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RESEARCH ARTICLE

WWOX expression in colorectal cancer—a real-time quantitative RT-PCR study

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Abstract The *WWOX* gene is a tumour suppressor gene affected in various types of malignancies. Numerous studies showed either loss or reduction of the *WWOX* expression in variety of tumours, including breast, ovary, liver, stomach and pancreas. Recent study demonstrated that breast cancer patients exhibiting higher *WWOX* expression showed significantly longer disease-free survival in contrast to the group with lower relative *WWOX* level. This work was undertaken to show whether similar phenomena take place in colon tumours and cell lines. To assess the correlation of *WWOX* gene expression with prognosis and cancer recurrence in 99 colorectal cancer patients, we performed qRT-PCR analysis. We also performed analysis of *WWOX* promoter methylation status using MethylScreen method and analysis of loss of heterozygosity (LOH) status at two *WWOX*-related *loci*, previously shown to be frequently deleted in various types of tumours. A significantly better disease-free survival was observed among patients with tumours exhibiting high level of *WWOX* (hazard ratio=0.39; p=0.0452; Mantel–Cox log-rank test), but in multivariate analysis it was not an independent prognostic factor. We also found that although in colorectal cancer *WWOX* expression varies among patients and correlates with DFS, the exact mode of decrease in this type of tumour was not found. We failed to find the evidence of LOH in *WWOX* region, or hypermethylation in promoter regions of this gene. Although we provide the evidence for tumour-suppressive role of *WWOX* gene expression in colon, we were unable to identify the molecular mechanism responsible for this.

Keywords *BCL2* · Colorectal cancer · CpG methylation · LOH · Quantitative RT-PCR · *WWOX*

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Introduction

The *WWOX* (WW domain containing oxidoreductase) gene is located in the chromosome 16 region 16q23.3–24.1, also known as common fragile site FRA16D [1], an area which was found to be frequently affected by allelic losses in breast and other cancers [2]. *WWOX* expression was reported to be higher in the testis, ovary and prostate, i.e. tissues where its activity is regulated hormonally [1]. On this basis, *WWOX* was speculated to be involved in regulation of the steroids signalling pathways. Studies on biological role of *WWOX* in tumourigenesis showed that its function in cellular metabolism is likely to modulate gene expression by interactions with other proteins involved in cell cycle/apoptosis control and transcription factors. Up to now several partner proteins were identified, i.e. p73, AP-2γ[3], ErbB-4 [4], Runx2 [5] and members of Dvl protein family [6]. It was also shown that WWOX protein physically binds to two cytoplasmic regions of ErbB-4, which were previously verified to be responsible for interactions with Yap proteins. This competition for the

ErbB-4 binding sites may prevent ErbB-4 transactivation and may lead to dysregulation of cell signalling [4]. Regardless of its function in cell metabolism, *WWOX* is considered as a tumour suppressor gene in various types of malignancies, including: breast [7], ovarian and lung cancer [2]. The evidence for its tumour suppressor activity was demonstrated for the first time in several cancer cell lines [7]. Since then numerous studies showed either loss or reduction of the *WWOX* expression in a variety of human tumours of breast, ovary, liver, stomach, pancreas, oesophagus, lung and haematopoietic malignancies [8]. Latest studies showed that *WWOX* gene is a bona fide tumour suppressor gene (reviewed in [3]), however the most common mechanism of decreasing *WWOX* expression in cancer cells is through hemizygous deletions (especially in breast cancer), while point mutations are very rare [1]. Recently, a set of complex deletions was found at FRA16D in the HCT116 colon cancer cell line, which was responsible for removing fragments of *WWOX* gene [9].

Another mechanism of reducing *WWOX* transcriptional level which was vastly studied is CpG islands hypermethylation of *WWOX* promoter and coding region. It seems that this mechanism may play some role in downregulation of *WWOX* expression in several cancer cell lines, for example tumours of pancreas and prostrate [10], breast, lung and bladder [11]; however first reports on methylation at the *WWOX* promoter region in thirteen breast cancer cell lines revealed that despite dramatic difference in *WWOX* expression, there was no methylation present at this region in any studied cell line [7]. Płuciennik et al. have shown that breast cancer patients exhibiting higher levels of *WWOX* expression exhibited significantly longer DFS in contrast to the group with relatively lower *WWOX* transcript levels [12]. Similarly, Aqeilan et al. showed prognostic relevance of WWOX and ErbB4 proteins in breast cancer [13].

With all results cited above, the lack of studies regarding role of *WWOX* gene and its protein product in tumourigenesis in colon and especially homozygous deletions in *WWOX* region found in HCT116 colon cancer cell line, as reported by [9], prompted us to undertake present work. The aim of our research was to evaluate the role of deletions in *WWOX* gene, its expression and prognostic value in patients with CRC (colorectal cancer). We also evaluated methylation of *WWOX* gene promoter region and the correlations of *WWOX* expression level with other well-known cancer/cell cycle-related genes, as: pro-apoptotic *BAX*, anti-apoptotic *BCL2*, cell cycle regulators: cyclins D1 (*CCND1*) and E1 (*CCNE1*) both regarded as playing an important role in tumourigenesis, tumour suppressor gene *TP73* which encodes for the p73 protein, proliferation marker - *Ki-67* and one *ERBB4* isoform transcript—JM-a/CVT-1.

Materials and methods

Patients and samples

The CRC samples analysed herein were obtained from 99 cases of primary colorectal tumours treated at the Oncology Clinic, Medical University of Łódź. Only patients without previous familial history of CRC and those who did not receive preoperative radiotherapy were enrolled to this study. From these, only 50 had complete history of disease and reliable DFS observations (thus only these patients could be included to survival analysis). Experiments involving human subjects were conducted according to the Declaration of Helsinki: the study was approved by the Ethics Committee at Medical University of Łódź. The mean age of the patients was 61.3 years (median, 63 years; for women, 60 years; for men, 63 years; range, 30–86 years). Median follow-up period was 42.5 months. More detailed characteristics of the patients are shown in Table 1, together with WWOX mRNA level and results of Mann–Whitney *U* test. Tumours were classified according to the International Union Against Cancer staging and grading criteria. The tissue samples were examined histologically and stored at −80°C in RNAlater (Ambion, Inc.) until RNA extraction.

Cancer cell lines

We used cell lines derived from tumours of colon (HCT116, SW480, SW620, HT-29) and two breast cancer cell lines (MDA-MB-231, MCF-7), which served as a control of our results, as both were previously studied for *WWOX* expression [7, 14]. Cell culture was performed according to the vendor's protocol. In brief, HT-29 and HTC116 cell lines were grown in McCoy's 5a medium with addition of 1% L-Glutamine; SW480 and SW620 were cultured in RPMI1640; MCF-7 and MDA-MB-231 cell lines were cultured in DMEM Advanced Medium with 1% L-Glutamine; MCF-7 cells were also supplemented with addition of bovine insulin to the final concentration 0.01 mg/ml. All media were supplemented with 10% foetal bovine serum and 1% PSN antibiotic mixture (penicillin, streptomycin and neomycin; all ingredients Sigma, Germany). Atmosphere consisted of 95% of air and 5% of CO_2; incubation temperature was 37°C.

Real-time quantitative RT-PCR analysis

All RNA extractions and cDNA synthesis were performed as described elsewhere [12]. All real-time RT-PCR reactions were performed in duplicate, except the samples in which the analysis outcome was questionable. If this had happened, another two replicates were analysed. Detection of the amplification product was enabled with EvaGreen®

Table 1 Correlations of *WWOX* expression with clinical characteristics of the patients

Feature	*n*	WWOX mRNA median (range)	P (Mann–Whitney U)
Sex			
Women	51	1.49 (1.23–3.744)	0.3985
Men	48	1.51 (0.57–2.193)	
Localisation of the primary tumour[a]			
Rectum	34[a]	1.69 (0.29–3.74)	0.8301
Sigmoid colon	37[a]	1.42 (1.23–2.47)	
Descending colon	6[a]		
Splenic flexure	3		
Transverse colon	4		
Ascending colon	3		
Cecum	14		
Lymphocytic infiltration			
Absent	57	1.68 (1.23–3.87)	0.1908
Present	41	1.35 (0.29–3.46)	
Unknown	1		
Metastasis to the lymph nodes[#]			
Absent	55	1.65 (1.24–4.26)	0.1591
Present	34	1.57 (0.57–3.52)	
Unknown	10		
Grading (differentiation)			
G1	10	1.09 (0.01–21.38)	0.7331 (G1/G2)
G2	60	1.51 (1.24–3.66)	0.7643 (G2/G3)
G3	29	1.60 (0.57–4.26)	0.7153 (G1/G3)
Dukes' stage			
A	26	1.119 (0.21–3.66)	0.6489 (A/B)
B	29	1.858 (0.32–4.78)	0.4133 (B/D)
C	26	2.475 (1.31–7.35)	0.8777 (A/C)
D	16	1.342 (0.86–4.89)	0.3166 (A/D)
Unknown	2		
Relapse during follow-up			
No	34	2.10 (0.97–5.729)	0.1008
Yes	27	1.32 (0.162–2.06)	
Unknown	38		
Demise during follow-up			
No	46	2.10 (1.26–5.729)	0.2370
Yes	45	1.40 (0.57–2.475)	
Unknown	8		

[a]The localisation of primary tumours from three patients was ambiguous, thus they were qualified to two groups

dye (Biotium Inc., Hayward CA, USA), according to the manufacturer's recommendations in Corbett Research RG-3000 platform (Corbett Life Science, Sydney, Australia), in total reaction volume of 10 or 25 μl. Expression levels were normalised using the panel of four genes: β2-microglobulin *B2M,* histone *H3F3A*, ribosomal proteins *RPS17* and *RPLP0*, which were selected using the geNorm applet [15]. Relative expression was calculated with the mathematical model allowing for correction of reaction efficiency and using the Universal Human Reference RNA (Stratagene, La Jolla, CA, USA) as a reference. Primer sequences used in this study are shown in Table 2; detailed PCR protocols are available upon request from the corresponding author.

LOH analysis

In order to determine the LOH status of the 16q23.3–24.1 region, we used two sequence-tagged site (STS) markers: D16S3096 and D16S518. They are located at: eighth intron and second intron, respectively, of *WWOX* gene. The D16S518 marker is the most frequently affected with LOH in breast cancer (up to 77% in some populations, as described in [16]). Primer sequences used were according to UniSTS database (http://www.ncbi.nlm.nih.gov/). HRM analysis of ampilification products was performed in a LightCycler 480 (Roche Diagnostics, Poland) with EpiTect HRM PCR Kit (Qiagen, Germany).

Table 2 Real-time RT-PCR primers and reaction conditions used for expression analysis of specified genes

Gene name	Gene primers (For/Rev) (5′→3′)	Annealing temperature (°C)	Detection temperature (°C)	PCR product size (bp)
BAX	For: AGAGGTCTTTTTCCGAGTGGCAGC Rev: TTCTGATCAGTTCCGGCACCTTG	56	81	137
BCL2	For: TTGGCCCCCGTTGCTTTTCCTC Rev: TCCCACTCGTAGCCCCTCTGCGAC	56	81	122
B2M	For: TGAGTGCTGTCTCCATGTTTGA Rev: TCTGCTCCCCACCTCTAAGTTG	50	81	88
CCND1	For: GTCCTACTACCGCCTCACACGCTTCCTCTCCAG Rev: TCCTCTTCCTCCTCCTCGGCGGCCTTG	63	86	160
CCNE1	For: TTCTTGAGCAACACCCTCTTCTGCAGCC Rev: TCGCCATATACCGGTCAAAGAAATCTTGTGCC	68	68	138
ERBB2	For: TGACCTGCTGGAAAAGGGGGAGCG Rev: TCCCTGGCCATGCGGGAGAATTCAG	63	83	150
ERBB4	For: ACACAGCCCTCCTCCTGCCTACAC Rev: AGGGCACAGACACTCCTTGTTCAGC	56	76	95
H3F3A	For: AGGACTTTAAAACAGATCTGCGCTTCCAGAG Rev: ACCAGATAGGCCTCACTTGCCTCCTGC	65	72	76
Ki-67	For: TCCTTTGGTGGGCACCTAAGACCTG Rev: TGATGGTTGAGGCTGTTCCTTGATG	56	81	156
RPLP0	For: ACGGATTACACCTTCCCACTTGCTGAAAAGGTC Rev: AGCCACAAAGGCAGATGGATCAGCCAAG	65	72	69
RPS17	For: AAGCGCGTGTGCGAGGAGATCG Rev: TCGCTTCATCAGATGCGTGACATAACCTG	64	72	87
TP73	For: AACCACGAGCTCGGGAGGGACTTCAAC Rev: TTCCGTCCCCACCTGTGGTGGCTC	63	81	159
WWOX	For: GAGCTGCACCGTCGCCTCTCCCCAC Rev: TCCCTGTTGCATGGACTTGGTGAAAGGC	63	77	150

For, forward primer; *Rev*, reverse primer

Analysis of *WWOX* methylation status

To assess the methylation status of one 5′-upstream region involved in regulation of *WWOX* expression (from −508 to −174 bp) and region adjacent to and containing *WWOX* promoter (from −171 to +239 bp) we used novel bisulfite-free alternative technology MethylScreen, utilising the real-time quantitative PCR assay on templates generated by combined restriction digest using: methylation-sensitive restriction enzymes (MSRE), methylation-dependent restriction enzymes (MDRE), combined double digest (both MSRE and MDRE) and mock digestion [17]. The enzymes used in this study were: HhaI, HpaII (MSRE) and McrBC (MDRE; New England Biolabs, Ipswich, MA, USA); all digestions were performed according to the manufacturer's instructions on 500 ng of patient's DNA. All PCRs were performed in total volume of 50 μl, with 4 μl of respective digested sample DNA, 1 μl of each primer (10 mM). The sequences of the primers used were as follows: −508 bp region; For—5′-ACAGAAGCCCAGGACAACAGCATGG-3′; Rev—5′-ACCACGAAGCTGAAATCCAGTCTCCG-3′; −171-bp region; For—5′-AGACTGGATTTCAGCTTCGTGGTCG-3′; Rev—5′-AAGCTCCTTAACAGTTACTTTCACTTTG CAC-3. Cycle conditions were: 95°C for 5 min followed by 55 cycles of 94°C for 30 s, 55°C/30 s, 72°C/90 s and 77°C/15 s (fragment −508 bp) or 95°C for 5 min followed by 50 cycles of 94°C for 30 s, 50°C/30 s, 72°C/90 s and 80°C/15 s (fragment −171 bp).

Statistical analysis

Spearman's rank correlation test was used to analyse possible linear associations between all the gene expression levels. Disease-free survival was estimated with the Kaplan–Meier method. The significance of differences between survival rates was verified using the log-rank (Mantel–Cox) test. Disease-free survival was calculated according to Kaplan–Meier method. Multivariate survival analysis was performed using Cox's regression model. Values of $p<0.05$ (confidence level>95%) were considered statistically significant.

Results

Correlation of *WWOX* expression with clinical parameters

Relative *WWOX* expression in CRC tissues ranged from 0 to 123.18 (median 7.66). Results of statistical analysis of *WWOX* expression in groups of patients divided according to the classical clinical markers are presented in Table 1. We did not find any statistically significant relations between groups of patients stratified according to their basic clinicopathological features; however, we found a tendency for higher relative *WWOX* expression in samples from patients without relapse during the follow-up period (2.10 versus 1.32 units; $p=0.1008$). This finding was then confirmed by analysis of DFS based on relatively high and low *WWOX* expression, which showed significant differences. The cut-off point for discrimination between 'high' and 'low' expression of *WWOX* was 2.70 (units of relative expression). This point was selected with the X-tile software [18]. We found that this cut-off value applied to the standard Kaplan–Meier DFS analysis yielded a significantly better DFS observed among patients with tumours in which the level of *WWOX* mRNA was classified as high (hazard ratio; HR=0.39; $p=0.0452$; Cox–Mantel log-rank test; Fig. 1), however in multivariate analysis it was not an independent prognostic factor ($p=0.8027$). We also conducted survival analysis of patients stratified according to the localisation of primary tumour. Although we found disparity between DFS in patients with primary tumour localised in rectum versus all other localisations, this was not statistically significant (HR=0.48; $p=0.1566$).

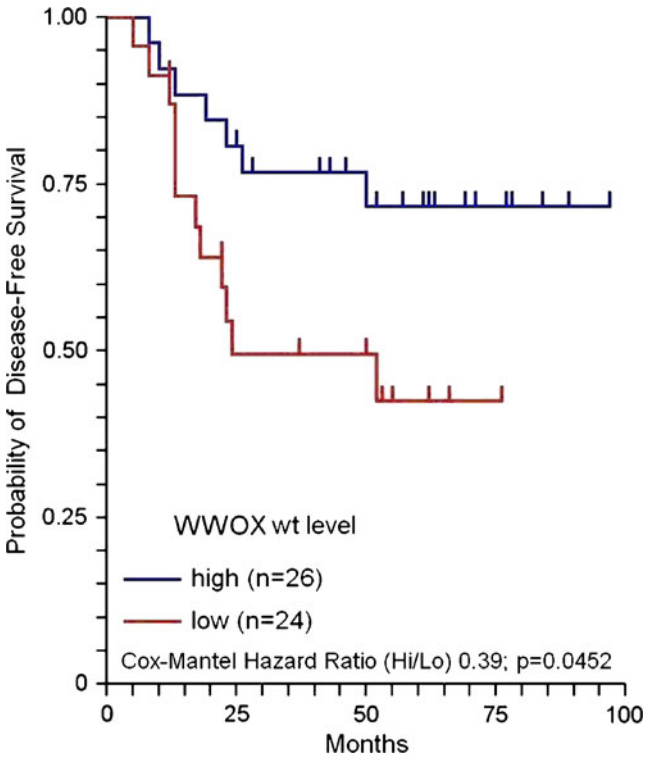

Fig. 1 Results of DFS analysis in patients stratified according to the WWOX mRNA level (Kaplan–Meier test)

Analysis of *WWOX* expression in cell lines

HCT116 colon cancer cell line, although previously found to harbour homozygous deletion in *WWOX* gene, surprisingly showed 70% level of *WWOX* expression, in comparison with MCF-7 cells (all results in Table 3). Reason for this results is suggested by the work of Alsop et al. [9]; authors imply that HCT116 homozygous deletions are within *WWOX* intron, so they should not affect its expression (apart for the supposed role of *WWOX* Δ6–8 transcript in decreasing *WWOX*wt transcription, which nowadays is not supported by solid evidence). Indeed, they previously found two *WWOX* transcripts: variant 1 (*WWOXwt*) and variant 4 (*WWOX* Δ6–8) in this cell line [2], although Northern blots presented showed that *WWOX*wt appears in low abundance. This discrepancy could be in part linked to different techniques used, as suggested by Ding et al.: the correlation between the Northern and qRT/PCR results for 24 genes studied was $r=0.39$; after excluding outlier genes the correlation coefficient rised to 0.72, still far from ideal [19]. We also found that HT-29 cell line, originating from a colorectal adenocarcinoma, showed very low level of *WWOX* expression. The difference in *WWOX* expression between SW480 and SW620 cell lines suggest that there is a room for stating that *WWOX* has some role (not fully identified yet) in the progression of CRC tumours: SW480 cells, originating from primary tumour showed 0.230 of *WWOX* relative expression; whereas in SW620 cells, from metastatic tumour of the same patient, relative *WWOX* expression was even lower, 0.175. This result is similar to the difference in *WWOX* relative expression among patients with lymph node metastases and patients in which there was no nodal metastasis present (1.65 vs. 1.57), however that relationship was not statistically significant ($p=$

Table 3 *WWOX* expression in the studied cell lines

Cell lines	Average *WWOX* relative expression
HCT116	1.075
HT29	0.080
SW480	0.230
SW620	0.175
MCF-7	1.497
MDA-MB-231	0.025

0.1591). MCF7 cells showed the highest expression of the studied cell lines; accordingly, aggressive and highly metastatic breast cancer cell line MDA-MB-231 had the lowest *WWOX* expression (60-fold lower than MCF-7), which is in accordance with previous reports [2, 3].

Analysis of methylation and LOH status of *WWOX* gene locus

In the studied population of patients, we did not find any significant hemizygosity suggesting LOH at the investigated loci in CRC tumours samples. Cell lines exhibited differences in surveyed markers (Table 4), however this had no connection to the expression level of *WWOX*. For instance SW620 cells, which showed retention of both alleles as the only one cell line studied, exhibited almost tenfold lower *WWOX* expression than MCF-7 cells, which expression was the highest observed here.

There was no significant methylation of *WWOX* promoter in patients' samples: we found that only eight (8.1%) of patients had low methylation and two (2%) had moderate methylation at 5′-upstream region (−508 to −174 bp), whereas only seven (7.1%) of patients exhibited low methylation at region adjacent to and containing *WWOX* promoter (from −171 to +239 bp). Of the patients, 12.1% had non-informative results of analysis at 5′ upstream region, while at the promoter region the number of non-informative cases was 8.1% (all results in Table 5). None of the cell lines used in this study showed methylation of *WWOX* promoter region (data not shown). Statistical analysis of correlations between methylation level or LOH at studied *loci* and *WWOX* relative expression did not prove that there was any relationship between those parameters in our study.

Correlation of *WWOX* transcript level with expression of other genes

We found that *WWOX wt* expression is correlated (with statistical significance; Spearman rank correlation test used) with number of surveyed genes (presented in order of lowering probability): significant negative correlation with *CCNE1* expression (−0.3579; $p=0.0005$), which in general is regarded as a marker of bad prognosis and is directly associated with tumourigenesis. We also found a significant positive correlation with *BCL2/BAX* ratio (0.3480; $p=0.0006$). Positive correlations were found between *WWOX* expression and *ERBB4* and *BCL2* (all results in Table 6).

Discussion

In the presented study, we analysed the expression of *WWOX* gene in 99 tumours from patients with colorectal cancer. In several reports it was shown that *WWOX* expression is lowered in various types of tumours (mentioned above). Moreover, many authors have shown that suppressed transcription of *WWOX* is associated with more aggressive phenotype of breast cancer [12], non-small cell lung cancer [20] and ovarian cancer [21]. Here, we show that relatively high *WWOX* expression corresponds with better disease-free survival of CRC patients hazard ratio (HR=0.39; $p=0.0452$; Mantel–Cox log-rank Test, Fig. 1) in comparison with those with lowered *WWOX* transcription. This supports the view that loss of *WWOX* expression is associated with tumourigenesis in different types of cancers. Such an idea was additionally proven by in vitro and in vivo studies which showed that elevated *WWOX* expression suppresses tumourigenicity of different cancer cell lines: breast [7], lung [22] and prostate [23]. However, *WWOX* expression cannot be used as an independent prognostic marker in CRC, since results of multivariate analysis excluded this marker from analysis on early stages (results not shown). Despite the frequent suppression of *WWOX* expression in many cancers, complete gene inactivation by deletion of one allele and second mutation or homozygous deletion is very rare [9]. Based on the observations, it was postulated that *WWOX* inactivation is driven by hemizygous deletions, which was recently proven with mouse model using targeted deletion of *WWOX* gene [24]. In our analysis of 16q23.3–24.1 region we did not find any evidence for LOH in the two studied *WWOX*-associated loci in CRC. We used two STS (sequence-tagged site) markers (D16S3096 and D16S518) which are most often afflicted by hemizygous deletions in all kinds of cancers, for instance: breast ductal carcinoma in situ lesions [16], breast cancer metastases [25], hepatocellular carcinoma [26], non-small cell lung cancer [27], oesophageal squamous cell carcinoma [28], gastric carcinoma [29], but none of the STS markers displayed LOH in our set of colorectal cancer samples.

Table 4 LOH analysis in two *WWOX* gene regions in human cancer cell lines

STS marker	Cell lines					
	HCT116	HT-29	SW-480	SW-620	MCF-7	MDA-MB-231
D16S3096	R	LOH	R	R	LOH	LOH
D16S518	LOH	R	LOH	R	LOH	LOH

LOH, loss of one allele; *R*, retention of both alleles; *NI*, non-informative result

Table 5 Results of the *WWOX* promoter regions methylation analysis in CRC patients

Methylation status	Number of cases (%)	
	5′-Upstream region (−508 to −174 bp)	*WWOX* promoter region (−171 to +239 bp)
'0'	77 (77.8%)	84 (84.8%)
'1'	8 (8.1%)	7 (7.1%)
'2'	2 (2.0%)	0 (0%)
NI	12 (12.1%)	8 (8.1%)
Sum	99	99

'0', no methylation found (difference between the Mock and the MDRE less than one cycle); *'1'*, low methylation (difference between the Mock and the MDRE 1≥1.49 cycle); *'2'*, intermediate methylation (difference between the Mock and the MDRE >1.5 cycle); *NI*, non-informative result

We also tested the status of methylation in the promoter region of *WWOX* gene, presumably resulting in lowered *WWOX* expression, which was shown in several studies [11, 22]. Nevertheless, there are data showing that the methylation status of *WWOX* promoter region does not contribute to the decrease of *WWOX* expression in breast cancer cell lines and prostate tumours [7, 30] which is also in the case of CRC patients studied herein. To our knowledge, this is the first report on methylation status of *WWOX* gene in CRC patients or CRC cell lines. Nevertheless, results of our MethylScreen analysis were very similar to the previously cited work by Bastian et al. [30], who analysed CpG island hypermethylation in a set of 13 gene loci (including *WWOX*) in 78 prostate carcinomas, 32 benign prostate hyperplasias and four prostate cell lines (LNCaP, DU145, PC3, BPH-1) using MethyLight PCR. They found only one case showing *WWOX* promoter region methylation; none of the benign samples were methylated in *WWOX* locus [30]. Moreover, none of the cell lines surveyed (LNCaP, DU145, PC3, BPH-1) exhibited methylation of *WWOX* [30]. Interestingly, previous studies showed loss of *WWOX* expression in as much as 84% (37 of 44 tumour samples) [23] and involvement of promoter methylation in decreasing of *WWOX* expression in prostate cancer cell lines LNCaP, DU145 and PC-3 [23]. We hypothesise that this striking discrepancy between the two abovementioned papers could arise because of the two different strategies of study: Bastian et al showed the exact methylation status of *WWOX* by using MethyLight PCR, whereas Qin et al. used methylation-specific PCR (MSP). One should remember that MSP is gel-based technique and provides rather qualitative results, whereas PCR-based techniques are able to discriminate between different levels of methylation. Qin et al. also assumed that increased *WWOX* mRNA and protein expression in prostate cancer-derived cells after treatment with 5-aza-2′-deoxycytidine (AZA; a DNA methyltransferase inhibitor) and trichostatin A (a histone deacetylase inhibitor), is a result of demethylation of only *WWOX* promoter region. However, one should be aware of the fact that these agents are not specific and they change the global methylation/acetylation status of the cell, including all hypothetical and/or unknown regulators of *WWOX* expression.

Table 6 Correlations between *WWOX wt* expression and other genes (Spearman test)

Gene name	Spearman rank correlation coefficient	*p* Value
CCNE1	−0.3579	0.0005
BCL2/BAX ratio	0.3480	0.0006
BAX/BCL2 ratio	−0.3308	0.0012
Ki67	−0.2913	0.0046
ERBB4	0.2473	0.0242
BCL2	0.2066	0.0372
ERBB2	−0.1957	0.0709
BAX	−0.1684	0.0906

Recently, a paper by Kosla et al. showed that both methylation of *WWOX* promoter region and LOH at D16S518, D16S3096 and D16S504 have influence on *WWOX* expression in glioblastoma multiforme tumours [31]. In this work, we did not find any evidence for such a relationship, which may suggest that these mechanisms are tissue specific.

We found that in population of Polish patients studied herein *WWOX* expression correlated with several genes involved in cell cycle/apoptosis or interacting with *WWOX*. The strongest correlation found was negative association of *WWOX* expression level with that of *CCNE1* (−0.3579; $p=0.0005$). Cyclin E1 is thought to be a potential predictor of systemic therapy, because of the cell cycle alterations induced by its overexpression: decreased length of the G1 phase, faster transition from G1 to S phase and increased genomic instability [32]. Moreover, overexpression of *CCNE1* and amplification in breast cancer human breast epithelial cells results in chromosomal instability and worse prognosis [32]. In colorectal cancer cells it was found that combined treatment of these cells with various cytotoxic drugs (e.g. c-myc antisense phosphorothioate oligonucleotides, taxol, 5-fluorouracil (5-FU), doxorubicin and vinblastine) resulted in growth arrest of these cells in the G2/M and S phases, noticeable apoptotic effect and the reduction of mRNA levels of *BCL2, BCLxL, CDK2, cyclin E1, CDK1* and *cyclin B1*, while increasing the mRNA levels of p21, p27, *BAX* and caspase-3 [33].

We also found correlation of *WWOX* transcription level with the *BCL2/BAX* expression ratio (0.3480 $p=0.0006$). This relationship would mean that in CRC patients with higher *WWOX* expression, the tumours/its cells are less

prone to apoptosis. This seemingly paradoxical finding has been also recently reported by Reeve's group in CRC patients. The impact of tumour proliferation on the grade of malignancy in CRC is not clear, especially when markers well established for breast cancer are used (e.g. Ki-67, PCNA) that is why the group used a self-devised colon-specific gene-proliferation signature (GPS) [34], including 36 genes commonly expressed (upregulated) in an exponentially growing in vitro CRC model and in human colon proliferative crypt compartments. Among the GPS genes, there are 15 cell cycle related, for instance *CCNA2* (cyclin A2), *CDC2* (cell division cycle 2, G1 to S and G2 to M, transcript variant 1). After stratification of colorectal tumours into high and low GPS groups by K-means clustering method, authors found that reduced GPS expression was associated with shorter DFS in CRC patients [34]. Authors also validated the GPS on public microarray data from two independent breast cancer experiments and found that in breast cancer group with increased GPS had significantly shorter DFS [34]. It is worth mentioning that among the 36 GPS genes there are only two involved in apoptosis—*MADL2*, which is anti-apoptotic and *ITGB3BP* (integrin beta 3 binding protein, *NRIF3*) shown to induce rapid and profound apoptosis in various breast cancer cell lines [35]. In a previous report, it was found that in breast cancer patients, the median expression of *WWOX* was almost 13-fold lower in tumours exhibiting *BCL2/BAX* ratio lower than 2 [12]. Similarly, in the presented work: colorectal tumours in which *BCL2/BAX* ratio was lower than 2, showed *WWOX* median expression 0.791, whereas in samples with higher *BCL2/BAX* ratio it was 4.590 (5.81-fold difference; $p=0.0025$). We also hypothesise that *WWOX* expression regulation in CRC, or in colon tissue/cell lines in general, could be similar to the E-cadherin (*CDH1*) gene/E-Cad protein. This well-known tumour suppressor, which is located in the vicinity of *WWOX* locus (16q22.1) was reported to have decreased expression in various cancers, including CRC. However, the exact mode of *CDH1* expression regulation was largely unknown when studies were performed to identify the 'classical' ways of downregulating gene expression. Early works on down-regulation of E-Cad expression due to the mutations in *CDH1* gene showed that the mutation rate in this gene was low [36]. Also, polymorphisms found in the *CDH1* and its promoter region seems to have at least ambiguous significance in regulation of E-Cad expression, because studies on greater number of patients showed no such associations [37]. Epigenetic changes (methylation status) in *CDH1* gene region in tumours were also studied, but results of these analyses are also unclear and seem to depend mostly on the technique used in the survey (this situation is very much alike to the one of *WWOX* methylation). Once again, when methylation was studied using MSP-based methods, it seemed that this kind of regulation has great influence on E-cad protein level [38], whereas study done using the qPCR-based method (*MethyLight*) showed extremely modest level of *CDH1* promoter methylation and there was no correlation between DNA methylation and E-cad protein level (neither in tumour tissues nor the normal mucosae; total 142 pairs of matching tissues) [39]. Also in a paper mentioned earlier, Bastian et al. described differences between the *CDH1* promoter methylation status they found in prostate carcinomas and previously published results of such analyses in this kind of tumour [30]. The next step in resolving this complexity was the showing of different repressor proteins that contribute to E-cad transcription regulation. Up to this date many of these transacting factors were discovered, including: Snail, Slug, Twist, SIP1/ZEB2, deltaEF1/ZEB2, as reviewed in [38]. Recently, a paper by Guler et al. described a relationship between the "triple negative" breast tumours phenotype and reduced expression of WWOX with elevated expression of AP-2γ (as shown by using tissue microarrays), although the authors did not find direct correlation between WWOX neither AP-2α nor Ap-2γ expression levels [40]. In summary, in this study we found that *WWOX* expression varies among patients and correlates with DFS, however we were unable to identify the molecular reason of lowered *WWOX* transcription. Our data suggest that unlike other tumours, *WWOX* expression in colorectal cancer is affected by different mechanisms than small deletions or methylation of promoter region. These findings, the ambiguous nature of role of the *WWOX* promoter methylation in expression regulation and the previous studies showing a wide array of proteins interacting with WWOX (e.g. YAP, ErbB-4, Dvl family) seem to suggest that there is a place to hypothesise that phenomena similar to *CDH1* expression regulation may occur in *WWOX* expression regulation in colon.

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Conflicts of interest None

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