovarian cysts who underwent radical surgical ovarian and uterine resection in the Clinic of Gynecological Surgery, Poznań University of Medical Sciences, Poland. Histopathological classification including stage, grade and tumor type was performed by an experienced pathologist. Women with cervical cancer and controls were Caucasian, collected from the same area of Poland. 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.

2.2. Antibodies and reagents

Rabbit polyclonal anti-PHD2 (H-40) antibody (Ab), goat anti-rabbit horseradish peroxidase (HRP)-conjugated Ab, and anti-actin HRP-conjugated Ab (clone I–19) were provided by Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Reverse transcription and real-time quantitative polymerase chain reaction (RQ-PCR) analysis of the PHD2 transcript levels

The total RNA from cancerous and corresponding normal tissues was isolated according to the method of Chomczyński and Sacchi (1987) [15]. RNA samples were treated with DNase I, quantified, and reverse-transcribed into cDNA. RQ-PCR was carried out in a Light Cycler real-time PCR detection system from Roche Diagnostics GmbH (Mannheim, Germany) using SYBR® Green I as detection dye. The target cDNA was quantified by relative quantification method using a calibrator. The calibrator was prepared as a cDNA mix from all samples and successive dilutions were used to create a standard curve as described in Relative Quantification Manual Roche Diagnostics GmbH (Mannheim, Germany). For amplification, 2 μl of cDNA solution was added to 18 μl of QuantiTect® SYBER® Green PCR Master Mix QIAGEN GmbH (Hilden, Germany) and primers (Supplementary data, Table 1S, Fig. 1S). The quantity of PHD2 transcript in each sample was standardized by the porphobilinogen deaminase (PBGD) cDNA levels (Supplementary data, Table 1S). The PHD2 transcript levels were expressed as multiplicity of these cDNA concentrations in the calibrator.

2.4. Western blotting analysis

Cancerous and corresponding normal tissues were treated with lysis RIPA buffer, and 30 μg of protein were resuspended in sample buffer and separated on 10% Tris-glicyne gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel proteins were transferred to PVDF, which was blocked with 5% milk in Tris buffered saline/Tween. Immunodetection was conducted with rabbit polyclonal anti-PHD2 (H-40) Ab, followed by incubation with goat anti-rabbit HRP-conjugated Ab, respectively. Bands were revealed using enhanced chemical luminescence (ECL) and Hyperfilm ECL Amersham (Piscataway, NJ). After stripping, the membranes were incubated with anti-actin HRP-conjugated Ab to ensure equal protein loading of the lanes. Densitometric analysis of the bands was then performed, followed by normalization β-actin loading to calculate the PHD2- to-β-actin ratio.

Table 1
Clinical characteristics of patients with cervical cancer and controls.

Characteristics	Patients with cancer ($n = 27$)	Controls ($n = 27$)
Mean age (years) ± SD	52.7 ± 11.6	50.7 ± 13.1
Tumor stage		
II	1 (3.7%)	
III	25 (92.6%)	
IV	1 (3.7%)	
Histological grade		
G1	1 (3.7%)	
G2	16 (59.2%)	
G3	10 (37.0%)	

2.5. Determination of mutation in PHD2 promoter regions and their effect on transcription factor binding.

Genomic DNA was isolated using DNA Mammalian Genomic Purification Kit purchased from Sigma-Aldrich Co. (St. Louis, MO). The binding sites of transcription factors and the effect of mutations on binding of transcription factors to promoter regions (Supplementary data, Fig. 1S) were determined by online programs: http://www-bimas.cit.nih.gov/molbio/proscan/ and http://zlab.bu.edu/~mfrith/cister.shtml. Transcription factor binding sites were found at −5967 bp to −5295 bp and in the first exon of the *PHD2* gene from 2439 bp to 2689 bp, the latter located in the 5'untranslated region (5'UTR) (Supplementary data, Fig. 1S). These DNA fragments containing transcription factor binding sites were amplified using appropriate primer pairs (Supplementary data, Fig. 1S, Table 1S). PCR amplification was performed by FastStart Taq DNA Polymerase from Roche Diagnostic GmbH (Penzberg, Germany). The PCR products were purified using Agarose Gel DNA Extraction Kit Roche (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 ten positive bacterial clones was used for commercial sequencing of the cloned fragments of DNA.

2.6. Statistical analysis

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 and two samples with equal variance at the $P < 0.05$ level. The normality of the observed patient data distribution was assessed by Shapiro-Wilk test and unpaired, two-tailed t-test or Mann-Whitney test was used to compare the mean values. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Reduced PHD2 expression in uterine cervical carcinoma was associated with accumulation of mutation in promoter regions having binding sites for transcription factors

To compare PHD2 transcript and protein levels in advanced uterine cervical carcinoma and normal uterine cervical tissues, we used RQ-PCR and Western blotting analysis, respectively. We found significantly lower levels of PHD2 transcript ($P = 0.0088$) and protein ($P = 0.0095$) in cancerous tissues than in corresponding normal tissue (Table 2 and Fig. 1A–C). There were no significant differences between PHD2 transcript and protein levels between patient groups with different tumor and histological stage.