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

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Received: 28 October 2010 / Accepted: 14 December 2010 / Published online: 25 February 2011
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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

**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 prostate [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]. Pluciennik 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 tumouriogenesis in colon and especially homzygous 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 tumouriogenesis, 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 RNAalater (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 CO2; 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®...
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 amplification products was performed in a LightCycler 480 (Roche Diagnostics, Poland) with EpiTect HRM PCR Kit (Qiagen, Germany).

| 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 tumoura   |    |                           |                   |
| Rectum                                | 34 | 1.69 (0.29–3.74)          | 0.8301           |
| Sigmoid colon                         | 37 | 1.42 (1.23–2.47)          |                   |
| Descending colon                      | 6  |                           |                   |
| 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 nodesb        |    |                           |                   |
| 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  |                           |                   |

*The localisation of primary tumours from three patients was ambiguous, thus they were qualified to two groups*
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′-ACAGAAGCCAGGACACAGCAGGGC-3′; Rev—5′-ACCACGAAGCTGAAATCCAGTCTCCAG-3′; −171-bp region; For—5′-AGACCTCTTACCCAGGATGATTCG-3′; Rev—5′-AAGCTCCTTACCCAGGATGATTCG-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).

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 *WWOXwt* 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* =

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**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                             |

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

| 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   |

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

LOH, loss of one allele; R, retention of both alleles; 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 MethyScreen 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.

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* \((r = -0.3579; \ p = 0.0005)\). 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

| 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 \(\geq 1.49\) cycle); ‘2’, intermediate methylation (difference between the Mock and the MDRE >1.5 cycle); NI, non-informative result

| 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 |

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 MethyScreen 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.

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CDH1 expression regulation may occur in WWOX expression regulation in colon.

Acknowledgements This study was founded by Polish Ministry of Science and Higher Education grants N N401 233934 and N N402 195635.

We would like to thank Ms. Ewa Łatowska for her excellent technical support, also Mrs. Agnieszka Piastowska-Ciesielska and Mrs Magdalena Nowakowska for the cell lines cultures studied in this paper.

Conflicts of interest None

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