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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Molecular analysis of *WWOX* expression correlation with proliferation and apoptosis in glioblastoma multiforme

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Abstract Glioblastoma multiforme is the most common type of primary brain tumor in adults. *WWOX* is a tumor suppressor gene involved in carcinogenesis and cancer progression in many different neoplasms. Reduced *WWOX* expression is associated with more aggressive phenotype and poor patient outcome in several cancers. We investigated alternations of *WWOX* expression and its correlation with proliferation, apoptosis and signal trafficking in 67 glioblastoma multiforme specimens. Moreover, we examined the level of *WWOX* LOH and methylation status in *WWOX* promoter region. Our results suggest that loss of heterozygosity (relatively frequent in glioblastoma multiforme) along with promoter methylation may decrease the expression of this tumor suppressor gene. Our experiment revealed positive correlations between *WWOX* and *Bcl2* and between *WWOX* and *Ki67*. We also confirmed that *WWOX* is positively correlated with *ErbB4* signaling pathway in glioblastoma multiforme.

Keywords *WWOX* · *ErbB4* · Glioblastoma multiforme · Methylation · LOH

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Introduction

Glioblastoma multiforme (GBM) is the most common type of primary brain tumor in adults. This highly lethal neoplasm is particularly difficult to treat due to its specific features, i.e., poor permeability of blood–brain barrier, that prevents chemotherapeutics from reaching their target, and low ability of brain tissue to renew, that makes surgical intervention very difficult and venturesome. The major molecular changes are well defined and differentiate GBM into primary and secondary tumor subtypes. The main known alterations are LOH 10q, *EGFR* amplification, $p16^{INK4a}$ deletion, *PTEN* and *p53* mutations [1]. However, the molecular basis of brain tumorigenesis still requires further intensive study, since neoplastic transformation is a complicated process resulting from both genetic and epigenetic alterations of the genome.

The tumor suppressor gene *WWOX* is localized in a common fragile site FRA16D (*locus* 16q23.3–24.1). Protein encoded by *WWOX* is an oxidoreductase containing two WW protein interaction domains. The biological role of the protein is not yet defined, although there are suppositions that it may play a part in steroid hormones metabolism and ErbB4 receptor signaling pathway [2, 3]. Modified expression of *WWOX* suppressor gene was observed in many types of tumors [4–6]. Absence of point mutations necessitates searching for other mechanisms which can deregulate *WWOX* expression. Loss of heterozygosity in *WWOX locus* was reported in many types of cancer (breast, esophagus, and lungs, among others [7–9]). Some authors observed a correlation between *WWOX* promoter methylation status and its expression level [10–13]. Promoter methylation is one of the key mechanisms silencing tumor suppressor genes. It has been well demonstrated that expression of genes such as *p27* [14, 15],

BRCA1 [16, 17], and *CDH1* [18] is blocked by hypermethylation in some cancer types.

Futile traditional methods of glioblastoma multiforme treatment have provoked an intensive search for a better understanding of molecular aberrations underlying the formation and progression of this kind of cancer. The aim of our experiments was to elucidate the role of the *WWOX* suppressor gene in GBM. Here, we report on our investigation into correlations between expression of *WWOX* and genes involved in cellular functions such as proliferation (*Ki67*), signal transduction (*EGFR*, *ErbB4*), and apoptosis (*Bax*, *Bcl2*). Moreover, we assessed the methylation state of *WWOX* promoter and examined the association between methylation and expression level. Additionally, we evaluated frequency of loss of heterozygosity in *WWOX* locus and its influence on gene expression.

Materials and methods

Patients

The study included 67 cases of glioblastoma multiforme, diagnosed according to the World Health Organization criteria for classification of brain tumors. The group consisted of 20 females, 35 males and 2 not specified. The patients' mean age was 58.6 years (range 27–76). The mean follow-up was 11.8 months (range 1–26).

Total RNA isolation, cDNA syntesis and DNA isolation

RNA was extracted from frozen brain tissues, stored at −80°C in RNAlater (Ambion), using TRIzol reagent (Invitrogen). cDNA synthesis was performed from 10 μg of total RNA at volume of 100 μl using ImProm RT-II™ reverse transcriptase (Promega). Reverse transcription was carried out under the following conditions: incubation at 25°C for 5 min and 42°C for 60 min, heating at 70°C for 15 min. cDNA samples were diluted with sterile deionized water to a total volume of 150 and 2 μl was added to a PCR reaction.

DNA was recovered from organic rests of TRIzol after RNA isolation using manufacturer's protocol.

Real-time RT–PCR analysis

Real-time RT–PCR was performed using Rotor-Gene™ 3000 (Corbett Research). PCR products were detected using SYBR® Green I and qPCR Core kit for SYBR® Green I (Eurogentec). All reactions were performed in duplicate. We analyzed relative expression level of 6 genes (*Bax*, *Bcl2*, *EGFR*, *Ki67*, *WWOX*, *ErbB4* isoforms *JM-a* and *JM-b*). The expression levels of investigated genes were normalized to three reference genes (*RPS17*, *H3F3A*, and *RPLP0*).

Due to a relatively low level of *WWOX* mRNA, we used a semi-nested RT–PCR for detection of *WWOX* expression level. First, PCR reaction was performed with primers 5′-TGCAACATCCTCTTCTCCAACGAGCTGCAC-3′ and 5′-TCCCTGTTGCATGGACTTGGTGAAAGGC-3′ in 50 μl reaction volume. Subsequently, 2 μl of 200-fold-diluted PCR product (171 bp) was used as a template for semi-nested PCR. The cycling protocol consists of: 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 72°C.

Sequences of primers, annealing and detection temperatures, and the length of products are presented in Table 1.

Relative gene expression was calculated with the Roche guidebook according to a previously published algorithm [19]. Universal Human Reference RNA (Stratagene) composed of 10 cell lines was used as a calibrator.

All primers were designed to be intron-spanning to avoid amplification of genomic DNA. Detection temperature was determined above unspecific/primer-dimer melting temperature.

LOH analysis

Loss of heterozygosity detection was performed using "high" resolution melting of Rotor-Gene™ 3000 (Corbett Research). Allelic losses were analyzed by PCR amplification with three sets of primers for microsatellites D16S518 (intron 1 of *WWOX* gene), D16S3096 and D16S504 (intron 8). The primer sequences were obtained from the Genome database. PCR cycling programs included 1 cycle with 95°C for 10 min followed by 35 cycles consisting of 94°C for 30 s, 56°C (for D16S3096 and D16S504) or 55°C (for D16S518) for 30 s, 72°C for 60 s.

MethylScreen

To assess the level of promoter methylation we preformed the MethylScreen assay [20]. The method is based on a set of restriction digestions coupled with subsequent real-time PCR. DNA was isolated from frozen brain tumor tissue. Next, 2 μg of DNA of every sample were divided into four 500-ng parts and each underwent different treatment. The first, called Mock, was a positive control, incubated with digestion buffer, yet without addition of any enzymes. The second was treated with two methylation-sensitive restriction enzymes (MSRE), *Hha*I and *Hpa*II (cutting only unmethylated template). The third was incubated with methylation-dependent restriction enzyme (MDRE), *McrBC* (cutting only methylated DNA). The fourth, a negative control, was simultaneously double digested with

Table 1 Real-time PCR reactions characteristics

Gene primers (5′ → 3′)	PCR product size (bp)	Annealing temp. (°C)	Detection temp. (°C)
H3F3A	76	65	72
AGGACTTTAAAAGATCTGCGCTTCCAGAG			
ACCAGATAGGCCTCACTTGCCTCCTGC			
RPLPO	69	65	72
ACGGATTACACCTTCCCACTTGCTAAAAGGTC			
AGCCACAAAGGCAGATGGATCAGCCAAG			
RPS17	87	64	72
AAGCGCGTGTGCGAGGAGATCG			
TCGCTTCATCAGATGCGTGACATAACCTG			
Bcl2	122	56	81
TTGGCCCCCGTTGCTTTTCCTC			
TCCCACTCGTAGCCCCTCTGCGAC			
Bax	137	56	81
AGAGGTCTTTTTCCGCGTGGCAGC			
TTCTGATCAGTTCCGGCACCTTG			
Ki67	156	56	81
TCCTTTGGTGGGCACCTAAGACCTG			
TGATGGTTGAGGCTGTTCCTTGATG			
WWOX	150	63	77
GAGCTGCACCGTCGCCTCTCCCCAC			
TCCCTGTTGCATGGACTTGGTGAAAGGC			
EGFR	106	58	81
AGCTTCTTGCAGCGATACAGCTCAGAC			
TGGGAACGGACTGGTTTATGTATTCAGG			
JM-a ErbB4	95	56	76
ACACAGCCCTCCTCCTGCCTACAC			
AGGGCACAGACACTCCTTGTTCAGC			
JM-b ErbB4	82	56	76
AGAGCAAGAATTGACTCGAATAGGAACC			
AGGGCACAGACACTCCTTGTTCAGC			

MSRE and MDRE enzymes. Each reaction consisted of equivalent amounts of 1× NEB buffer 2 (10 mM Tris–HCl, 55 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT), 1 μg/ml bovine serum albumin (BSA), 2 mM guanosine-5′-triphosphate (GTP), water and appropriate enzymes. Digestions were incubated for 4 h at 37°C and then 20 min at 65°C to halt enzyme activity. Following enzyme digestion, samples were analyzed by real-time PCR using the RotorGene 3000™ system. Using locus-specific PCR primers, intact genomic DNA was amplified, while genomic DNA that had been cleaved by restriction endonucleases was not. A change in the number of amplifiable loci was detected in the fluorescence cycle threshold among the separate digests. Standard curve data was created for each patient by setting the Mock positive control as 100 standard and double digest negative control as 0.001 standard. The threshold was set by maximizing the regression fit to the standard curve. The DNA sample was scored as methylated if the difference between Mock and MSRE/MDRE digestions Ct was >1.

Two sets of PCR primers were designed for the amplification of two genomic DNA fragments at the promoter and first exon of the *WWOX* gene. The PCR products were 384 and 413 bp long and contained %GC = 66.5. The forward primer sequence for the first (−508 to −174 bp) region of the promoter was 5′-ACAGAAGCCCAGGACAACAGCATGG-3′, and the reverse primer sequence was 5′-ACCACGAAGCTGAAATCCAGTCTCCG-3′. The forward primer sequence for the second region (from –171 bp to +239 bp) was 5′-AGACTGGATTTCAGCTTCGTGGTCG-3′, and the reverse primer sequence was 5′-AAGCTCCTTAACAGTTACTTTCACTTTGCAC-3′.

For the *WWOX* distal promoter fragment, the reaction mix consisted of 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. Fluorescence reading was performed after each cycle at 77°C. For the second *WWOX* promoter fragment (covering the 3′ end of the promoter and part of exon 1), the reaction mix consisted of 2.5 µl of SYBR® Green I, qPCR Core kit for SYBR® Green I reagents, 10 nM of each primer, 4 µl of digested DNA template, and 70 mM of betaine. Real-time PCR was conducted at the following conditions: 95°C for 5 min, followed by 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. Fluorescence reading was performed after each cycle at 83°C.

Statistical analysis

Correlation analysis between the expression levels of *WWOX* and other genes and clinical factors was performed using non-parametric Spearman Rank Correlation test. Differences between transcription level of *WWOX* gene in relation to its methylation status, LOH and patients' sex were estimated by Student's *t* test. All results were recognized statistically significant at confidence level $> 95\%$ ($P < 0.05$).

Results

The methylation analysis of 61 GBM tissue samples revealed that the first, distal fragment of *WWOX* promoter was methylated in 18% (11 of 61) of cases, while the second, nearby TATA box in 15% (9 of 61). Further analyses revealed that methylation state of the distal promoter region had no influence on *WWOX* expression. Hence, to clarify the presentation, it has been excluded from further consideration in the current report. The mean *WWOX* expression level in samples with methylated second promoter fragment was noticeably lower than in unmethylated (11.71 vs 21.93; $P = 0.15$), although this difference was not statistically significant in the Aspin–Welch mean test. The medians were 6.23 for methylated and 12.04 for unmethylated samples ($P = 0.23$). Despite the absence of statistical significance in the obtained results, there was a noticeable tendency of promoter methylation to lower *WWOX* gene expression.

We observed a relatively high percentage of LOH for two out of three analysed microsatellites. The level of hemizygosity was D16S3096 (38.5%) and D16S504 (54.5%) which is significantly higher than homozygosity observed in the population. The two microsatellites are located adjacent to the end of *WWOX* gene, in intron 8. The details of the LOH analysis are presented in Table 2. Statistical analysis revealed no correlation between LOH and reduction of *WWOX* expression. In D16S3096, mean *WWOX* expression was 18.84 for heterozygous samples and 24.91 for homozygous ($P = 0.63$). The medians were 12.81 and 8.97, respectively ($P = 0.58$). For the D16S504 microsatellite marker, these values were: means 26.94 versus 15.25 ($P = 0.24$) and medians 9.78 versus 10.9 ($P = 0.71$).

We also analyzed the *WWOX* expression level concurrently with reference to promoter methylation and microsatellite markers state. This revealed a difference in *WWOX* expression in homo- and heterozygotes, which was not noticeable in the summary analysis without considering methylation state. The highest expression was demonstrated by unmethylated, heterozygous samples, while the lowest by methylated, homozygous samples. Loss of heterozygosity lowered expression levels in unmethylated samples (with the exception of D15S504). Promoter methylation considerably reduces *WWOX* expression both in hetero- and homozygous cases. The details are presented in Table 3.

In an experiment on 67 patients with GBM, we noticed a positive correlation between expression level of *WWOX* suppressor gene and marker of proliferation *Ki67* ($R_s = 0.5440$; $P < 0.0001$). Correlation analysis of *WWOX* gene expression level with apoptotic pathway revealed a positive association with antiapoptotic gene *Bcl2* mRNA level ($R_s = 0.7092$; $P < 0.0001$) but not with proapoptotic *Bax* mRNA level and *Bcl2/Bax* ratio ($P > 0.05$). Furthermore, there was a positive correlation with transcription level of *WWOX* and *JM-a* isoform of *ErbB4*. We did not find any statistically significant association between *WWOX* and *EGFR* expression level ($P > 0.05$), or between *WWOX* and *JM-b* isoform of *ErbB4* ($P > 0.05$). The analysis of expression correlation between *WWOX* and other investigated genes is presented in Table 4.

There was no correlation between *WWOX* expression and factors such as age, sex and time of survival.

Discussion

LOH

Lowered expression level of the *WWOX* gene has been observed in many types of cancer [8, 9, 11, 12, 21–27]. Among other possibilities, the reason might be the loss of heterozygosity or epigenetic changes such as methylation of CpG islands in promoter region. Several researchers have revealed LOH of *WWOX* locus in gastric [21], pancreatic [12], esophageal [8] and lung [9] cancer. In our study on GBM, we noticed no LOH in *WWOX* intron 1 (observed hemizygosity and populational homozygosity were nearly the same) and relatively frequent incidence of

Table 2 An analysis of LOH in *WWOX* region

	D16S518 (%)	D16S3096 (%)	D16S504 (%)
Observed hemizygosity in GBM	18	38.5	54.5
Populational homozyogosity[a]	17	26	36.7
Predicted loss of heterozygosity	1	**12.5**	**19.8**

[a] From Genome database

Table 3 Median *WWOX* expression in LOH and promoter methylation status differing subgroups

	D16S518	D16S3096
Heterozygous, unmethylated	12.69	13.17
Heterozygous, methylated	6.63	6.23
Hemizygous, unmethylated	7.32	9.37
Hemizygous, methylated	5.66	5.85

The differences between the subgroups' median *WWOX* expression were statistically insignificant

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

Gene	Correlation coefficient R_s	*P*
EGFR	0.2413	NS (0.0611)
ErbB4 JM-a	0.7102	<0.0001
ErbB4 JM-b	0.2108	NS (0.1029)
Ki67	0.5440	<0.0001
Bax	0.2301	NS (0.0745)
Bcl2	0.7092	<0.0001
Bcl2/Bax	0.1995	NS (0.1442)

hemizygosity at intron 8 (38.5 and 54.5%). Although we did not observe a correlation of LOH with *WWOX* expression level, adding data on promoter methylation state to our analysis allowed us to observe such a connection. That indicates that loss of heterozygosity concurrently with promoter methylation affects the expression of *WWOX* gene in glioblastoma multiforme.

Promoter methylation

Hypermethylation of gene regulatory regions is a common event in cancer cells. In most cases, promoter methylation reduces or completely silences affected gene expression. *WWOX* promoter methylation has been reported to be a possible cause of reducing *WWOX* expression in breast, lung, bladder, pancreas and prostate cancers [10, 12, 13]. As shown in our experiment, methylation of the *WWOX* promoter region in the examined GBM samples was infrequent: 18% for the distal and 15% for the nearby TATA box promoter fragment. If it occurred, methylation slightly correlated with reduction of *WWOX* mRNA level, although the difference in mean expression for methylated and unmethylated samples was not statistically significant, probably because of the small number of methylated samples (11 and 9 of 61). Nevertheless, a tendency of *WWOX* promoter methylation to reduce its expression can be seen.

Expression correlations

Numerous authors have shown significant dependence of recurrence and drug resistance on alterations of the apoptosis pathway in glioma tumors. In patients with GBM, up-regulation of antiapoptotic genes *Bcl2*, *Bcl-xl*, *MCL-1* and down-regulation of proapoptotic *Bax* gene were associated with recurrence of the disease [28]. Other authors have demonstrated that increasing the *Bcl2* protein level correlates with higher grading in the glioma tumor cell line [29]. Moreover, up-regulation of antiapoptotic *Bcl-xl* gene with concurrent overexpression of *EGFRvIII* can be a cause of resistance to cisplatin agent [30]. Usage of *Bcl2* specific antisense oligonucleotides activates apoptosis and may be helpful in treatment [31]. In our study, we found a positive correlation between expression of *WWOX* and the antiapoptotic gene *Bcl2*. However, we did not observe any connection of *WWOX* expression either with proapoptotic *Bax* gene expression or with a *Bcl2/Bax* ratio. These results are consistent with previously reported breast cancer analysis [32]. Interestingly, in vitro experiments conducted on MDA-MB-231 breast cancer cell line showed elevated apoptosis immediately after transfection with vectors harboring *WWOX* cDNA [33], though the stable transfectants showed elevated *Bcl2* expression level, induced migration in Matrigel, and invasion through basal membrane, but inhibition of anchorage-independent growth [34]. Ovarian cancer cell lines showed similar behavior when transfected with *WWOX* cDNA; apoptosis was induced in cells growing in suspension but not in adherent cells [35].

Additionally, we found that *WWOX* expression was positively correlated with *Ki67* mRNA level, considered as a marker of cell proliferation. A similar correlation of *WWOX* and *Ki67* was observed in breast cancer samples [32]. Analysis of proliferation in ovarian cancer cell lines transfected with *WWOX* cDNA showed no correlation of

WWOX and *Ki67* [35]. There are reports presenting results of experiments which have proved that there is no association between *Ki67* and factors such as age, Karnofsky index, and survival in human glioblastoma. However, there is a positive correlation with *p53* expression [36, 37]. There was no connection observed between proliferation activity in cells of anaplastic glioma with other molecular proliferation markers such as *p16*, *Rb*, *EGFR* [37]. Nevertheless, *Ki67* exhibited statistically significant differences in staining between the tumor types of astrocytoma, anaplastic astrocytoma, and glioblastoma [38]. The prognostic value of *Ki67* in glioblastoma multiforme is currently strongly controversial [36, 39].

Unfortunately, we have less than 50% patient survival, tumor size and localization data, and therefore we were unable to perform reliable survival analysis.

WWOX involvement in ErbB4 signalling pathways

Two *ErbB4* splice isoforms differ in a short juxtamembrane sequence. JM-a form is prone to sequential proteolytic cleavage conducted by tumor necrosis factor α converting enzyme (TACE) and γ-secretase. That intramembranous proteolysis releases intracellular domain (ICD) which might be translocated to the nucleus, where it transactivates genes expression [40]. As was shown by Aqeilan et al., ErbB4 might be regulated by the WWOX tumor suppressor protein [2, 41]. The WWOX-ErbB4 interaction occurs with both full-length membrane-anchored ErbB4 and cleaved ICD fragment. When associated to uncleaved ErbB4, WWOX stabilizes the receptor in cellular membrane. Linked with ICD, WWOX prevents it from reaching the nucleus. The YAP protein has an opposite effect on ICD function. It facilitates ICD translocation to the nucleus and gene activation. Probably, YAP and WWOX compete for association with ICD fragment [41]. JM-b splice variant of *ErbB4* harbors changes in a short sequence containing the cleavage site and is resistant to proteolytic enzymes activity. There is no evidence of WWOX association with JM-b. Our results showed a functional difference between JM-a and JM-b isoforms. While JM-a expression strongly correlated with *WWOX* ($R = 0.7102$), which is consistent with breast cancer results obtained by Aqueilan et al. [2], JM-b did not show such a correlation. As shown elsewhere [41], WWOX associates with two PP*x*Y motifs of JM-a by its first WW domain. Subcellular localization of transcription cofactors is one of the major ways to regulate gene expression activation. Our study confirmed that *WWOX* is an important player in the *ErbB4* signaling pathway although details of its action still wait to be revealed. The mechanism of the *WWOX* tumor suppressor function is not explained, but it seems that interactions with ErbB4 receptor might be one element.

Conclusion

Our study indicated that genomic deletions in the 3′ end of the *WWOX* gene occurs with high frequency in glioblastoma multiforme. LOH correlates with reduction of *WWOX* expression. Although without statistical confirmation, our results suggest that promoter methylation may also differentiate expression of *WWOX*. Moreover, *WWOX* transcription in GBM is strongly associated with proliferation and apoptosis, and is involved in a signal transduction through the ErbB4 pathway. *WWOX* genetic alternations and expression correlation with other cancer-related genes in GMB are similar to those observed in breast and ovarian cancer. Therefore, *WWOX* gene inactivation seems to play an important role in GBM carcinogenesis.

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