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The correlation analysis of *WWOX* expression and cancer related genes in neuroblastoma— a real time RT-PCR study

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Neuroblastoma is one of the most common paediatric cancers, described as unpredictable due to diverse patterns of behaviour. *WWOX* is a tumour suppressor gene whose expression is reduced in many tumour types. Loss of its expression was shown to correlate with more aggressive disease stage and mortality rate. The aim of this study was to investigate the role of the *WWOX* tumour suppressor gene in neuroblastoma formation. We performed real-time RT-PCR to analyse levels of *WWOX* expression in 22 neuroblastic tumour samples in correlation with genes involved in cell cycle regulation (*CCNE1, CCND1*), proliferation (*MKI67*), apoptosis (*BCL2, BIRC5, BAX*) and signal transduction (*EGFR, ERBB4*). We also evaluated two potential mechanisms — promoter methylation (MethylScreen method) and loss of heterozygosity (LOH) status, which could be connected with regulation of *WWOX* gene expression. We found a positive correlation between *WWOX* gene and *BCL2* and *HER4 JM-a* and negative with cyclin D1 and E1. Our observations are consistent with previous findings and emphasise the role of *WWOX* in cell cycle and apoptosis regulation. Moreover, strong positive association with *HER4 JM-a* in this tumour type may indicate a role for *WWOX* in neuroblastoma cell differentiation. The presented results indicate that LOH in locus D16S3096 (located in intron 8) may be involved in the regulation of *WWOX* mRNAexpression. However, no association between methylation status of *WWOX* promoter and its expression was observed.

Key words: neuroblastoma, *WWOX* gene, LOH, real time RT-PCR

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INTRODUCTION

Neuroblastoma is one of the most common paediatric cancer (Maris & Matthay, 1999; Heck *et al.* 2009; Kaatsch, 2010). This malignant tumour is an embryonic cancer consisting of undifferentiated neuroectodermal cells derived from the neural crest (Schwab *et al.*, 2003). In most cases neuroblastoma arises in the adrenal glands, but can also appear in the abdomen, chest or pelvis (Heck *et al.,* 2009). This type of tumour is often characterized as enigmatic or unpredictable due to contrasting patterns of behaviour from life-threatening progression, development to ganglioneuroblastoma or ganglioneuroma to spontaneous regression (Schwab *et al.*, 2003). Neuroblastoma classification is still based on age, histologic stage and Shimada pathology (Shimada *et al.*; 1999; Schwab *et al.*, 2003). The most common molecular marker of neuroblastoma pathology is *MYCN* amplification which is regarded as a prognostic factor in determining treatment. Moreover, chromosomal abnormalities, including chromosome gain or LOH within chromosome 1p, 17q appear quite frequently. Other chromosomes affected in neuroblastoma formation include chromosomes 2q, 3p, 4p, 11q, 14q, 16p and 19q (Schwab *et al.*, 2003).

WWOX is a tumour suppressor gene located on the long arm of chromosome 16 (Bednarek *et al.*, 2000). This area is known as a common fragile site FRA16D, which is affected in many cancers (Chen *et al.*, 1996; Latil *et al.,* 1997; Bednarek *et al.*, 2000). Genomic alteration within the *WWOX* region and its differential expression has been found in variety of tissues and tumour types (Kuroki *et al.,* 2002; Aqeilan *et al.,* 2004; Guler *et al.,* 2004; Kuroki *et al.,* 2004). The reduction of *WWOX* expression in many cancers was found to correlate with more aggressive disease stage and higher mortality rate (breast, gastric, bladder, lung cancer) (Nunez *et al.*, 2005; Pluciennik *et al.*, 2006; Aqeilan *et al.*, 2007; Ramos *et al.*, 2008; Maeda *et al.*, 2010).

It was previously shown that ectopically induced *WWOX* overexpression in different cell lines (breast, lung) promoted apoptosis, suppressed anchorage-independent growth and inhibited colony formation in Matrigel (Bednarek *et al.*, 2001; Kuroki *et al.*, 2004; Fabbri *et al.*, 2005; Qin *et al.*, 2006; Lewandowska *et al.*, 2009; Xiong *et al.*, 2010; Zhou *et al.*, 2010). WWOX as a partner of several transcription factors participates in controlling expression of genes which are responsible for tissue morphogenesis and cell differentiation (Ramos *et al.*, 2008; Aqeilan *et al.*, 2008; Lewandowska *et al.*, 2009). More recently, Gourley and coworkers showed that WWOX restoration or its overexpression in an ovarian cell line resulted in decreased attachment and reduced cell migration on fibronectin, which as an ECM component is associated with peritoneal metastasis (Gourley *et al.,* 2009). Several studies on animal models not only confirmed the role of *WWOX* in tumourigenesis (Aqeilan *et al.*, 2007; Ludes-Meyers *et al.*, 2007; Aqeilan *et al.*, 2008; Ludes-Meyers *et al.*, 2009), but also revealed its potential role in steroidogenesis and proper gonadal function (Aqeilan *et al.*, 2009).

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Abbreviations: ECM, extracellular matrix; HRM; high resolution melting; LOH, loss of heterozygosity; RT-PCR, reverse transcriptase polymerase chain reaction

The aim of our study was to evaluate the role of WWOX in neuroblastoma carcinogenesis. We examined *WWOX* expression, methylation status and the frequency of LOH within its genomic region in neuroblastoma samples and analyzed the correlation between its expression level and genes involved in cell cycle regulation (*CCNE1, CCND1*), proliferation (*MKI67*), apoptosis (*BCL2*, *BIRC5*, *BAX*) and signal transduction (*EGFR, ERBB4*).

MATERIALS AND METHODS

Patients. Tissue samples were obtained from children treated in the Department of Paediatric Oncology and Hemtology, Medical University of Lodz, Poland, and in the Department of Bone Marrow Transplantation, Pediatric Oncology, and Haematology, Medical University, Wroclaw, Poland.

The study included 22 neuroblastic tumour samples: 18 neuroblastoma (8 described as poorly differentiated) and 4 ganglioneuroblastoma. The group consisted of 12 males and 10 females. There were 8 patients aged under one year, and the remaining group were patients older than one year old (mean age 2.86). According to clinical records 5 out of 22 tumours were metastatic. MYCN status was also determined, as well as stage, risk of tumour development and prognosis according to INPC.

More detailed information is shown in Table 1. This study was conducted after receiving patients' family consent and approved by the Institutional Bioethics Committee

RNA, DNA isolation and cDNA synthesis. RNA was isolated from neuroblastic tissues stored at –80°C in RNAlater (Ambion), using TRIzol reagent (Invitrogen). 10 μg of total RNA was used to cDNA synthesis, at the total volume of 100 μl with ImProm RT-II reverse transcriptase (Promega). The conditions of reverse transcription were as follows: 5 min incubation at 25°C, and 60 min at 42°C, heating at 70°C for 15 min. The synthesised cDNA was diluted with sterile deionised water to 150 μl and 2 μl of cDNA was used in a PCR reaction.

DNA was recovered using back extraction buffer (BEB, 1 M Tris Base, 4 M guanidinium thiocyanate, and 50 mM sodium citrate) from organic remains of TRIzol after RNA isolation and was performed according to the manufacturer's protocol.

Real-time RT-PCR analysis. Real-time RT-PCR was performed with Light Cycler 480 II (Roche). SYBR Green I and qPCR Core Kit for SYBR Green I (Eurogentec) was used to detect PCR products. Reactions were performed in duplicate. We analyzed relative expression of 11 genes (*BAX, BCL2, BIRC5, EGFR, MKI67, WWOX, HER4 (isoforms JM-a and JM-b), CCNE1, CCND1, CDH1)*. The expression level of the studied genes was normalized to two reference genes (*RPLP0, RPS17)*.

As the level of *WWOX* mRNA was relatively low, we performed a semi-nested RT-PCR for assessing the *WWOX* expression level. In the first PCR reaction we used primers: 5'-TGCAACATCCTCTTCTCCAACGAGCTGCAC-3' and 5'-TCCCTGTTGCATGGACTTGGTGAAAGGC-3' in 50 μl volume. Next, 2 μl of 200-fold diluted PCR product (171 bp) was a template for semi nested PCR. The cycling protocol included 2 min at 94°C, 30 s denaturation at 94°C, 30 s annealing at 63°C, 1 min extension at 72°C, repeated for 77 cycles, and additional extension for 7 min at the same temperature as previous step.

Table 1. Patient's characterization.

Clinical factor	Number of samples
Sex	
Male	12
Female	10
Age	
<1	8
>1	15
INSS	
I	3
II	8
III	6
IV	5
Risk	
low	7
Intermediate	6
high	9
MYCN status	
Positive	6
Negative	15
No data	1
INPC-prognostic category	
favourable	11
Unfavourable	11

INSS — International Neuroblastoma Staging System; INPC — International Neuroblastoma Pathology Classification

Sequences of used primers and the PCR reaction conditions, as well as the length of received products are presented in Table 2.

Relative expression level was calculated according to Roche algorithm (Pfaffl *et al.,* 2002), which includes the differences in efficiency and crossing point (Cp) of each sample versus calibrator sample and normalize the value to a reference gene.

The Universal Human Reference RNA (Stratagene) was used as a calibrator for each reaction.

To avoid amplification of genomic DNA, all primers were designed to be intron spanning. Detection temperature was designated above the non-specific/primer-dimer melting temperature.

LOH analysis. Loss of heterozygosity was analysed with high resolution melting (HRM) of Light Cycler 480 (Roche). We used two microsatellite markers D16S518 and D16S3096 located on chromosome 16; on intron 1 and intron 8 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 s, annealing at 56°C (for D16S3096) or 55°C (for D16S518) for 30 s, elongation at 72°C for 60 s.

Analysis of WWOX methylation status. We performed a MethylScreen assay which is based on a set of restriction digestions and subsequently performed Real-time PCR according to (Holemon *et al.*, 2007). We analysed two fragments which comprise the promoter and the first exon of the *WWOX* gene.

The PCR for the first fragment of the *WWOX* gene (-508 to -174 bp) region was performed with the following primers: the forward primer sequence was:

Table 2. Real-time RT-PCR primers and reaction conditions.

Primer sequence (5′-3′)	Product size (bp)	Annealing temp. (°C)	Detection temp. (°C)
RPLPO ACGGATTACACCTTCCCACTTGCTAAAAGGTC AGCCACAAAGGCAGATGGATCAGCCAAG	69	65	72
RPS17 AAGCGCGTGTGCGAGGAGATCG TCGCTTCATCAGATGCGTGACATAACCTG	87	64	72
BCL2 TTGGCCCCCGTTGCTTTTCCTC TCCCACTCGTAGCCCCTCTGCGAC	122	56	81
BAX AGAGGTCTTTTTCCGCGTGGCAGC TTCTGATCAGTTCCGGCACCTTG	137	56	81
BIRC5 AGTGTTTCTTCTGCTTCAAGGAGCTGGAAG ACCGGACGAATGCTTTTTATGTTCCTCTATG	83	65	72
MKI67 TCCTTTGGTGGGCACCTAAGACCTG TGATGGTTGAGGCTGTTCCTTGATG	156	56	81
CCNE1 TTCTTGAGCAACACCCTCTTCTGCAGCC TCGCCATATACCGGTCAAAGAAATCTTGTGCC	138	68	68
CCND1 TGTCCTACTACCGCCTCACACGCTTCCTCTCCG TCCTCTTCCTCCTCCTCGGCGGCCTTG	160	63	86
CDH1 TCCCCCGGTATCTTCCCCGCCCTG AGTTCAGGGAGCTCAGACTAGCAGCTTCGG	168	63	81
EGFR AGCTTCTTGCAGCGATACAGCTCAGAC TGGGAACGGACTGGTTTATGTATTCAGG	106	58	81
JM-a HER4 ACACAGCCCTCCTCCTGCCTACAC AGGGCACAGACACTCCTTGTTCAGC	95	56	76
JM-b HER4 AGAGCAAGAATTGACTCGAATAGGAACC AGGGCACAGACACTCCTTGTTCAGC	82	56	76

5'-ACAGAAGCCCAGGACAACAGCATGG-3', and the reverse primer sequence was: 5'-ACCACGAAGCTGAAATCCAGTCTCCG-3'.

For the second region (from –171 bp to +239 bp) covering the 3' end of the promoter and part of exon 1 the following primers were used: forward primers: 5'-AGACTGGATTTCAGCTTCGTGGTCG-3', and the reverse primer sequence: 5'-AAGCTCCTTAACAGTTACTTTCACTTTGCAC-3'.

For the first analysed promoter fragment of the *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 s, 55°C for 30 s, and 72°C for 90 s, and 77°C for 15 s (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 betaine. Real-time PCR were performed at the following conditions: 95°C for 5 min, 50 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s, and 83°C for 15 s (additional temperature for reading only specific amplification product size 413 bp). All reactions were performed in duplicate.

Statistical analysis. Non-parametric Spearman Rank Correlation test was used to analyze correlations between expression of *WWOX* and other genes. Student's/Aspin-Welsch *t*-test was used to estimate differences between *WWOX* expression in relation to LOH, methylation status and clinical factors. All results were assumed as statistically significant at the confidence level >95% ($p<0.05$).

RESULTS

Methylation of the *WWOX* promoter (on both fragments) was observed only in 7 out of 22 neuroblastoma samples and had no influence on *WWOX* gene expression in our sample population.

Loss of heterozygosity for locus D16S3096 was observed in 50% (11 samples) and for microsatellite D16S518 in 16.6% (3 samples) of neuroblastoma samples. The information about observed LOH and comparison with populational homozygosity according to Genome databases are presented in Table 3.

Table 3. LOH analysis in neuroblastoma tumours.

	D16S3096	D16S518
Observed hemizygosity in neuroblastoma tumour	50%	16,6%
Populational homozygosity	26%	17%
Predicted loss of heterozygosity	24 %	0%

Moreover, an analysis of influence of LOH on *WWOX* gene mRNA level revealed a tendency to reduction of its expression via this mechanism ($p>0.05$). In D16S3096, mean *WWOX* gene mRNA level was 16.6 and 13.3 for heterozygous and homozygous samples, respectively ($p>0.05$). Due to the low number of homozygous samples in the second examined locus D16S518 the comparison between mean expression level had no statistical legitimacy.

We found a positive, statistically significant correlation between the *WWOX* expression level and antiapoptotic *BCL2* gene ($R_s=0.6838$, $p=0.0005$) and *HER4* isoform JM-a mRNA ($R_s=0.7165$, $p=0.0002$). Negative significant correlations were observed for *WWOX* mRNA and cyclins *CCND1* ($R_s=-0.4671$, $p=0.0284$), and *CCNE1* ($R_s=-0.4884$, $p=0.0211$). We did not find any statistically significant association between the *WWOX* expression level and the expression of proapoptotic *BAX* gene, nor with the *BCL2/BAX* ratio. Moreover, we did not find any correlation between the examined suppressor gene and proliferation marker *MKI67*, as well as with the other investigated genes. More detailed information on correlation levels is presented in Table 4 and correlation plots are presented on Fig. 1. There were no significant correlations between expression of investigated *WWOX* and clinical factors such as age, stage and histology (favourable/unfavourable) or MYCN status. However, we noticed elevated mean *WWOX* expression in association with INPC prognostic category and in accordance with tumour type. Detailed information is presented in Table 5.

Table 4. Spearman rank correlation between *WWOX* expression level and other tumour related genes.

Gene	Correlation coefficient Rs	P-value
BAX	0.3529	0.1072 *(NS)*
BCL2	**0.6838**	**0.0005**
CCND1	**–0.4671**	**0.0284**
CCNE1	**–0.4884**	**0.0211**
EGFR	0.2705	0.2234 (NS)
MKI67	0.1090	0.6293 (NS)
HER4 Jm-b	0.2603	0.2420 (NS)
BIRC5	–0.0920	0.6837 (NS)
CDH1	–0.3525	0.1076 (NS)
HER4 Jm-a	**0.7165**	**0.0002**
BCL/BAX	0.0909	0.6874 (NS)

Bold indicates statistically significant correlation.

DISCUSSION

WWOX is a tumour suppressor gene with decreased expression in many tumour types (Yendamuri *et al.*,

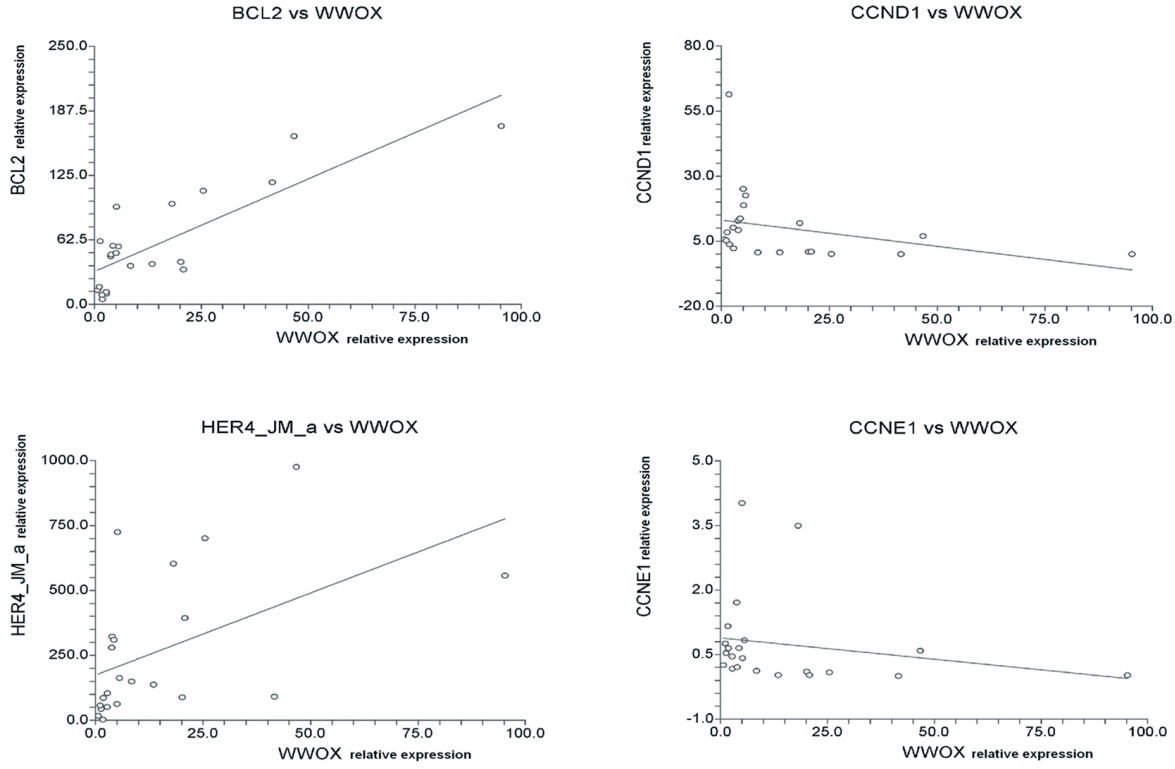

Figure 1. Correlation analysis of *WWOX* and cancer related genes expression in neuroblastic tumours.
Both axes represent the relative expression level of investigated genes.

Table 5. *WWOX* gene expression alterations according to clinical factors.

Clinical factor	Sample count	*WWOX* mean expression ±S.E.
Tumour		
NBL	10	20.02±9.4
NBL PD	8	9.8±3.6
GNBL	4	12.4±3.6
INPC prognostic category		
Favourable	11	20.1±8.9
Unfavourable	11	9.9±2.8

NBL — neuroblastoma; NBL PD — neuroblastoma poorly differentiated; GNBL — ganglioneuroblastoma; INPC — International Neuroblastoma Pathology Classification; SE — standard error

2003; Aqeilan *et al*., 2004; Nunez *et al*., 2005; Nunez *et al*., 2005; Dias *et al*., 2007; Ramos *et al*., 2008; Maeda *et al*., 2010). One of the possible mechanisms inactivating its expression is loss of heterozygosity and methylation of CpG islands within the promoter region. LOH of *WWOX* locus has been found in few tumour types like Wilm's tumours (Skotnicka-Klonowicz *et al*., 2000), prostate (Carter *et al.,* 1990), breast (Bednarek *et al*., 2000), gastric (Aqeilan *et al.,* 2004), oesophageal (Kuroki *et al*., 2002) and pancreatic (Kuroki *et al*., 2004) cancer. In our study, we observed LOH on both examined microsatellite markers. However, the observed hemizygosity in intron 1 was the same as populational homozygosity. As for intron 8, hemizygosity was relatively frequent. Similar frequency for the same marker was observed in glioblastoma multiforme tumour samples (Kosla *et al.,* 2011) and intronic deletions spanning several hundred base pairs of intron 8 were reported in many other cancers (Paige *et al*., 2000). Loss of heterozygosity is an abnormality frequently appearing in neuroblastoma which mainly affects other chromosomes than 16. However, LOH at this chromosome but within region 16p12-p13 was previously found to be connected with both sporadic and familial neuroblastoma (FNB locus) (Furuta *et al*., 2000; Weiss *et al*., 2000). In our study we did not observe any correlation of LOH with prognostic variables. However, such a frequent LOH at the *WWOX* gene region in our neuroblastoma population, seems to require further studies. The second above mentioned inactivating mechanism i.e. methylation of the *WWOX* region appears to be an insignificant mechanism in neuroblastoma tumours.

In our study we also assessed the correlation of *WWOX* expression with the expression of genes with different cell functions i.e. apoptosis, cell cycle, adhesion, proliferation and signal transduction.

The observed positive correlation with antiapoptotic *BCL2* is consistent with previous findings in glioblastoma and colon cancer (Kosla *et al*., 2011; Zelazowski *et al.,* 2011). The influence of WWOX on apoptosis pathways is not well defined. In vitro studies conducted on MDA-MB231 cells transiently transfected with adenovirus harbouring *WWOX* cDNA resulted in increased cell death (Iliopoulos *et al*., 2007). On the other hand, the same cell line but stably transfected with *WWOX* cDNA had upregulated *BCL2* expression and was characterized by increased invasion through basal membranes, suppressed anchorage independent growth and higher migration in Matrigel (Lewandowska *et al*., 2009). Gourley et al. found similar discrepancies after ovarian cancer cell lines transfection. Adherent cells modified with *WWOX* cDNA did not have higher apoptotic potential in contrast to those grown in suspension (Gourley *et al*., 2009). The highest rate of spontaneous regression is characteristic for neuroblastoma tumours (Oue *et al*., 1996). The molecular process underlying and explaining this phenomenon is still not well defined, although it is believed that programmed cell death and apoptosis may regulate its appearance. BCL2 as the apoptosis pathway regulator does not have a well defined role in neuroblastoma. Some research has shown that its overexpression correlated with favourable histology whereas others found that increased expression of this gene leads to tumour progression (Abel *et al*., 2005). Overall, there seems to be no correlation between this gene and prognostic variables (Ramani *et al*., 1994; Maris & Matthay, 1999).

In our study we did not observe any correlation between *BCL2* expression and prognostic variables, thus the association found between this gene and the *WWOX* only confirms the correlation of their expressions also observed in other tumours.

Moreover, we observed a significant negative correlation of expression between *WWOX* and both cyclins (*CCND1* and *CCNE1*). Similar correlation of *WWOX* and cyclin expression has been recently reported in Wilm 's tumours and colon cancer (Zelazowski *et al*., 2011; Pluciennik *et al*., 2012).

Both cyclins are phase G1/S specific and their increased expression was reported in neuroblastoma tumour and was associated with unfavourable histology (Hiyama *et al*., 2004). Cyclin E1 has been also associated in this tumour with stage 4 and poor prognosis in neuroblastoma patients (Mao *et al*., 2012). The second cyclin, *CCND1* was shown to be overexpressed in unfavourable, malignant neuroblastomas (Hiyama *et al*., 2004; Molenaar *et al*., 2008) and was also connected with an undifferentiated phenotype (Molenaar *et al*., 2008). Thus, acquired negative correlation between *WWOX* tumour suppressor gene and both cyclins may indicate that this gene negatively regulates the cell cycle in tumour cells.

During our study we also examined differences in the expression of signal transduction genes, i.e. *EGFR* and both isoforms of *HER4*. We only observed a strong positive correlation between *WWOX* and *HER4 JM-a*, which is consistent with previous findings. Aqelian and coworkers established the competition between WWOX and YAP protein for interaction with HER4, this interaction modulates signal transduction of the HER pathway (Aqeilan *et al*., 2005). The sequestration of JM-a in cytoplasm as result of WWOX-HER4 interaction has been confirmed in breast cancer (Aqeilan *et al*., 2007), Wilm's tumours (Pluciennik *et al.,* 2012), glioblastoma (Kosla *et al*., 2011) and colon cancer (Zelazowski *et al*., 2011). The HER family receptors are involved in embryonic development of the sympathetic system (Britsch *et al*., 1998; Casalini *et al*., 2004), however, their role in neuroblastoma is not well understood (Izycka-Swieszewska *et al*., 2011). There have been conflicting reports in terms of HER4 expression alterations in cell lines and primary tumours. Low expression of HER4 has been established in some neuroblastoma cell lines, and primary tumours, indicating no correlation with tumour stage (Ho *et al*., 2005). However, Richards et al. consistently detected expression of HER 4 in 7 out of 9 examined neuroblastoma cell lines and in all examined 20 tumour samples, indicating its potential role in promotion of neuroblastoma growth (Richards *et al*., 2010).

More recently, Izycka-Swieszewska and coworkers (2011) evaluated *via* immunohistochemistry the expression of HER1-4 receptors in 103 NT and their prognostic significance and clinicopathological correlations. HER4 expression was found in 87% of all tumours, however, its low expression was more frequent in poorly differentiated neuroblastomas, but it did not correlate with the histological risk groups. Moreover, HER4 inversely correlated with the MKI index, and high expression was more frequent in children older than 18 months. On the other hand, expression of this receptor was characteristic for high risk group tumours and for metastatic rather than localized stage of disease (Izycka-Swieszewska *et al*., 2011).

Nevertheless, taking together the above mentioned publications describing the role of HER4 in neuroblastoma and observed in our study the strong correlation between expression of *WWOX* and *HER4* we may suggest a potential role of this tumour suppressor gene in neuroblastoma cell differentiation regulation. We noticed decreased *WWOX* mean expression in poorly differentiated neuroblastoma samples and in samples which were assigned to the unfavourable prognostic category.

In conclusion, our study conducted on neuroblastoma samples revealed the potential role of LOH in altering *WWOX* expression. Moreover, the negative correlation observed with both cyclins and the positive correlation with *BCL2*, suggest its role in the regulation of the cell cycle and apoptosis of neuroblastoma cells. This results are consistent with previous findings.

Our observations for *WWOX* in neuroblastoma samples also outlined its potential role in the regulation of cell differentiation, however, further studies ought to be conducted.

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