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WWOX, A NEW POTENTIAL TUMOR SUPPRESSOR GENE

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Background: WWOX (WW domain-containing oxidoreductase) gene, located on chromosome 16q 23.3-24.1 in the region recognized as the common fragile site FRA16D is considered to be a tumor suppressor gene involved in various cancers: breast, ovarian, prostate, esophageal, lung, pancreatic, gastric and hepatic. The aim of this study was to describe (i) putative protein interactions of WWOX (ii) the molecular mechanisms of tumor suppressor activity (iii) present an overview of WWOX in relation to nervous system and breast, prostate and ovarian cancers.

Methods and Results: WWOX expression is up-regulated in endocrine organs indicating its importance in these tissues. In many cancers WWOX expression is down-regulated and low WWOX expression is related to poor prognosis.

Conclusion: All the evidence suggest that WWOX can be considered as a new tumor suppressor gene and target for gene therapy due to the association of high WWOX expression with improved disease free survival.

INTRODUCTION

Chromosomal and genomic abnormalities affecting chromosome 16q have been frequently reported in cytogenetic and allelotypic studies of various epithelial tumors. Several authors have shown, that LOH of 16q, is often observed in breast carcinomas[1–5]. Other tumor types, such as prostate, ovarian, esophageal, lung, pancreatic, gastric and hepatic carcinomas, exhibit similar abnormalities of 16q[6, 7, 8, 14–19].

Bednarek et al.[8] cloned and described a gene mapping a genomic region of more than 1 million nucleotide bp located on chromosome 16q23.3-24.1, in a region recognized as the common fragile site FRA16D[8]. The gene was named **WWOX** (synonym name: fragile site FRA16D oxido-reductase, symbol: **FOR).** The WWOX gene is composed of nine exons and encodes a 46 kDa protein that possesses two NH_2-terminal WW domains and a short chain dehydrogenase domain (SDR) found in the SDR family of enzymes. WW domains are characterized by the presence of highly conserved proline and tryptophan residues[9–11] and occur in diverse types of proteins, such as Yes-associated protein, Nedd4, dystrophin, Rsp5, Pub1, FE65, Pin1, and FBPs. In common with the SH3 domain, the WW domain is characterized by its interaction with proline-containing ligands and mediating protein–protein interactions[12, 13].

The SDR family of enzymes encompasses a wide spectrum of enzymes. One important group among SDR proteins is the family of **hydroxysteroid dehydrogenases.** These short-chain dehydrogenase/reductases are usually involved in the metabolism of steroid hormones such as androgens and estrogens. Further, WWOX expression is up-regulated in endocrine organs such as **testis, ovary and breast**, indicating the importance of WWOX in these tissues[8].

A Drosophila model has been developed for the investigation of WWOX in vivo. The Drosophila DmWWOX orthologue shares 49 % identity with human WWOX and has a conserved domain structure of tandem WW domains and oxidoreductase homology. DmWWOX null mutants are viable and fertile with no obvious phenotype. However, they show increased sensitivity to ionising radiation[20]. Protection from radiation can be restored by reintroduction of either Drosophila or human WWOX. The radiation sensitivity phenotype is similar to that previously observed for Drosophila mutants p53 (ref.[21, 22]).

PUTATIVE INTERACTIONS OF WWOX, MOLECULAR MECHANISMS OF TUMOR SUPPRESSOR ACTIVITY

2.1. WWOX-p73

Aqeilan et al.[38] showed that WWOX interacts via its first WW domain with the PPPY motif of p73. The tyrosine kinase, Src, phosporylates WWOX at tyrosine 33 in the first WW domain and enhances its binding to p73.

The p73 protein is a structural and functional homologue of the p53 tumor suppressor protein[39, 40]. The p73 protein localizes in the nucleus, and not only recognises and binds to p53-responsive elements in the promoter regions of diverse p53-target genes such as $p21^{Waf1/Cip1}$ and Bax[41], but can also transactivate the transcription of these target genes. WWOX localizes to the cytoplasm,

and consequently overexpression of WWOX causes the sequestration of p73 in cytoplasm and increases its proapoptotic activity[38]. It is likely that loss of WWOX in tumor cells may result in reduced p73 apoptotic activity in cytoplasm[38]. Although mutations of p73 in cancer are not frequent[41] and p73-defficient mice exhibit no predisposition to tumors[43], several reports have established the role of p73 in apoptosis. Its modulation of p53 function may be important in malignity development[42]. Further studies will be necessary for evaluation of the biological consequences of this association in normal and cancer cells. Interestingly, human WWOX does not bind with p53[38] whereas the murine WWOX ortholog, Wox1, interacts with p53 and Jnk1 under stress conditions[45]. Wox1 also interacts synergistically with p53 during TNF-mediated apoptosis. This proapoptotic role of Wox1 is negatively regulated by interaction with JNK [44, 45].

2.2. WWOX-*activating protein-2λ* (AP-2 **λ**)

Aqeilan et al.[46] demonstrated a functional association between AP-2λ transcription factor and the WWOX protein. AP-2λ at 20q13.2 encodes a transcription factor and is frequently amplified in breast carcinoma. WWOX binds to the PPPY motif of AP-2λ via its first WW domain. Alterations of tyrosine 33 in the first WW domain of WWOX or the proline-rich motif in AP-2λ dramatically reduce this interaction. This shows that WWOX expression triggers redistribution of nuclear AP-2λ to the cytoplasm and hence suppresses its transactivating function. It is suggested that WWOX tumor suppressor protein inhibits AP-2λ oncogenic activity by its sequestering into the cytoplasm.

2.3. WWOX-YES-associated protein (YAP)

Yes-associated protein (YAP) also containing WW domains was shown to associate with p73 and enhance its transcriptional activity[47]. YAP competes with WWOX for interaction with ErbB-4 and modulates its transcriptional function. YAP interacts with ErbB-4 receptor tyrosine kinase and acts as a transcriptional coactivator of the COOH-terminal fragment (CTF) of ErbB-4. Interaction of WWOX and ErbB-4 suppresses transcriptional coactivation of CTF by YAP in a dose-dependent manner. A mutant form of WWOX lacking interaction with ErbB-4 has no effect on ErbB-4 coactivation ErbB-4. Further, WWOX is able to inhibit coactivation of p73 by YAP. These data indicate that WWOX antagonizes the function of YAP by competing for its interaction with ErbB-4 and other targets and thus affects their transcriptional activity[47].

WWOX IN RELATION TO CANCER

Low, undetectable expression or aberrant transcripts of WWOX have been reported in different types of cancer and several tumor cell lines of different origin[23]. The frequent deletion of WWOX in multiple tumors suggests that WWOX may act as a tumor suppressor gene[46].

Bednarek et al.[8] evaluated WWOX expression in normal human tissue by Northern blot analysis: high levels of WWOX RNA were detected in endocrine organs such as prostate, testis and ovary and weak levels were detected in small intestine, spleen and colon. Thymus, leukocytes and breast have to the contrary very low levels of WWOX RNA.

3.1. WWOX and breast cancer

In breast carcinoma, LOH on 16q was found in 50–55 % of early tumors [19, 22]. Allelic imbalances of 16q have been reported in up to two-thirds of all sporadic breast carcinomas and have been detected even in the absence of other genetic alterations. Consequently, such losses have been considered very early events in breast carcinogenesis[25]. Some authors have described 16q loss as a discriminating factor among low-grade ductal invasive, tubular, and tubulolobular carcinomas[26, 27]

Bednarek et al. [28] showed that WWOX suppresses the tumorgenicity of breast cancer cells in vitro as well as in vivo. Stable expression of WWOX in MD-MB-435 and T47D breast carcinoma cells resulted in a dramatic reduction in colony number in soft agar. Increased WWOX expression after transfection of WWOX into MDA-MB-435 breast cancer cells dramatically reduced tumorigenicity in nude mice. The breast cancer cell line expressing the highest levels of WWOX mRNA/protein are the ER+ (estrogen receptor positive) MCF7 cells. The ER- breast cancer lines MDA435 and MDA231 were found to have practically undetectable levels of WWOX protein. This suggests, there is a possible corelation between WWOX protein expression and ER status. Furthermore, other authors have also described a statistically significant correlation between WWOX expression and ER status in breast carcinomas[29, 32]. Nunez et.al.[29] also studied PR (progesterone receptor) status, but due to the limited number of ER-PR+ breast carcinomas samples, they could not confirm that PR+ status alone correlates with high WWOX expression.

Pluciennik et al.[30] analyzed levels of WWOX expression in 132 cases of breast cancer. They showed that WWOX expression was higher in patients under 50, in ER and PR positive tumors and in tumors without lymph node metastasis. WWOX mRNA levels were also higher in tumors with a higher apoptotic index (Bcl2/Bax ratio).

Guler et al.[32] evaluated WWOX expression immunohistochemically in 97 archived breast carcinoma specimens. Decreased WWOX expression was found in 63.2 % of invasive tumors. Reduced WWOX staining was found more frequently in ER (–) or scanty positive tumors ($p = 0.033$) and WWOX expression in adjacent normal tissue was reduced in 32.9% of specimens, especially in patients with higher stage disease ($p = 0.033$). Highly reduced WWOX staining (> 10 %) in normal breast tissue was found in post-menopausal women or in breast cancer patients exposed to neoadjuvant chemotherapy, suggesting that WWOX expression may be associated with level of steroid hormone expression and can be affected by chemotherapy.

Guler et al. [35] also compared expression of WWOX, ErbB2 and p53 in 44 pure ductal carcinoma in situ (DCIS) cases and 31 DCIS lesions adjacent to invasive

tumors. Statistically significant loss of WWOX expression was found in 68,2 % DCIS, followed 61,3 % invasive tumors and 54,8 % DCIS adjacent to invasive tumor.

Further studies will be necessary to prove whether loss of WWOX expression is an early causative event of breast cancer, for at least in a subset of tumors or, whether this event predominantly occurs during the progression of breast carcinomas.

3.2. WWOX and prostate cancer

Chang et al.[34] postulated that progression from normal prostate to hyperplasia and non-invasive/invasive cancer stages positively correlate with up-regulation and activation of WWOX[34].

Activated Cdc42-associated kinase (Ack1) primarily phosphorylates WWOX at tyrosine 287, resulting in WWOX being targeted for ubiquitination mediated degradation. Therefore WWOX protein can be activated or inactivated via ubiquitination, by tyrosine phosphorylation at either Tyr 33 or Tyr 287 respectively. Primary androgen-independent prostate tumors but not benign prostate showed increased tyrosine-phosporylated Ack1 and decreased WWOX. These results suggest that Ack1 stimulates prostate tumorigenesis at least in part by negative regulation of the tumor suppressor WWOX[35].

3.3. WWOX and ovarian carcinoma

Nunez et al.[36] analyzed the WWOX protein expression in normal ovaries and ovarian carcinomas (n = 444). Immunoblotting analysis of normal ovarian samples demonstrated consistently strong WWOX expression, whereas 37 % of ovarian carcinomas showed reduced or undetectable WWOX protein expression In addition, immunohistochemistry of ovarian tissue showed either no or barely detectable levels of WWOX expression in 30 % of tumors. The remaining ovarian carcinomas (70 %) were moderately to strongly positive for this protein. Significant loss of WWOX expression was detected in 70 % of the mucinous and 42 % of clear cell carcinomas. Reduced WWOX expression significantly correlated with clinical stage IV (FIGO – International Federation of Gynecology and Obstetrics) (p = 0.007), negative progesterone receptor (PR) status (p = 0.008) and shorter overall survival (p = 0.03). Association with ER status was not significant.

3.4. WWOX and other carcinomas

Kuroki et al.[37] showed similar tumor suppressor effect of WWOX in AsPc1 and Panc1 pancreatic carcinoma cells.

Allelic losses and homozygous deletions at the FRA16D common fragile site were also reported in gastric cancer[50]. Kuroki et al.[51] examined 81 primary gastric adenocarcinomas immunohistochemically for WWOX protein expression and found a lack of WWOX protein expression in 65 %. They also found a significant correlation between WWOX expression and tumor histological grade, with higher-grade tumors being significantly more likely to be negative for Wwox protein expression.

Several reports have described the frequent allelic losses on chromosome 16q23–24 around the FRA16D locus in hepatocellular carcinoma[16]. Park et al.[52] found that loss of the DNA copy-number confined to 16q23 was detected by comparative genomic hybridization in several hepatocellular carcinoma cell lines, and that WWOX protein expression was absent or diminished in 72 % of cell lines.

The WWOX gene is also altered in lung cancer[53]. In nonsmall cell lung cancer, transcripts lacking WWOX exons were detected in 26 % of tumors and in five of eight cell lines[55]. WWOX allele loss occurred in 37 % of tumors, and the promoter was hypermethylated in 62.5 % of squamous cell lung carcinomas[17, 54] Fabri et al.[53] examined the tumor suppressor function of WWOX in preclinical lung cancer models. They performed restoration of WWOX levels with the help of adenovirus infection in endogenous WWOX protein-negative cell lines (A549, H460, and H1299). Restored expression of WWOX caused dramatic suppression of tumorigenicity of A549, H460, and H1299 cells in nude mice. A549 and H460 cells lines underwent apoptosis through activation of the intrinsic apoptotic caspase cascade after WWOX expression.

WWOX AND THE NERVOUS SYSTEM

4.1. The role of Wox1 (murine homolog of human WWOX) in the developing nervous system

Hyaluronidases involved in murine embryonic development such as PH-20, Hyal-1 and Hyal-2 induce expression of Wox1. Wox1 significantly down-regulates the apoptotic

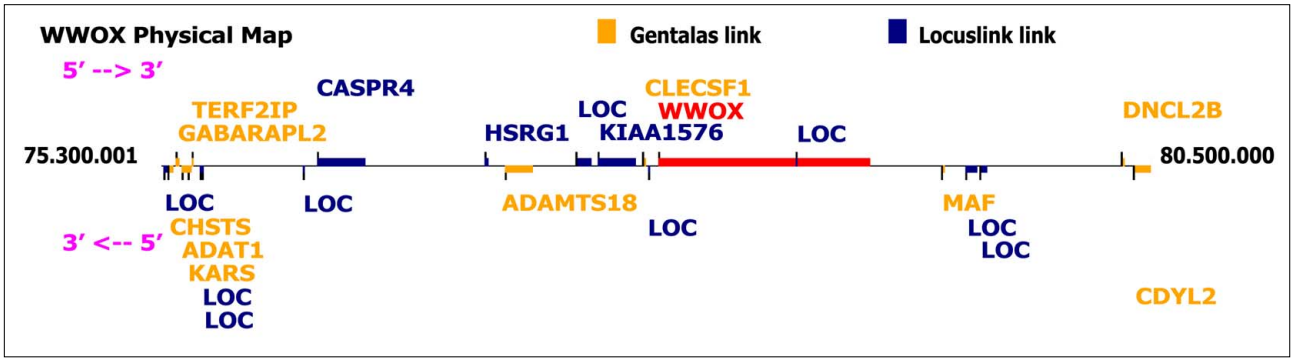

Fig. 1. Physical map of WWOX gene.

inhibitors Bcl-2 and Bcl-xL, and up-regulates p53 expression and TNF cytotoxicity[44]. Chen et al.[49] also reported, that WOX1 is expressed in the developing murine nervous system. WOX1 is differentially expressed during various stages of brain development. High levels of WOX1 protein have been observed in neural crest-derived structures such as cranial and spinal ganglia, pigmented skin cells, suggesting a potential role of WOX1 in promoting neuronal differentiation and maturation.

4.2. WWOX and neurodegenerative diseases

WWOX is likely involved in the pathogenesis of Alzheimer‘s disease (AD) and other neurodegenerative diseases involving neurofibrillary tangles. WWOX interacts with Tau proteins. Tau is a microtubule-associated protein that occurs mainly in neurons and is involved in neurite extension and maintenance. Although tau is a highly soluble protein and has a natively unfolded structure, it generates insoluble and hyperphosphorylated aggregates in Alzheimer's disease, which is characterized by degenerating neurons. WWOX binds Tau via its COOH-terminal short-chain alcohol dehydrogenase/reductase domain and most likely prevents 17βestradiol and enzyme- mediated Tau phosporylation *in vivo.* Down-regulation of WWOX in AD neurons of hippocampi induces Tau hyperphosporylation *in vivo*, leading to the formation of neurofibrillary tangles (NFTs) which characterize Alzheimer’s disease. This suggests a protective role of WWOX in neurodegenerative disseases[48].

CONCLUSION

Loss of WWOX expression is associated with a number of cancers including breast, ovary, testis, prostate, lung, pancreas, gastric and hepatocellular carcinomas. The role of WWOX has not yet been studied in brains tumors. In our earlier study we determined androgen receptor expression in brains tumor cell lines. It would be interesting to analyse, whether the WWOX plays a role in brain tumors.

The association of high WWOX expression to improved disease free survial, means that WWOX can be considered as a tumor suppresor gene and a new target for gene therapy[30].

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