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Review

WWOX tumor suppressor gene

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Summary. Loss of heterozygosity and chromosomal rearrangement of the WWOX gene, which is located at 16q23.3-24.1, have been detected in ovarian, breast, hepatocellular, and prostate carcinomas and in other neoplasias. This gene, which spans the common chromosomal fragile site 16D, contains 9 exons and encodes a 46 kDa WWOX protein that contains 414 amino acids. The evidence from cancer cell lines and primary tumor tissues suggests that WWOX is a tumor suppressor gene and that its inactivation contributes to cancer development. The results from studies of WWOX gene knockout cancer cells and a WWOX knockout mouse model partly confirm this hypothesis. The nature of the various proteins that the WWOX protein can interact with, such as c-Jun, TNF, p53, p73, AP-2gamma, and E2F-1, suggests that WWOX plays a central role in tumor suppression through transcriptional repression and apoptosis, with its apoptotic function the more prominent of the two. However, there is not universal agreement that WWOX is a tumor suppressor gene. Further analysis is needed to reveal the true nature of WWOX.

Key words: WWOX gene, Tumor suppressor gene, Apoptosis

Introduction

The WW domain-containing oxidoreductase (WWOX) gene is located at 16q23.3-24.1, a region that spans the second most common human fragile site, FRA16D. The name for this newly identified gene comes from the fact that it has two WW domains coupled to a region with high homology to the short-chain dehydrogenase/reductase family of enzymes (Bednarek et al., 2000). Genomic analysis has revealed that WWOX contains 9 exons encoding an mRNA that is 2.2 kb long, which encodes a 46 kDa WWOX protein containing 414 amino acids (Bednarek et al., 2000; Ried et al., 2000). The gene is also called FOR, which stands for FRA16D oxidoreductase. Alternative spliced WWOX transcripts (variants 1-8) encode proteins that share N-terminal WW domains in common but differ at their C-terminus, with variant 3 having a truncated oxidoreductase domain. It has been suggested that proteins encoded by these variants interfere with normal WWOX function in a dominant negative fashion (Gourley et al., 2005).

In a comprehensive immunohistochemistry analysis of WWOX protein expression in normal tissues done using utilizing a very specific anti-WWOX polyclonal antibody (Nunez et al., 2006), WWOX was found to be highly expressed in secretory epithelial cells of the reproductive, endocrine, and exocrine organs, as well as in ductal epithelial cells in specific segments of the urinary system. Significant WWOX protein expression has also been seen in various types of cells of neural origin, including neurons, ependymal cells, and astrocytes. WWOX expression has not been detected in adipose, connective, and lymphoid tissues; in myelinized structures; or in blood vessels.

Alterations in the WWOX gene and its expression in carcinomas

Two observations indicate that WWOX is a tumor suppressor gene. The first is that the FRA16D fragile site is a frequent site of loss of heterozygosity (LOH) and chromosomal rearrangement in ovarian, breast, hepatocellular, and prostate carcinomas and in other neoplasias. Recent studies have also shown that WWOX protein expression is reduced or lost in tumor cells compared with normal cells and that this finding could be associated with certain clinical or pathological parameters (Bednarek et al., 2000; Ried et al., 2000; Aqeilan et al., 2004a; Gourley et al., 2005).

In one such study, Nunez et al. (2005) evaluated the relationship between WWOX protein expression and clinico-pathological characteristics in the setting of breast cancer and found that all normal breast epithelial

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samples expressed WWOX protein abundantly, whereas 34% (69/203 cases) of invasive tumors were completely negative for WWOX expression and 26% (52/203) of tumors expressed WWOX very weakly. Of the 15 ductal carcinoma in situ samples, five showed either negative or weak staining for WWOX. These data indicate that loss of WWOX expression is a common event in breast cancer (Nunez et al., 2005a). Pluciennik et al. (2006), who evaluated the association of WWOX expression in breast cancer samples with diagnostic and prognostic markers, found that WWOX was expressed at higher levels in patients younger than 50 years old than in those 50 years and older, in estrogen receptor (ER)- and progesterone receptor (PR)-positive tumors than in tumors negative for these receptors, and in tumors without lymph node metastasis than in tumors with lymph node metastasis. WWOX mRNA levels were also higher in tumors with a higher apoptotic index, and a high expression level of WWOX was associated with better disease-free survival rates. In contrast, the presence of WWOX Delta6-8 transcripts was accompanied by a lower level of wild-type WWOX mRNA (Pluciennik et al., 2006). From these data, it seems that the expression and status of the WWOX gene correlate with the outcome and degree of malignancy of breast tumors.

In non-small cell lung cancer (NSCLC), transcripts missing WWOX exons were detected in 25.9% of primary tumors (7/27) and 62.5% (5/8) of cell lines (Yendamuri et al., 2003). Loss of heterozygosity at the WWOX locus was observed in 37.0% of primary tumors (10/27). From this, it is clear that WWOX alterations occur in a significant fraction of lung cancers and thus may contribute to the pathogenesis of NSCLC (Yendamuri et al., 2003). Immunohistochemistry studies showed that WWOX protein expression was lost or reduced in the vast majority of NSCLC tumors. The loss of WWOX expression was strongly related to high tumor aggressiveness and to tumor histopathology (i.e., squamous cell carcinomas vs. adenocarcinomas). The latter finding suggests that WWOX plays different roles in the tumorigenesis of different histological types and subtypes of NSCLC (Donati et al., 2007).

In prostate cancer, Qin et al. (2006) found that WWOX mRNA and protein expression were significantly reduced in the prostate cancer-derived LNCaP, DU145, and PC-3 cells compared with the noncancerous PWR-1E prostate cells. They also found that WWOX expression was reduced in 84% of primary prostate cancers. These researchers further found that this down-modulation of WWOX expression in the prostate cancer-derived cells was due to DNA hypermethylation in the WWOX regulatory region (Qin, et al., 2006).

In ovarian cancer, while immunoblotting analysis of normal ovarian samples showed consistently strong WWOX expression, expression was reduced or undetectable in 37% of ovarian carcinomas. Further immunohistochemistry analysis of normal human ovarian tissue showed strong WWOX expression in ovarian surface epithelial cells and in epithelial inclusion cysts within the cortex, whereas 30% of ovarian carcinomas showed no or barely detectable WWOX expression. The remaining 70% of tumors stained moderately to strongly for this protein. Significant loss of WWOX expression was found in two histotypes of ovarian carcinomas: the mucinous and clear cell. Reduced WWOX expression was also found to be significantly associated with clinical Stage IV (FIGO) disease and shorter overall survival. Subsets of ovarian tumors have also shown a loss of WWOX that is potentially associated with patient outcome (Nunez et al., 2005b). Gourley et al. (2005) demonstrated significantly lower expression of the WWOX variant 1 in tumors than in normal ovaries. This was also the case for variant 4, which was found to be significantly associated with high-grade and advanced-stage ovarian cancer. Furthermore, tumors co-expressing variant 4 and relatively high levels of variant 1 were found to be significantly associated with a worse survival than tumors expressing variant 1 alone. These results suggest that WWOX variant 1 is a suppressor of ovarian tumorigenesis (Gourley et al., 2005).

In oral squamous cell carcinomas (OSSCs), Pimenta et al. (2006) detected an altered WWOX gene in 50% (10/20) of tumors. Using nested RT-PCR, they found that mRNA transcription was altered in 35% of the tumors, with a complete absence of transcripts in two samples as well as an absence of exons 6-8, exon 7, and partial loss of exons 8 and 9. Although only the normal protein was detected, these alterations in mRNA transcription correlated with the reduced expression of the protein in 40% of the tumors as compared with normal mucosa. These results therefore showed that an altered WWOX gene may contribute to the carcinogenesis of oral cancer (Pimenta et al., 2006). To elucidate the potential role of such a gene in esophageal squamous cell carcinomas, Kuroki et al. (2002) examined 36 tumors for genetic alterations in the WWOX gene. They found LOH at the WWOX locus in 14 (39%) tumors. In addition, a tumor-specific missense mutation plus a lack of the other allele were found in one tumor; aberrant WWOX gene transcripts missing exons 6-8 were detected in two tumors; and complete absence of the transcript was found in one tumor. These results suggest that alteration and inactivation of the WWOX gene plays a role in the carcinogenesis of esophageal squamous cell tumors (Kuroki et al., 2002).

The same alterations as those seen in OSSCs were also seen in hematopoietic malignancies and pancreatic cancers (Ishii et al., 2003; Kuroki et al., 2004). In particular, WWOX transcripts were aberrant or missing in 51% of the primary cases and 55% of leukemic cell lines, with three WWOX nucleotide variants detected among the leukemic cell lines. In addition, while wild-type transcripts of the gene were expressed in normal blood cells along with a small fraction of short transcripts, a DNA blot study showed that the WWOX gene was deleted in two of 18 cases of primary acute leukemia and not expressed in the same 2 cases (Ishii, et

al., 2003). In addition, Kuroki et al. (2004) observed that WWOX expression was significantly reduced in all pancreatic cancer cell lines examined and in six primary tumors (40%). Western blot analysis further showed a significant reduction in WWOX protein expression in all cell lines examined (Kuroki et al., 2004).

Several homozygous deletions within the WWOX gene locus have been found in cancer-derived cell lines. For example, four different homozygous deletions were found in Col15, a colon carcinoma cell line (Finnis et al., 2005). In addition, three distinct regions of homozygous deletion in intron 8 of WWOX were found in AGS, a stomach carcinoma-derived cell line (Ried et al., 2000). LOH in the WWOX gene was also observed in a number of cell lines and primary tumors, including breast, prostate, esophageal, lung, pancreatic, and stomach carcinoma (Bednarek et al., 2000; Paige et al., 2001; Kuroki et al., 2002;Yendamuri et al., 2003; Aqeilan et al., 2004b; Kuroki et al., 2004).

Promoter region methylation may also be responsible for reduced WWOX expression in human tumors, suggesting that the loss of WWOX expression in cancer cells such as lung, squamous cell, breast, and bladder transitional cell carcinomas can result from genetic or epigenetic alterations as well (Kuroki et al., 2004; Iliopoulos et al., 2005). All these aberrations of the gene and of gene expression have been correlated with tumorigenesis, suggesting that the WWOX protein is capable of suppressing tumor growth.

From the above, it is clear that the genetic alteration of WWOX in cancer-derived cell lines and primary cancers include homozygous deletion, LOH, DNA methylation, somatic mutations such as S329F in OSCCs, and other types of DNA instability (Ried et al., 2000; Bednarek et al., 2000; Driouch et al., 2002; Kuroki et al., 2002, 2004; Iliopoulos et al., 2005; Pimenta et al., 2006).

WWOX gene function as a tumor suppressor

Although considerable data have shown that WWOX is inactivated in a variety of human malignancies, early evidence of WWOX's potential ability to suppress tumor growth came from overexpression studies (Aqeilan and Croce, 2007). Subsequent studies revealed the tumor-suppressing ability of WWOX gene.

In a study done by Aqeilan et al. (2007b), who studied WWOX (+/-) and WWOX (+/+) mice, inactivation of the WWOX gene appeared to enhance esophageal/forestomach tumorigenesis induced by the carcinogen N-nitrosomethylbenzylamine. Specifically, forestomach tumors developed in 96% (25/26) of WWOX (+/-) mice but only 29% (10/34) of WWOX (+/+) mice, a difference that was statistically significant. In addition, 27% of WWOX (+/-) mice had invasive squamous cell carcinomas in the forestomach, while none of the wild-type controls has such tumors. These findings provide convincing *in vivo* evidence of the tumor suppressor function of WWOX in carcinogenesis (Aqeilan et al., 2007b).

WWOX protein expression has also been observed to be restored by infection with a recombinant adenovirus carrying WWOX cDNA (Ad-WWOX) or by treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine, both of which activate the endogenous WWOX gene. Fabbri et al. (2005) studied the effect of both of these strategies in a lung cancer model. After the WWOX gene was restored in vitro in the endogenous WWOX protein-negative NSCLC cell lines A549, H460, and H1299, the A549 and H460 cells, underwent apoptosis through activation of the intrinsic apoptotic caspase cascade. Similarly, the ectopic expression of WWOX dramatically suppressed the tumorigenicity of A549, H460, and H1299 cells in nude mice after Ad-WWOX infection. Tumorigenicity and the in vitro growth of U2020 (WWOX-positive) lung cancer cells were unaffected by WWOX overexpression, however. This study confirmed that WWOX is a tumor suppressor gene and is highly effective in preventing the growth of lung cancer xenografts (Fabbri et al., 2005).

Similar experiments involving pancreatic, prostate, leukemia, and breast cancer cell lines have further supported these results (Kuroki et al., 2004; Qin et al., 2006; Iliopoulos et al., 2007). For example, the restoration of WWOX expression led to the suppressed growth of WWOX-deficient breast cancer-derived cells through activation of the intrinsic caspase pathway but did not affect the growth of WWOX-sufficient MCF7 cells. Similarly, the intratumoral restoration of WWOX suppressed tumor growth in nude mice by inducing apoptosis. These results confirm that the overexpression of exogenous WWOX inhibits breast cancer cell growth *in vitro* and *in vivo* (Iliopoulos et al., 2007). These studies further revealed that WWOX overexpression resulted in caspase-mediated apoptosis. Thus, multiple lines of evidence from both cell culture and mouse model experiments have suggested that WWOX functions as a tumor suppressor.

Ultimate proof of the tumor suppressor function of a given gene is the development of tumors in knockout mice. In such a study of the tumor suppression function of WWOX, Aqeilan et al. (2007b) observed that osteosarcomas developed in four of 13 juvenile WWOX-/- knockout mice. Histological examination of the knockout mice showed focal lesions along the bone diaphysis plus increased numbers of osteoblasts and enlarged cells with multiple nucleoli in the cartilage matrix, findings suggestive of the proliferation of the progenitors arising from the periosteum. Analysis of adult tumors was precluded by the death of WWOX-/- knockout mice by 4 weeks of age. Of the WWOX+/- mice, spontaneous tumors developed in 10 of 58 mice, while tumors developed in only 1 of 60 wild-type (control) mice. The tumor spectrum in the WWOX+/- mice included lymphomas, lung papillary carcinomas, liver tumors, and gastric squamous cell carcinomas. These data collectively indicate that WWOX is a tumor suppressor and that inactivation of one WWOX allele is enough to allow tumorigenesis to occur (Aqeilan et al.,

2007b).

The mechanism of the tumor suppressor function of the WWOX gene

In several studies examining the molecular mechanism of action of WWOX, the WWOX gene was found to function as a modular protein partner of transcription factors such as c-Jun, TNF, p53, p73, AP-2gamma, and E2F-1, among others, or to enhance the function of some carcinogens.

Carcinogens

Thavathiru et al. (2005) reported that the environmental carcinogens ultraviolet (UV) light and BPDE significantly down-regulated the expression of the WWOX gene, which correlated with S-phase delay in the cell cycle. Treatment of the UV-irradiated cells with caffeine both abrogated the S-phase delay and overcame the down-regulated expression of WWOX. This suggests the involvement of unique cell cycle checkpoint mechanisms in this phenomenon. On the basis of such findings, it has been hypothesized that the protracted down-regulation of the putative tumor suppressor gene WWOX by environmental carcinogens constitutes an additional tumorigenic mechanism (Thavathiru et al., 2005).

C-Jun

WWOX contains two WW domains that mediate protein-protein interactions. Gaudio et al. (2006) showed that WWOX specifically associated with the proline-rich motif of the c-Jun proto-oncogene via its first WW domain. This group further observed that the phosphorylation of c-Jun caused by overexpression of the mitogen-activated protein kinase kinase kinase 1 (MEKK1), an upstream activator of c-Jun, in turn enhanced the interaction of c-Jun with WWOX. In addition, they found that exposure to UVC radiation resulted in the formation of endogenous WWOX-c-Jun complexes that mainly occurred in the cytoplasm. Conversely, expression of WWOX reversed the ability of MEKK1 to increase the activity of a c-Jun-driven activating protein-1 (AP-1)-luciferase reporter plasmid. These findings reveal a functional cross-talk between the c-Jun transcription factor and WWOX tumor suppressor protein (Gaudio et al., 2006).

TNF, Bcl-2, Bcl-xL, p53, p73, and other apoptosis-associated factors

WWOX is mainly located in the mitochondria, and the mitochondrial targeting sequence has been mapped to within the ADH domain. As there is now so much evidence from cancer cell lines and animal models supporting a tumor suppressor function for the WWOX gene, it seems very likely that WWOX chiefly suppresses tumor growth by promoting apoptosis.

Chang et al., (2001, 2003a,b, 2005, 2007), who has performed serial investigations exploring the molecular partners involved in this process, found that the induction of mitochondrial permeability transition by TNF resulted in WWOX release from mitochondria and its subsequent nuclear translocation (Chang et al., 2001; Driouch et al., 2002). Of particular importance was the finding in L929 cells that WWOX enhanced TNF cytotoxicity via its WW and ADH domains and that this enhancement was partly due to the significant down-regulation of the apoptosis inhibitors Bcl-2 and Bcl-xL and up-regulation of the pro-apoptotic p53 by the ADH domain. When overexpressed, the ADH domain mediated apoptosis, probably as a result of the modulation of expression of these proteins (Chang, 2002). Further investigation by Chang et al. (2003a,b, 2005, 2007) showed that WWOX co-localized with p53 in the cytosol and bound to the proline-rich region of p53 via its WW domain. The transient co-transfection of cells with both p53 and WWOX induced apoptosis in a synergistic manner. On the other hand, p53-induced apoptosis was abolished when WWOX expression was blocked by antisense mRNA. Transiently activated JNK1 also induced an anti-apoptotic response that inhibited WWOX-induced apoptosis. It can be concluded from these findings that WWOX is involved in the apoptotic response by regulating the activation of both p53 and JNK1 (Chang et al., 2003a,b, 2005, 2007). Aqeilan et al. (2004b) showed that, unlike in mice, in humans WWOX physically interacts via its first WW domain with the p73 gene, which was discovered as a member of p53 gene family and its overexpression can inhibit cell growth and induce cell apoptosis. This occurs as the result of the tyrosine kinase, Src, phosphorylating WWOX at tyrosine 33 in the first WW domain, which enhances its binding to p73 (Aqeilan et al., 2004b; Chang et al., 2007).

AP-2gamma

In breast carcinoma, WWOX binds to the PPPY motif of AP-2gamma via its first WW domain. Alterations in tyrosine 33 in the first WW domain of WWOX or the proline-rich motif in AP-2gamma dramatically reduce this interaction. More importantly, WWOX expression triggers the redistribution of AP-2gamma from the nucleus to the cytoplasm, thereby suppressing its transactivating function. These findings suggest that the WWOX protein inhibits AP-2gamma oncogenic activity by sequestering it in the cytoplasm (Aqeilan et al., 2004a).

E2F-1

Ishii et al. (2004) found that ectopic E2F-1 expression led to an increase in FHIT and WWOX expression in allele-retaining tumor cells, which induced apoptosis in these cells. A reporter assay showed that the E2F-1 site in the FHIT 5' region was involved in the downstream transcription after the introduction of

exogenous E2F-1. These data suggest that E2F-1 overexpression plays a role in tumor suppression, at least in part through the transcriptional regulation of FHIT and relevant activation of WWOX (Ishii et al., 2004).

Other mechanisms such as promoter region hypermethylation may also be responsible for reducing WWOX expression in lung, breast, bladder, and pancreatic cancers, suggesting that the loss of WWOX expression in cancer cells could be due to genetic or epigenetic alterations (Kuroki et al., 2004; Iliopoulos et al., 2005).

The controversies and the unanswered questions

Although WWOX is frequently inactivated in human malignancies and is considered a tumor suppressor, there are several controversies regarding this view. One differing view is that the loss of WWOX expression in several cancer types is due mainly to its location within FRA16D and that it might be a secondary event not associated with tumorigenesis. In keeping with this, Watanabe et al. (2003) found that WWOX protein levels were elevated rather than decreased in gastric and breast carcinoma, a finding that provides a novel insight into aspects of human WWOX function in both normal and malignant cells and that clearly challenges the notion that WWOX is a classic tumor suppressor. Along similar lines, Guler et al. (2004) found that reduced WWOX staining was more common in invasive breast carcinoma with a less favorable estrogen receptor status than in normal breast tissue. Intriguingly, these researchers found that WWOX expression in normal tissue was reduced in 32.9% of specimens, especially in patients with higher-stage disease. In an in vivo study, Aqeilan et al. (2007) found that the near-normal epithelium of the forestomachs from WWOX(+/-) mice stained moderately strongly for the WWOX protein, but staining of the squamous cell carcinoma in the same tissue section was weak and diffuse. These results suggest that inactivation of one allele of WWOX augments the predisposition of normal cells to malignant transformation but WWOX is haploinsufficient for the initiation of tumor development (Aqeilan et al., 2007a). In keeping with this, a higher incidence and number of ethyl nitrosourea-induced tumors were seen in WWOX+/- mice than in wild-type mice. The tumor spectrum in these WWOX+/- mice included lymphomas and lung papillary carcinomas, among others. Of particular note, these tumors still expressed the WWOX protein, suggesting that the haploinsuffiency of WWOX is a cancer-predisposing characteristic (Aqeilan et al., 2007b). At the same time, in an experiment examining the targeted deletion of the WWOX gene in mice, osteosarcomas developed in four of 13 juvenile WWOX-/- knockout mice, but whether loss of the WWOX gene causes the development of osteosarcomas in mice and humans is unknown. Future studies need to focus on showing whether the WWOX gene is a logical target of therapy, such as the p53 gene is now regarded as an important target of therapy. Thus, the function of the WWOX gene as a tumor suppressor and its mechanism need more investigation in different kinds of tumors.

Conclusions

WWOX gene alteration and a decrease or loss of its expression have been seen in a number of cancers, which suggests that WWOX inactivation is a contributing factor in cancer development and the WWOX gene is a tumor suppressor. Findings in WWOX gene knockout cancer cells and a WWOX mouse knockout model partly confirm this belief. The nature of the various interacting partners, such as c-Jun, TNF, p53, p73, AP-2gamma, and E2F-1, with which the WWOX protein can physically associate, suggest that WWOX plays a central role in different signal transduction pathways, especially transcriptional repression and apoptosis. However, there are also opposing views regarding the function of the WWOX tumor suppressor gene. Further investigation of WWOX will provide further insights into the mechanism of WWOX's molecular action.

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