

Genetic alterations of *WWOX* in Wilms' tumor are involved in its carcinogenesis

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Received March 22, 2012; Accepted May 18, 2012

DOI: 10.3892/or.2012.1940

Abstract. Loss of heterozygosity (LOH) in 16q appears in ~20-30% cases of Wilms' tumor. Within this region, known as common fragile site FRA16D, the *WWOX* tumor suppressor gene is located. Abnormalities of *WWOX* gene expression levels were observed in many tumor types and were associated with worse prognosis. The purpose of this study was to investigate the role of the *WWOX* tumor suppressor gene in Wilms' tumor samples. We evaluated the correlation between expression of *WWOX* and genes involved in proliferation (*Ki67*), apoptosis (*BCL2*, *BAX*), signal transduction (*ERBB4*, *ERBB2*, *EGFR*), cell cycle (*CCNE1*, *CCND1*), cell adhesion (*CDH1*) and transcription (*TP73*) using real-time RT-PCR in 23 tumor samples. We also analyzed the potential causes of *WWOX* gene expression reduction i.e., promoter methylation status (MethylScreen method) and loss of heterozygosity (LOH) status. We revealed a positive correlation between *WWOX* expression and *BCL2*, *BCL2/BAX* ratio, *EGFR*, *ERBB4 isoform JM-a*, *TP73* and negative correlation with both cyclins. Loss of heterozygosity of the *WWOX* gene was observed only at intron 8, however, it had no influence on the reduction of its expression levels. Contrary to LOH, methylation of the region covering the 3' end of the promoter and part of exon 1 was associated with statistically significant reduction of *WWOX* gene expression levels. In the present study we reveal that in Wilms' tumors the *WWOX* expression levels are positively associated with the process of

apoptosis, signal transduction through the *ERBB4* pathway and *EGFR* and negatively with the regulation of the cell cycle (by cyclin E1 and D1). Moreover, our analysis indicates that in this type of tumor the expression of the *WWOX* gene can be regulated by an epigenetic mechanism - its promoter methylation.

Introduction

Wilms' tumor (nephroblastoma) arises from embryonal blastema cells, which should arrest in cell cycle and differentiation. It is one of the most common pediatric abdominal tumors and usually occurs in 1:10,000 children. The most cases of Wilms' tumors are sporadic, however, 5% of incidences arise with three congenital syndromes WAGR, Denys-Drash, Beckwith-Wiedemann (1). Despite of high cure rate of patients with Wilms' tumor (90% of cases), it is necessary to search for new prognostic factors, which will help better understand the biology of this tumor and to create better target therapy. Moreover, in majority of Wilms' cases the primary genetic causes are still unknown.

The development of genetic and epigenetic events in multiple loci is characteristic of Wilms' tumors. Gene expression studies revealed the influence of *C/EBPB*, *H4FG* up-regulation and *p21*, *CF542255* down-regulation on this tumor relapse (2). High hTERT expression also correlated with Wilms' recurrence. However, alterations in chromosomal regions appear to play crucial role in nephroblastoma development. Molecular biology analysis revealed imbalances in chromosomal regions spanning 1p, 7p, 17q, 16q, 11q13 (*WT1* gene), 11p15 (*WT2* gene).

The most interesting of the affected chromosomes appears to be 16q. LOH at that region has been connected with Wilms' development, poor prognosis and increased risk of relapse and death (3-5). Moreover, at 16q the common fragile site FRA16D is located, and also at 16q23.3-24.1 tumor suppressor gene *WWOX* is spanning approximately one million base pairs.

WWOX encodes protein containing 414 amino acids (46 kDa) that possesses two N-terminal WW domains of interaction with protein and C-terminal short-chain dehydrogenase domain (SDR) (6). *WWOX* protein through its first WW domain interacts with many partners such as *ERBB4* (7,8), *TP73* (9), transcription factor *AP2γ* (10,11), *YAP* (7), *Jun* (12). The role of *WWOX* protein is not well determined, however, it probably participates

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Abbreviations: *WWOX*, WW domain containing oxidoreductase; LOH, loss of heterozygosity; MSRE, methylation-sensitive restriction enzyme; MDRE, methylation-dependent restriction enzyme; DD, double-digest; ICD, intracellular domain

Key words: Wilms' tumor, *WWOX* gene

in hormonal metabolism. The relative high expression of *WWOX* was detected in reproductive, endocrine and exocrine organs (for example mammary epithelium, ovaries, endometrium, prostate gland, testes, liver, stomach, thyroid, parathyroid, adenohypofysis, and brain cells). *In vivo* studies indicated that *WWOX*^{-/-} mice display abnormalities in growth, survival and bone metabolism (13). Simultaneously, mice with phenotype *WWOX*^{+/-} more frequently develop tumors (14). In many types of cancers alterations in *WWOX* expression were observed due to loss of heterozygosity (15-20) and its promoter methylation (15,21,22). Inactivation of one allele of tumor suppressor *WWOX* gene is sufficient for tumorigenesis initiation (mechanism of haploinsufficiency) (14).

The aim of our study was to evaluate the role of *WWOX* gene in Wilms' tumors. In our experiment we analyzed correlation between expression level of *WWOX* gene and genes involved with proliferation (*Ki67*), apoptosis (*BCL2*, *BAX*), transduction signal (*ERBB4*, *ERBB2*, *EGFR*), cell cycle (*CCNE1*, *CCND1*), cell adhesion (*CDH1*) and transcription (*TP73*). Moreover, we evaluated loss of heterozygosity and methylation status of *WWOX* promoter and examined correlation between those events and gene expression.

Patients and methods

Patients. This study was performed on 23 Wilms' tumor samples obtained from children at the age between 0.25 and 8.92 years (mean 3.41 years).

The children were treated in the Department of Paediatric Oncology and Hematology, Medical University of Lodz and in the Department of Bone Marrow Transplantation, Pediatric Oncology, and Hematology, Medical University, Wrocław. The experimental group consisted of 10 males and 13 females. According to International Classification of renal tumors in childhood, 7 tumors were of high risk, 1 low and the remaining group were of intermediate risk (23). Tumor division according to stage was as follows: stage I, 5 cases; stage II, 6 cases; stage III, 3 cases; stage IV, 3 cases; stage V, 1 case. Samples of 4 cases had no stage characterisation. According to clinical records 7 out of 23 tumors were metastatic. Disease recurrence was observed in the group of 5 patients and the mortality was at 17% (4 cases). Before tumor resection, children were subjected to chemotherapy according to SIOP scheme therapy. This study was conducted after receiving patients' family consent.

Isolation of RNA, DNA and cDNA synthesis. Resected tumor samples were stored in -80°C in RNAlater (Ambion), RNA was isolated using TRIzol reagent (Invitrogen, USA). cDNA synthesis was performed from 10 µg of total RNA at the total volume of 100 µl with ImProm RT-II reverse transcriptase (Promega). Reverse transcription was performed under following conditions: 5-min incubation at 25°C and 60 min at 42°C, heating at 70°C for 15 min. Then, synthesized cDNA was diluted with sterile deionized water to 150 and 2 µl of cDNA was used in PCR reaction. Organic remains of TRIzol after RNA isolation were used for DNA isolation, according to the manufacturer's instructions.

Real-time RT-PCR analysis. Real-time RT-PCR was performed with LightCycler 480 II (Roche). PCR products were detected with SYBR Green I and qPCR Core kit for SYBR Green I

(Eurogentec). Reactions were performed in duplicate. We analyzed relative expression of 11 genes (*BAX*, *BCL2*, *EGFR*, *Ki67*, *WWOX*, *ERBB4* (isoforms *JM-a* and *JM-b*), *CCNE1*, *CCND1*, *CDH1*, *TP73*). The expression level of studied genes was normalized to two reference genes (*BMG2*, *RPS17*).

WWOX mRNA level was relatively low, therefore we performed a semi-nested RT-PCR for assessing *WWOX* expression level. Following primers in the first PCR reaction were used: 5'-TGCAACATCCTCTTCTCCAACGAGCTGCAC-3' and 5'-TCCCTGTTGCATGGACTTGGTGAAAGGC-3' in 50 µl volume. Next, after 200-fold PCR product dilution (171 bp), 2 µl was a template for semi-nested PCR. The cycling protocol was as follows: 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, additional extension for 7 min at the same temperature. Primer sequences, the PCR reaction conditions and the length of received products are available upon request.

Roche algorithm was used for relative expression level calculation. The Universal Human Reference RNA (composed of 10 cell lines) was used as a calibrator for each reaction. All primers were designed to be intron spanning in order to exclude genomic DNA amplification. Detection temperature was designated above non-specific/primer-dimer melting temperature.

LOH analysis. Loss of heterozygosity was analyzed with high resolution melting (HRM) of LightCycler 4800 (Roche). We used two microsatellites markers D16S3096 and D16S504 located on chromosome 16; on intron 8 and intron 1 of *WWOX* gene, respectively. The primer sequences were obtained from the Genome database. PCR conditions were as follows: initial denaturation 95°C for 10 min; 35 cycles of repeated denaturation at 94°C for 30 sec, annealing at 56°C (for D16S3096) or 55°C (for D16S518) for 30 sec, elongation at 72°C for 60 sec.

Analysis of *WWOX* methylation status. We performed MethylScreen assay, which is based on a set of methylation specific restriction digestions; subsequently associated with real-time PCR (24). DNA of samples (2 µl) were divided into four parts and treated with different digestions: MSRE, two methylation-sensitive enzymes *HhaI* and *HpaII*, which are cutting only unmethylated DNA; MDRE, one methylation-dependent restriction enzyme *McrBC*, which is cutting only methylated DNA; MOCK, positive control without enzymes; DD, negative control of reaction with both MSRE and MDRE enzymes.

The digestions reactions contained 1X NEB buffer 2 (10 mM Tris-HCl, 55 mM NaCl, 10 mM MgCl₂, 1 mM DTT), 1 µg/ml bovine serum albumin (BSA), 2 mM guanosine-50-triphosphate (GTP), appropriate enzymes. Next, this mixture was incubated for 4 h at 37°C and then 20 min at 65°C to restrain enzyme activity. Afterwards, samples were analyzed with real-time PCR using the RotorGene 3000™ system. We analyzed two fragments containing the promoter and first exon of the *WWOX* gene. The PCR for the first region of *WWOX* gene (-508 to -174 bp) was performed using the following primers: the forward primer sequence was 5'-ACAGAAGCCCAGGACAACAGCATGG-3', and the reverse primer sequence was 5'-ACCACGAAGCTGAAATCCAGTCTCCG-3'. For the analysis of the second region (from -171 to +239 bp) covering the 3' end of the promoter and



Figure 1. Western blot analysis demonstrating WWOX protein expression in tumor samples with low and high relative gene expression. Samples 3, 15 and 88 represent tumors with low WWOX relative gene expression with values 1,480, 0,214 and 0,957, respectively. Samples 89, 91 and 92 represent tumors with high WWOX expression and relative expression values: 58,304, 36,491 and 49,213 respectively.

part of exon 1 following primers were used: forward primer was 5'-AGACTGGATTTTCAGCTTCGTGGTTCG-3', and the reverse primer sequence was 5'-AAGCTCCTTAACAGTTACTTTCACCTTGCAC-3'.

For the first analyzed promoter fragment of WWOX gene, the PCR mix included 2.5 µl of SYBR Green I, qPCR Core kit for SYBR Green I reagents (Eurogentec), 10 nM of each primer, 4 µl of digested DNA template. Real-time PCR was conducted at the following conditions: 95°C for 5 min, followed by 50 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec, and 77°C for 15 sec (additional temperature for reading only specific amplification product size 384 bp). The PCR mix for the second WWOX promoter fragment consisted of 4 µl of digested DNA template, 10 nM of each primer, 2.5 µl of SYBR Green I, qPCR Core kit for SYBR Green I reagents, 70 mM of betaine. Real-time PCR was performed in the following conditions: 1 cycle of 95°C for 5 min, followed by 50 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 90 sec, and 83°C for 15 sec (additional temperature for reading only specific amplification product size 413 bp). All reactions were performed in duplicate.

To analyze methylation status of WWOX gene, we made standard curve for each patient by setting the MOCK positive control as 100 standard and DD negative control as 0.001 standard. If the differences between Mock and MSRE/MDRE digestions Ct was >1 this DNA samples, it was defined as methylated.

Statistical analysis. For the analysis of correlation between expression of WWOX and other genes we used non-parametric Spearman rank correlation test. Estimation of differences between WWOX expression in relation to LOH, methylation status and clinical factors were performed with Mann-Whitney t-test. All results were assumed as statistically significant at the confidence level >95% (p<0.05).

Real-time RT-PCR validation. In order to validate real-time RT-PCR we performed western blot analysis. We have chosen representative tumor samples with low and high relative WWOX gene expression. The results are presented on Fig. 1.

Protein isolation and immunoblotting assay. Total protein was extracted from frozen tissues using RIPA protein extraction buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Na Doc, 0.1% NP-40, 0.1% SDS, 2 mM EDTA, supplemented with protease, phosphatase inhibitor cocktail and 1 mM PMSF (Sigma-Aldrich, Germany). The Bradford method (Bio-Rad Laboratories) for determination of protein concentration was used, according to the manufacturer's instructions. The

Table I. LOH analysis in Wilms' tumor samples.

| | D16S3096 (%) | D16S518 (%) |
|--------------------------------------|--------------|-------------|
| Observed hemizygoty in Wilms' tumors | 30.4 | 6.7 |
| Population homozygoty | 26 | 17 |
| Predicted loss of heterozygoty | 4.4 | 0 |

amount of 30 µg of sample protein were run on 10% SDS-PAGE gel electrophoresis and transferred to PVDF membrane (Sigma-Aldrich). Membranes were blocked in 5% non-fat milk in TBST (20 mM Tris-HCl, 500 mM NaCl, Tween-20, pH 7.5) for 1 h at room temperature. Then, the membranes were incubated for 19 h at 4°C in primary antibodies (Santa Cruz Biotechnology Inc., USA) in 1:200 concentration. Then, 3 times wash with TBST was performed and one hour incubation in secondary antibodies conjugated with alkaline phosphatase (Sigma-Aldrich). Post-incubation washes were repeated in the same conditions as previously. Novex® AP Chromogenic Substrate (Invitrogen) was used for the induction of colour reaction. Band visualization was performed on membranes. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) was used as the reference protein.

Results

LOH analysis. In all 23 samples we were able to examine loss of heterozygoty for locus D16S3096 and in 30.4% cases LOH was observed. The level of noticed hemizygoty in intron 1 of WWOX gene (marker D16S518) was lower then homozygoty observed in the population (according to Genome databases). Detailed information on LOH analysis is presented in Table I. Analysis of correlation between LOH for microsatellite locus D16S3096 and expression level of WWOX gene did not reveal statistically significant reduction of this gene transcription.

Analysis of WWOX methylation status. The methylation analysis of WWOX gene in Wilms' tumor samples revealed that the first region from -508 to -174 bp was methylated in 13.64% cases and the second region in 8.7% cases. We noted statistically significant reduction of WWOX gene expression level in samples with

Table II. Correlation between expression of the *WWOX* gene and other analyzed genes.

| Gene | Correlation coefficient R_s | p-value |
|--------------------------|-------------------------------|-------------|
| <i>BAX</i> | -0.2219 | 0.3089(NS) |
| <i>BCL2</i> | 0.8676 | <0.0001 |
| <i>BCL2/BAX</i> | 0.8368 | <0.0001 |
| <i>CCND1</i> | -0.6594 | 0.0006 |
| <i>CCNE1</i> | -0.6594 | 0.0006 |
| <i>EGFR</i> | 0.4812 | 0.0201 |
| <i>ERBB4 JM-a</i> | 0.5720 | 0.0054 |
| <i>ERBB4 JM-b</i> | 0.3521 | 0.0994 (NS) |
| <i>TP73</i> | 0.9229 | <0.0001 |
| <i>CDH1</i> | -0.1127 | 0.6087 (NS) |
| <i>Ki67</i> | -0.1887 | 0.3884 (NS) |

Bold indicates statistically significant correlation.

methyated region covering the 3' end of the promoter and part of exon 1. The mean was 15.94 for unmethylated and 4.01 for methylated samples ($p=0.036$). Also in the second promoter region of *WWOX* gene its expression decreased after methylation (means were 14.92 vs 0.83; $p=0.0037$, for unmethylated vs. methylated samples, respectively).

Expression. Our experiment revealed correlations between *WWOX* expression level and expression levels of examined genes. Spearman rank correlation analysis showed that *WWOX* expression level positively correlated with anti-apoptotic *BCL2* gene ($R_s=0.8676$; $p<0.0001$) and *BCL2/BAX* expression ratio ($R_s=0.8368$; $p<0.0001$). Correlation between *WWOX* and proapoptotic *BAX* was not statistically significant. Positive correlation was also found between examined suppressor gene and *ERBB4 isoform JM-a* ($R_s=0.5720$, $p=0.0054$), *TP73* ($R_s=0.9229$; $p<0.0001$), *EGFR* ($R_s=0.4812$; $p=0.0201$). On the other hand, *WWOX* gene expression was inversely associated with both cyclins *CCND1*, *CCNE1* ($R_s=-0.6594$, $p=0.0006$, $R_s=-0.6594$, $p=0.0006$, respectively). More detailed information on received correlation is shown in Table II. There were no statistically significant correlations between *WWOX* gene expression and clinical factors such as age, stage or risk.

Discussion

Reduced expression level of *WWOX* gene was demonstrated in many studies conducted on different types of tumors (breast, gastric, esophageal, pancreatic, lung cancer). One of the explanatory mechanism of this decrease is loss of heterozygosity (15-20). This is also a frequent event in Wilms' tumors comprising examined region of chromosome. The LOH on chromosome 16 in this embryonal tumor was associated with higher risk of recurrence and death (4,5,25). In our experiment we observed loss of heterozygosity only at intron 8 of *WWOX* in 30.4% samples. In the second analyzed microsatellite marker located at intron 1, the incidence of hemizyosity was at the general population level. However, LOH did not influence the *WWOX* expression level.

WWOX promoter methylation in nephroblastoma specimens was also examined. According to previous findings in Wilms' tumors, epigenetic mechanism appears frequently and regulates expression of *IGF2*, *H19* and *CDKN1C* ($p57^{KIP-2}$) genes (26). As for *WWOX*, this event has also been observed and methylation of its promoter correlated with reduction of its expression in breast, lung, bladder, gastric and hematopoietic cancers (15,20,21). On this basis we analyzed Wilms' samples in terms of *WWOX* promoter methylation and we observed this epigenetic change of -508 to -174 bp region in 13.64% cases and 8.7% for -171 bp to +239 bp region.

Contrary to LOH, methylation of region covering 3' end of the promoter and part of exon 1 was associated with statistically significant reduction of *WWOX* gene expression level. Taking into consideration these results, we may assume that LOH in Wilms' tumors does not play as vital role as methylation status in lowering expression of this tumor suppressor gene.

In this study we also focused on examining the *WWOX* expression correlation with other genes with definite function in proliferation, apoptosis, signal transduction, cell cycle, cell adhesion and transcription. First of all, the conducted analysis on nephroblastoma samples revealed negative correlation between *WWOX* mRNA and the expression level of the cyclins *CCNE1* and *CCND1*.

Cyclin E is a marker of cell cycle progression from G1 into S phase and controls RB protein phosphorylation by cyclin-dependent kinase 2 (Cdk2). Loeb *et al* identified in the promoter of *CCNE1* sites binding of WT1 gene (Wilms tumor suppressor gene) (27). Moreover, high protein level of cyclin E was associated with aggressiveness in this type of tumor (28). Whereas, overexpression of cyclin D1 was observed in 28% of cases, but authors indicated a role of cyclin D2, which was overexpressed in 86% of Wilms' tumors but did not correlate with clinicopathological factors and recurrence (29). Moreover, we revealed a positive correlation between expression of *WWOX* and *TP73*, which is a member of p53 family and *WWOX* partner at the same time.

In Wilms' tumors an interaction between *TP73* and *WT1* was demonstrated and it was suggested as a possible influence on the biological function of *TP73* gene (30). The balance between apoptosis and proliferation is essential for tumor cells growth and response to drugs. In this study a positive correlation between *WWOX* expression level and anti-apoptotic *BCL2* and ratio *BCL2/BAX* was found. The role of apoptosis in progression of Wilms' tumors is ambiguous. Ghanem *et al*, demonstrated significant relationship between blastemal *BCL2* expression, *BCL2/BAX* ratio and clinical progression, with no relevance to stage. Patients with low *BCL2/BAX* ratio (*BCL2/BAX*⁺) appeared to have better prognosis in comparison with three groups of patients with ratio *BCL2/BAX*, *BCL2*⁺/*BAX*, *BCL2*⁺/*BAX*⁺ (31). Other authors noted significant difference in the apoptotic index between stages I and IV and there was lower apoptotic process in patients with unfavorable histology. However, they did not observe correlation between apoptotic index and *BCL2* expression suggesting the role of other regulator genes in apoptosis (32).

Recently conducted studies revealed interaction of *WWOX* protein by the first WW domain with two PPxY motifs of *JM-a* isoform *ERBB4* protein. *WWOX* protein prevents cleaving

membranous ERBB4 through tumor necrosis factor α and γ -secretase enzymes to intracellular domain (ICD) fragment which in nucleus regulates gene transcription (7). In Wilms' tumors we have also observed a positive relation of *ERBB4 JM-a* expression level with *WWOX* and such coexpression with membranous ERBB4 was found in breast cancer patients with favourable prognosis (8).

In Wilms' tumors overexpression of HER family members i.e., EGFR (HER1) and HER2 was reported with indication on more frequent EGFR expression in intermediate- and low-risk tumors (33). Another study did not reveal any association between EGFR overexpression and gene amplification, stage, histopathology nor prognosis (34). In the present study we obtained positive correlation for *WWOX* and *EGFR* mRNA level.

In conclusion, the presented study conducted on Wilms' tumors samples revealed that expression of *WWOX* is positively associated with process of apoptosis, signal transduction through the ErbB4 pathway and EGFR and negatively with regulation of cell cycle (by cyclin E1 and D1). Moreover, our analysis demonstrated statistically significant correlation between *WWOX* gene promoter methylation and its expression.

Our data indicate that epigenetic mechanism can regulate *WWOX* gene transcription in Wilms' tumors. Furthermore, all obtained results are similar to those revealed for this gene in other cancer types and are of importance in Wilms' tumor and seems to influence the process of its development. However, further studies ought to be conducted.

Acknowledgements

The authors wish to thank Ewa Latkowska for her excellent technical support. This study was supported by the Polish Ministry of Science and Higher Education 2670/B/P01/2008/34 and 0757/B/P01/2009/37. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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