The *WWOX* tumor suppressor gene in endometrial adenocarcinoma

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Abstract. Endometrial cancer is a lethal malignancy, the causes of which remain to be determined. The aim of the present study, carried out on tumor samples from 79 patients, was to evaluate the role of the *WWOX* tumor suppressor gene in endometrial adenocarcinoma. The expression levels of *WWOX* and its protein content were assessed in normal endometrium and cancer samples. Quantitative PCR was used to assess the correlation between the expression levels of *WWOX* and the genes involved in the proliferation (*MKI67*), apoptosis (*BAX*, *BCL2*), signal transduction (*EGFR*), cell cycle (*CCNE1*, *CCND1*), cell adhesion (*CDH1*) and transcription regulation (*TP73*, *NCOR1*). The relationship between loss of heterozygosity (LOH) and *WWOX* mRNA levels was also investigated using high resolution melting. Results of the present study demonstrated a positive correlation of *WWOX* expression with *BCL2* and *CCND1* and a negative correlation with *BAX*, *CDH1*, *NCOR1* and *BCL2*/*BAX* ratio. The results also showed that loss of heterozygosity at two analyzed loci of the *WWOX* gene is frequent in patients with endometrial cancer and that *WWOX* expression levels are lower in tumor samples than in normal tissue. In conclusion, *WWOX* may be involved in endometrial cancer.

Introduction

Endometrial cancer is one of the most common and lethal gynecological malignancies in Poland. However, the causes of endometrial carcinogenesis remain to be clarified. Possible causes include the imbalance of endogenous estrogen and progesterone levels, obesity, polycystic ovarian syndrome and estrogen replacement therapy (1,2). Since the 1980s, endometrial cancer has been classified into two main subsets of sporadic endometrial cancer that differ in molecular genesis and prognosis (3). Type I endometrioid cancers are estrogen-dependent cancers that develop from hyperplasia and are usually low grade with a favorable prognosis. Endometrioid cancers account for ~70-80% of cases, affecting mainly younger, pre- and post-menopausal females. In this type of tumor, specific molecular aberrations such as *PTEN* gene inactivation by mutation and/or promoter methylation, mutation of protooncogene *H-RAS*, mutation of *CTNNB*, and microsatellite instability (MSI) are common (4). Type II sporadic endometrial cancer is non-endometrioid endometrial carcinoma (NEEC), which progresses from an atrophic endometrium, occurs in older females and has a more aggressive course of disease. This type of cancer is estrogen-independent and characterized by frequent molecular alterations in oncoprotein *HER2*/*neu*, TP53 mutation and inactivation of *CDH1* (4,5). Experiments conducted in this study focused on endometrioid adenocarcinoma, which is the type I endometrioid cancer.

Clinicopathologic variables such as age, FIGO stage, histological grade, myometrial invasion, metastasis to lymph node and histological type are crucial prognostic factors (6). However, new molecular markers should be identified to improve the prediction of therapy outcome and prognosis. Currently, the potential markers available remain controversial and intensely discussed (6).

WWOX is localized in the common fragile site, FRA16D (locus 16q23.3-24.1). It has been confirmed to be altered in various types of cancer including breast, lung, gastric, ovarian, Wilms' tumor and glioblastoma multiforme (7-13). Genetic and epigenetic alterations of this gene include loss of heterozygosity (LOH) and promoter methylation. The gene product is an oxidoreductase comprising two WW protein interaction domains. One of the roles of WWOX protein is participation in steroid hormone metabolism (14,15). Results of previous studies showed that *WWOX* is also associated with apoptosis, proliferation, adhesion and cell signaling pathways (16-19). Additionally, WWOX has been shown to bind to PPxY motif-containing proteins, and inactivate their transcription transactivation function by sequestering them in the cytoplasm (20,21). It is known that WWOX modulates the Ap2α/γ, p73, ErbB4, Met, Jun, Wnt signaling pathways.

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Moreover, Gourley *et al* identified its role in the decrease of integrin activity and adhesion of tumor cells to fibronectin (22).

The expression level of *WWOX* is known to be decreased in breast cancer cells and to correlate with poor prognosis (11). In a series of *in vitro* experiments, a *WWOX*-transfected MDA-MB-231 breast cancer cell line showed increased migratory ability. However, the results of a test of growth in Matrigel showed that the transduced cells had more 'normal' phenotype and formed mammary ducts. Control cells in Matrigel grew into spherical structures, typical of neoplastic cells (22). The improved differentiation evident in cells with an elevated *WWOX* level suggests its involvement in these type of processes. The tumor suppressor function of *WWOX* was confirmed in a soft agar growth test, where *WWOX*-transfected cells exhibited inhibition of anchorage-independent growth (23). MDA-MB-231 cells overexpressing *WWOX* were also significantly less tumorigenic *in vivo* (24). Experiments performed by Gourley *et al* on ovarian cancer cells confirmed that WWOX protein is an inhibitor of anchorage-independent growth also in this case. Moreover, *WWOX* silencing was found to result in enhanced adhesion to fibronectin (22).

No data is currently available on the role of the *WWOX* gene in endometrial cancer. The present preliminary qPCR-based study was conducted on 79 endometrial adenocarcinoma in relation to 28 tumor-free endometrial tissue samples. The aim of this study was to investigate the correlation of the expression levels of *WWOX* and nine other tumor-related genes: *MKI67*, *BAX*, *BCL2*, *EGFR*, *CCNE1*, *CCND1*, *CDH1*, *TP73* and *NCOR1*. Additionally, the implications of loss of heterozygosity with regard to the regulation of *WWOX* expression in endometrial cancer were also analyzed.

Materials and methods

In total, 79 samples of endometrial carcinoma (endometrioid adenocarcinoma) were collected at the Department of Gynecological Oncology, Medical University of Lodz, Poland. The tumors were classified according to the FIGO (International Federation of Gynaecology and Obstetrics) classification system. The mean age of the patients was 61 years (median 60, range 36-83 years). The samples were examined histologically and stored at -80˚C in RNAlater buffer (Ambion, Inc., Austin, TX, USA) until RNA extraction. Clinical characteristics of the patients are presented in Table I. Experiments involving human subjects were conducted according to the Declaration of Helsinki, and the study was approved by the Ethics Committee at the Medical University of Lodz. Control samples (n=28) were received from patients operated on for benign gynecologic disorders.

qPCR. RNA was extracted from frozen tissues, stored at -80˚C in RNAlater (Ambion), using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed from 10 μg of total RNA at a volume of 100 μl using ImProm RT-II™ reverse transcriptase (Promega, Madison, WI, USA). Reverse transcription was carried out under the following conditions: incubation at 25˚C for 5 min and 42˚C for 60 min, and heating at 70˚C for 15 min. cDNA samples were diluted with sterile deionized water to a total volume of 150 μl, and 2 μl was added to a PCR reaction. qPCR was performed using Rotor-Gene™ 3000 (Corbett Research, Australia). PCR products were detected using SYBR®-Green I and qPCR Core kit for SYBR-Green I (Eurogentec, Seraing, Belgium). Reactions were performed in duplicate. The relative expression levels of the following genes were analyzed: *WWOX*, *TP73*, *CCND1*, *CCNE1*, *BCL2*, *BAX*, *MKI67*, *CDH1*, *EGFR*, and *NCOR1*. The expression levels of the investigated genes were normalized to three reference genes (*RPS17*, *H3F3A*, *RPLP0*). Due to the presence of a relatively low level of *WWOX* mRNA, a semi-nested RT-PCR was used for the detection of *WWOX* expression levels. The first PCR reaction was performed with primers: 5'-TGCAACATCCTCTTCTCCAACGAGCTGCAC-3' and 5'-TCCCTGTTGCATGGACTTGGTGAAAGGC-3' in a 50 μl reaction volume. Subsequently, 2 μl of 200-fold diluted PCR product (171 bp) was used as a template for semi-nested PCR. The cycling protocol consisted of 2 min at 94˚C, 30 sec denaturation at 94˚C, 30 sec annealing at 63˚C, 1 min extension at 72˚C, repeated for 77 cycles, and additional extension for 7 min at 72˚C.

The primer sequences, PCR reaction conditions and the length of obtained products are available upon request.

Relative gene expression was calculated based on the Roche company guidebook according to the previously published algorithm (25). Universal Human Reference RNA (Stratagene, La Jolla, CA, USA) composed of 10 cell lines was used as a calibrator.

The primers were designed to be intron-spanning to avoid amplification of genomic DNA. The detection temperature was determined above the non-specific/primer-dimer melting temperature.

Loss of heterozygosity analysis. LOH detection was performed using the high-resolution melting method in a LightCycler480 (Roche Molecular Systems, Penzberg, Germany). Genomic DNA was recovered after RNA isolation using back extraction buffer (BEB, 1 M Tris Base, 4 M guanidinium thiocyanate, and 50 mM sodium citrate) according to the manufacturer's instructions. Allelic losses were analyzed by PCR amplification with two sets of primers for microsatellites D16S518 (intron 1 of *WWOX* gene) and D16S3096 (intron 8). The primer sequences were obtained from the Genome database. PCR cycling programs included one cycle with 95˚C for 10 min followed by 35 cycles consisting of 94˚C for 30 sec, 56˚C (for D16S3096) or 55˚C (for D16S518) for 30 sec, 72˚C for 60 sec. The high-resolution melting conditions involved a temperature increase of 50-95˚C, ramp rate 0.01˚C/sec and 40 acquisitions per ˚C.

Western blot analysis. The tissue fragments were lysed in RIPA protein extraction buffer supplemented with protease, phosphatase inhibitor cocktail and PMSF (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration was measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA), and 100 μg amounts were run on 10% SDS-PAGE gel electrophoresis and subsequently transferred to a PVDF membrane (Sigma-Aldrich). The membranes were blocked in 5% non-fat milk in TBST for 1 h at room temperature and then incubated for 19 h at 4˚C with primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following incubation, the membranes were washed three

times with TBST and incubated with secondary antibodies conjugated with alkaline phosphatase (Sigma-Aldrich) for 1 h. Membranes were washed three times in TBST and developed using Novex® AP Chromogenic Substrate (Invitrogen). Glyceraldehyde-3-phosphate dehyrogenase (GAPDH) was used as a reference protein. The relative protein amount was assessed with ImageJ software (National Institutes of Health, USA) based on integrated density of bands.

Statistical analysis. Data analysis was performed by using Statistica version 8.0 (StatSoft, Tulsa, OK, USA). Gene expression correlation analysis was performed using the non-parametric Spearman's rank correlation test. The Mann-Whitney t-test was used to determine differences between the transcription levels of the *WWOX* gene in relation to its hemi/heterozygosity, as well as differences between quantities of WWOX in different types of tissue. Results were recognized as being statistically significant at a confidence level of >95% (P<0.05).

Results

LOH. Loss of heterozygosity in the region of the D16S3096 microsatellite marker (localized in intron 8 of *WWOX* gene) was found to be a common event in endometrial cancer, with observed hemizygosity at 38%. Moreover, hemizygous samples exhibited decreased *WWOX* gene expression levels compared with heterozygous samples (medians: 0.66 vs. 1.04, P=0.066). The percentage of LOH for the microsatellite marker, D16S518, was 40%; however, hemizygosity did not reveal any tendencies to correlate with decreased *WWOX* expression levels (Fig. 1).

Expression correlation of WWOX and various tumor-related genes. The expression correlation between *WWOX* and nine other tumor-related genes is assessed. *MKI67* was connected with the rate of proliferation, *BAX*, *BCL2* and *TP73* are involved in the course of apoptosis, *CCNE1* and *CCND1* encode cyclins crucial for cell cycle progression, *EGFR* and *NCOR1* act as receptors and transcriptional factors, while *CDH1* is a gene of one of proteins responsible for cell-cell adhesion. A positive correlation was observed between the expression of *WWOX* and *BCL2* (R_s = 0.3822; P=0.0005), *CCND1* (R_s = 0.3821; P=0.0005) as well as with the *BCL2*/*BAX* anti-apoptotic ratio (R_s = 0.4496; P=0.0001). *WWOX* expression correlated inversely with *BAX* (R_s = -0.2302; P=0.0412), *CDH1* (R_s = -0.4126; P=0.0002) and *NCOR1* (R_s = -0.3061; P=0.0064) expression levels. Details of the expression correlations between *WWOX* and the nine investigated genes are presented in Table II.

No correlation was found between *WWOX* mRNA levels and clinicopathological factors such as grade, FIGO stage, lymph node metastasis or myometrial invasion (Table III). However, the highest expression of *WWOX* was observed in normal endometrium tissue (NT) in comparison to tumor tissue (median expression 2.826, NT vs. G1, P=0.003; NT vs. G2, P<0.0001; NT vs. G3, P=0.002).

Western blot analysis. To assess the level of WWOX protein in normal and cancer tissues, a western blot assay was conducted, which revealed that the protein amount was greater in normal endometrium tissue compared with cancer samples, although this tendency did not achieve statistical significance (P>0.05). There was no difference between tumor grades. Such results are reflected in the amounts of mRNA. However, in the qPCR analysis of gene expression the differences between normal and cancer tissues were statistically significant. The smaller differences in protein between normal and tumor tissues may depend on the low level of the WWOX protein, which hindered western blot sensitivity. The densitometric analysis and protein bands are presented in Fig. 2.

Table I. Clinical characteristics of endometrial cancer patients.

Factor	No. of patients
FIGO stage	
I	44
II	16
III	10
NS	9
Lymph node metastasis	
Negative	65
Positive	8
NS	6
Histological grade	
I	27
II	39
III	12
NS	1
Myometrial invasion	
<1/2	41
>1/2	33
Without	4
NS	1

NS, not specified.

Table II. Correlation analysis between the expression levels of *WWOX* and other tumor-related genes.

Gene	Spearman's rank correlation	P-value
BCL2/*WWOX*	0.3822	0.0005
BAX/*WWOX*	-0.2302	0.0412
CCND1/*WWOX*	0.3821	0.0005
CDH1/*WWOX*	-0.4126	0.0002
NCOR1/*WWOX*	-0.3061	0.0064
BCL2_BAX ratio/*WWOX*	0.4496	0.0001
EGFR/*WWOX*	0.1004	0.3786
CCNE1/*WWOX*	-0.0162	0.8872
MKI67/*WWOX*	-0.1132	0.3314

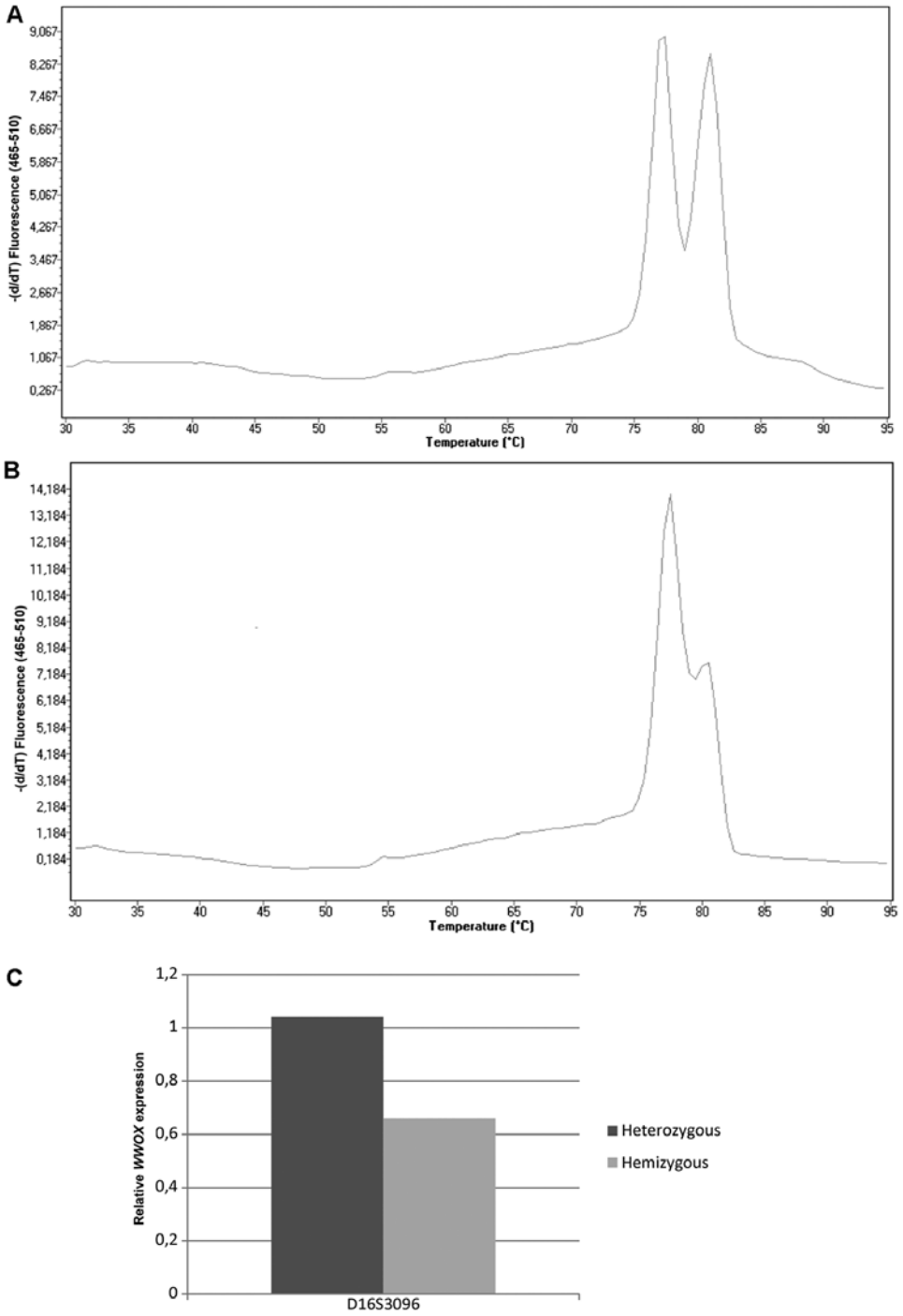

Figure 1. Correlation of loss of heterozygosity (LOH) with *WWOX* expression levels. (A) Heterozygous sample melting peaks. (B) Hemizygous sample melting peaks. (C) Relative *WWOX* mRNA expression in heterozygotes and hemizygotes.

Discussion

Apoptosis is a natural process for the elimination of senescent cells from a layer of endometrium during late secretory and menstrual phases. The mRNA levels of apoptosis-related genes change during phases of the ovulation cycle. The expression level of the antiapoptotic *Bcl2* gene increases during the late proliferative phase but decreases during the late secretory and menstruating phases when the expression of the *Bax* proapoptotic gene increases. Anomalies in the expression of apoptosis-related genes may lead to pathological changes including endometriosis and cancer (26).

In the present study, positive correlations were found between *WWOX* and the antiapoptotic *BCL2* gene as well as between *WWOX* and the *BCL2*/*BAX* ratio. Findings of a previous study demonstrated the increasing expression of the proapoptotic *BAX* gene and decreasing expression of the antiapoptotic *BCL2* gene during progression from endometrial hyperplasia to cancer (27). Geisler *et al* (28) demonstrated that a higher expression level of the Bcl2

Table III. Dependence of the *WWOX* expression levels and clinical characteristics of endometrial cancer patients.

Factor	n	Median of *WWOX* mRNA level (range)	P-value
FIGO stage			
I	44	1.004 (0.678-1.613)	0.701 (I/II)
II	16	0.716 (0.357-1.370)	0.527 (II/III)
III	10	0.871 (0.554-4.267)	0.982 (III/I)
NS	9		
Lymph node metastasis			
Negative	65	0.870 (0.613-1.295)	0.646
Positive	8	0.871 (0.554-1.436)	
NS	6		
Histological grade			
G1	27	1.125 (0.494-1.824)	0.386 (I/II)
G2	39	0.732 (0.613-0.974)	0.756 (II/III)
G3	12	0.722 (0.306-1.613)	0.523 (III/I)
NS	1		
Myometrial invasion			
<1/2	41	1.050 (0.641-1.563)	0.279
>1/2	33	0.856 (0.410-1.226)	
Without	4		
NS	1		

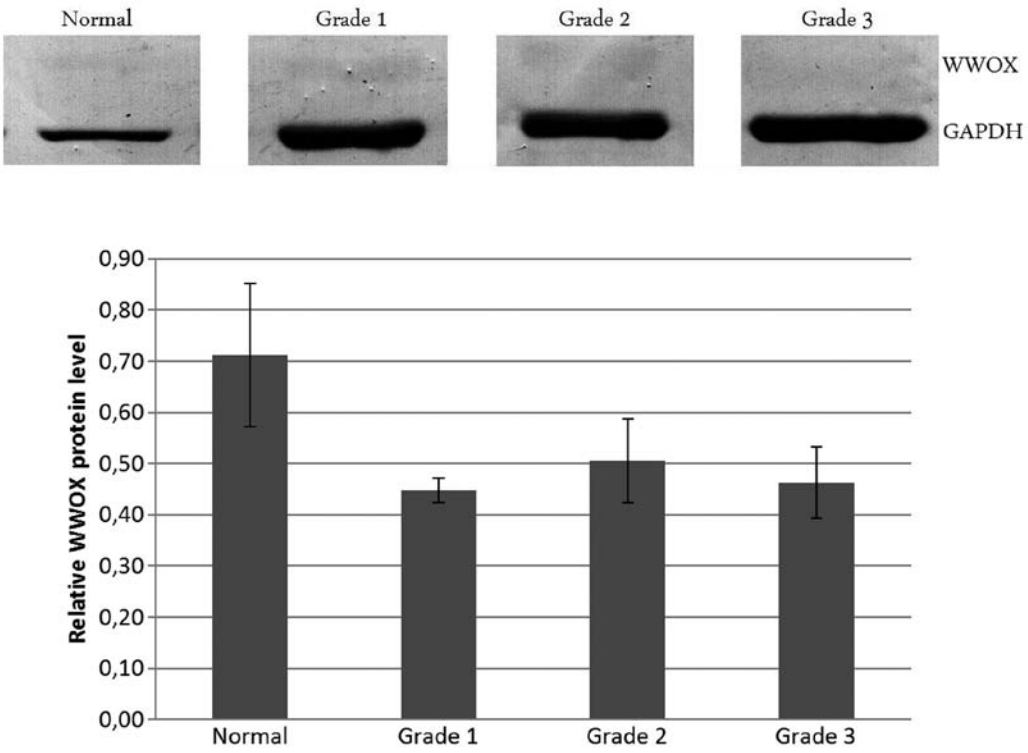

Figure 2. Expression of WWOX protein in representative normal endometrium tissues and grade 1-3 cancers.

protein correlates with favorable clinicopathological variables such as well-differentiated tumor cells, reduced FIGO stage, lack of invasion into lymph node and superficial myometrial invasion (28,29). Chao *et al* observed a correlation of *BAX* overexpression in endometrial cancer specimens in relation to normal endometrium and premalignant lesions (30). A negative correlation between *WWOX* and *BAX* gene was also identified in the present study. These results are similar to our

previously reported gene expression analysis conducted on breast cancer (11) and glioblastoma multiforme patients (9). Investigations on apoptosis of an A2780 ovarian cancer cell line transfected with the *WWOX* gene demonstrated a decreased ability for anchorage-independent growth with a simultaneous increase of apoptosis (31).

An important regulator of the cell cycle is cyclin D1. Expression of *CCND1* can be regulated by several signaling pathways, such as *RAS* or *PTEN*. In type I endometrioid cancer, expression of the *CCND1* gene is connected with the proliferation process Wnt signalling pathways (32). Findings of a previous study identified an increase in expression of cyclin D1 from normal endometria to hyperplasia and carcinoma (33). Moreno-Bueno *et al* suggest two different causes of cyclin D1 overexpression: an amplification of the gene in NEEC and a microsatellite instability in endometrioid cancer (34). In the present study, a positive correlation of *WWOX* and *CCND1* was observed.

Additionally, results of the present study demonstrate that *WWOX* expression level correlates negatively with the *NCOR1* (nuclear receptor corepressor 1) ERα corepressor gene. The corepressor suppressess transcription estrogen-responsive genes by modeling chromatin structures by incorporating histone deacetylases (HDAC) (35). Using a microarray method Moreno-Bueno *et al* noted a 2-fold higher expression of *NCOR1* in endometrioid cancer in comparison to NEEC samples (36). However, such differences between tumor groups were not observed by Kershah *et al*, although they demonstrated the upregulation of nuclear receptor coregulators including *NCOR1* in a malignant endometrium, as compared to a normal one. The ratio between coactivators SRC-1 and SRC-2 and corepressor NCOR1 decreased in malignant tissues. No significant differences were identified between tumor groups regarding NCOR expression, categorized on the basis of such clinical parameters as grades or stages (37). Upregulation of *NCOR1* was also observed in breast cancer, however, a low expression of this gene was associated with worse prognosis and serves as a potential predicting factor for tamoxifen treatment in estrogen receptor α-positive breast cancer (38). In a previous study on ER-positive breast cancer patients, a positive correlation was noted between the expression level of *WWOX* and *NCOR1* (data not shown).

The *CDH1* gene encoding the cell-cell adhesion protein E-cadherin is located near *WWOX* on chromosome 16 (*CDH1 locus* 16q22.1, *WWOX locus* 16q23.3-24.1). As previously shown, *CDH1* expression is often reduced or completely inactivated by promoter methylation. A low E-cadherin expression is associated with worse prognosis, higher stage and greater metastatic potential (39). Our previous experiments conducted on breast and colon cancer lines confirm that an increase of *WWOX* expression level results in changes in cell behavior (23, unpublished data). Cancer cells with a high *WWOX* express a higher motility, which has an effect on improved migration through the basal membrane. Additionally, they are less malignant due to the suppression of anchorage-independent growth. This change in cell motility may explain the observed correlation of *WWOX* expression with the reduced expression of the cell adhesion gene *CDH1*. A previous *in vivo* study revealed the role of WWOX protein in the attachment and adhesion of ovarian cancer cells. *WWOX*-transfected PEO1 cells showed a decrease in the adhesion to fibronectin in comparison to vector-transfected control cells, which suggests a *WWOX* influence on processes such as tumor invasiveness and spread (40). Gourley *et al* also confirmed these results on the ovarian cancer cell line, A2780, and showed that *WWOX* overexpression reduces adhesion through membranous integrin α3 protein (22).

In previous studies, a decrease in the expression of the *WWOX* gene was found to be associated with loss of heterozygosity (LOH) in gastric (7), pancreatic (18), esophageal (41) and lung cancer (13). In the present study, the percentage of hemizygosity at two analyzed loci of the *WWOX* gene was ~40%. LOH in microsatellite marker D16S3096, exhibited a tendency towards a correlation with the reduced expression level of the *WWOX* gene. This observation suggest that this process is involved in the regulation of the *WWOX* mRNA level.

In conclusion, to the best of our knowledge, this is the first study to demonstrate the potential role of *WWOX* in endometrial cancer through the regulation of Wnt (*CDH1* and *CCND1*), apoptosis (*BCL2* and *BAX*) and estrogen-related genes (*NCOR1*). Results of the present study have also shown that *WWOX* mRNA and protein levels decrease in transformed endometrial tissue. The fact that no significant differences exist between tumor grades suggests that *WWOX* silencing is an early event in endometrial cancerogenesis. Similar to other types of cancer, *WWOX* expression in endometrial adenocarcinoma correlates with the expression level of apoptosis and cell cycle regulators. The results of our preliminary experiment have shown that additional investigations should be conducted that may enable the better elucidation of the role of *WWOX* in endometrial cancer. Future experiments are to be conducted on endometrial cell lines, which may shed some light on the functions performed by the WWOX protein and its relevance to endometrial cancer promotion and progression.

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