Functional and Clinical Characterization of the Putative Tumor Suppressor WWOX in Non-small Cell Lung Cancer

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Introduction: The oxidoreductase WWOX was initially described as a putative tumor suppressor in breast cancer. Non-small cell lung cancers (NSCLCs) frequently show aberrant WWOX expression. Herein, we characterized WWOX at a functional level in preclinical NSCLC models and in primary NSCLC biopsies.

Methods: The human wild-type (wt) WWOX complementary DNA and a mutant WWOX A132T with structurally disrupted short-chain dehydrogenase/reductase domain were conditionally expressed at physiological levels in several human NSCLC models. Resulting transgenic cell populations were analyzed with respect to clonogenic survival and apoptosis sensitivity in vitro and tumor growth in immune-deficient mice. Tissue microarrays prepared from surgically resected primary human NSCLC tumors were studied to correlate intratumoral WWOX expression with patient outcomes.

Results: Conditional expression of wt WWOX, but not mutant WWOX A132T, suppressed clonogenic survival of NSCLC cells in vitro and tumor growth in vivo. In addition, preserved intratumoral WWOX expression was associated with improved outcome in a cohort of 85 patients with surgically resected NSCLC. Unexpectedly, wt WWOX failed to sensitize NSCLC cells to various apoptotic stimuli but robustly protected against apoptosis induced by inhibitors of growth factor signal transduction.

Conclusions: WWOX acts as a tumor suppressor in human NSCLC models in a short-chain dehydrogenase/reductase domain-dependent manner. This activity is independent of sensitization to apoptotic cell death. WWOX expression as detected by immunohistochemistry may be a prognostic biomarker in surgically resected, early-stage NSCLC.

Key Words: WWOX, Non-small cell lung cancer, Apoptosis, Biomarker.


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Non-small cell lung cancers (NSCLCs) are the leading cause of cancer mortality in the Western World. Although inhalative cigarette smoking has been established as the dominant risk factor for the development of NSCLC, the complete oncogenic process is less well characterized and certainly differs between various histologically and genetically defined NSCLC subsets. Recently, the concept of "genetic dependence" to specific oncogenic "driver mutations" has drawn much attention in lung cancer pathophysiology research. It has been paradigmatically demonstrated in pulmonary adenocarcinomas bearing activating mutations and amplification of the epidermal growth factor receptor or translocations of the anaplastic lymphoma kinase (ALK), both aberrations clustering in patients without smoking history.1–5 Functional dependence to gene amplification involving the fibroblast growth factor receptor 1 has been observed in a subgroup of pulmonary squamous cell carcinomas, thus extending the concept to smoking-associated NSCLC.6 In addition to such "druggable" oncogenic driver lesions, functional inactivation of tumor suppressor gene products is regarded an important step in lung carcinogenesis. Moreover, this is thought to contribute to resistance of NSCLC to DNA-damaging agents and radiation, which are still the mainstay of nonsurgical lung cancer treatment. A key example are inactivating mutations of the TP53 tumor suppressor, which are found in approximately 50% of NSCLC, and which may result in functional impairment of the treatment-induced DNA damage response.7,8

Next to the TP53 gene product, additional tumor suppressors have been implied in lung cancer pathophysiology. The putative tumor suppressor gene WW domain-containing oxidoreductase (WWOX) localizes at the common fragile site FRA16D on chromosome 16q, which is frequently affected by structural genetic aberrations in cancer.9 WWOX was originally described in breast cancer,10 but loss of heterozygosity was observed in 37% of lung cancers, and expression of aberrant WWOX transcripts was found in 25% of lung cancers studied.11 Immunohistochemical studies detected reduced or absent WWOX protein expression in 85% of cases, with highest rates in squamous cell carcinomas and undifferentiated carcinoma.12 Next to expression of aberrant transcripts, epigenetic inactivation by WWOX promoter hypermethylations and mutations of the short-chain dehydrogenase/reductase (SDR) domain have been reported in primary lung...
cancers. Gene targeting of Wwox in the mouse resulted in increased formation of juvenile osteosarcomas in homozygously deleted mice and papillary lung tumors in heterozygously deleted mice. Importantly, the latter exhibited enhanced carcinogen-induced lung tumorigenesis, supporting a role for WWOX as a lung cancer tumor suppressor. This was further bolstered by the induction of apoptosis following heterologous overexpression of WWOX complementary DNA (cDNA) in human lung cancer models.

Against this background, we have developed models of conditional expression of wild-type (wt) WWOX and a SDR domain-mutant WWOX cDNA at physiological levels in NSCLC. Although we confirm a tumor-suppressive activity of WWOX in vitro and in vivo, which depends on a structurally intact SDR domain, we fail to demonstrate proapoptotic activity of the transgene. Surprisingly, WWOX protects lung cancer cells against apoptosis induced by broad-spectrum kinase inhibitors. Studying WWOX protein expression in a series of surgically resected NSCLC reveals that low intratumoral WWOX staining correlates with dismal outcome. These findings support a role for WWOX as lung cancer tumor suppressor, which, however, is not explained by enhancing apoptotic cell death.

MATERIALS AND METHODS

Reagents

Etoposide, paclitaxel, and staurosporine were purchased from Sigma-Aldrich (Munich, Germany). KN-93 (Ca/CaM-dependent kinase II inhibitor) was obtained from Alexis Biochemicals (Switzerland). Wortmannin (PI3-kinase inhibitor), genistein (general tyrosine kinase inhibitor), U0126 (MEK inhibitor), Akt-inhibitor VIII, and BAFF-AM (cell permeable Ca²⁺ chelator) were purchased from Calbiochem (Schwalbach, Germany). 17AAG (heat shock protein 90 inhibitor) was obtained from LC Laboratories (Woburn, MA). protein kinase C (PKC) 412 (PKC inhibitor) was provided by Novartis (Basel, Switzerland), and ABT-737 was provided by Abbott (Abbott Park, IL).

Cell Lines and DNA Constructs

NSCLC cell lines A549, NCI-H460 and NCI-H1299, and the breast cancer cell line, MCF-7, were maintained in Dulbecco’s Modified Eagle Medium media supplemented with 10% FCS either in presence or absence of doxycycline (1 µg/ml) for 7 to 14 days. Colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/ml) for 2 hours at 37°C for photomicrographic documentation and enumeration.

Murine Tumor Model

Irradiated (150 rad) nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice received subcutaneous flank injections of 10⁷ A549-WWOX-FLAG or A549-WWOXΔ132T-FLAG cells. For induction of conditional WWOX expression in vivo, the drinking water was supplemented with doxycycline (1 mg/ml). Tumor growth was monitored by bidimensional measurements using a caliper. All animal studies were conducted in compliance with institutional guidelines and German Animal Protection Law.

Tissue Microarray Analyses

Demographic and clinical characteristics of the study population are summarized in Supplemental Table 1 (http://links.lww.com/JTO/A130). The construction of the tissue microarrays (TMA), immunohistochemical, and statistical analyses have been described previously. In brief, TMA sections were immunohistochemically analyzed using the
WWOX Ab-3 (Abcam, Cambridge, UK; rabbit polyclonal, cat. No ab14691, epitope AA 397-411) as primary antibody. Specific immunoreactivity was quantified by an immunoreactivity score (IRS), which is based on the product of staining intensity (0 = absent, 1–3 = weak, moderate, high) and fraction of positively staining tumor cells (0 = absent, 1 = 1–9%, 2 = 10–50%, 3 = 51–80%, and 4 > 80%). Hence, resulting IRS values range from 0 to 12.

Statistical Methods

Results obtained in cell biology experiments were analyzed using the GraphPad Prism 4 software (Student’s t test). Data from mouse experiments (analysis of variance) and survival data of patients with lung cancer (Mantel-Cox Log-rank test) were statistically analyzed by means of the SPSS software, version 18.

RESULTS

Tumor-Suppressive Activity of WWOX Requires a Structurally Intact SDR Domain

To study the functional consequences of WWOX expression in NSCLC cells, we devised a retroviral vector system equipped with a tetracycline-inducible promoter. We generated several constructs expressing the human WWOX cDNA and a WWOX cDNA fused to a FLAG tag-encoding sequence at its 3’ end. Similar vectors were designed encoding a mutant WWOX with an amino acid exchange at codon 132 (WWOX<sup>A132T</sup>), which was generated by random mutagenesis. The selection of this mutant for further study was based on biochemical considerations, which predicted a structurally altered SDR domain due to replacement of alanine at position 132 by differently charged and sized threonine. These constructs were used to transduce A549, NCI-H460, and NCI-H1299 NSCLC cells stably expressing a mutant rtTA-M2 transactivator, thus enabling conditional transgene expression on treatment with doxycycline. The resulting cellular systems allowed the validation of several antibodies, which had been reported to specifically detect endogenously expressed WWOX. Cell extracts were prepared from MCF-7 breast cancer cells, which express wt WWOX, and were subjected to immuno blotting. All three tested antibodies detected a band of the expected molecular mass of approximately 44 kDa. Surprisingly, only one of these antibodies (further denoted Ab-3) crossreacted with the conditionally expressed WWOX-FLAG fusion protein, which was readily detected using an anti-FLAG monoclonal antibody (Supplemental Figure 1, http://links.lww.com/JTO/A131). This antibody was used for all further experimentation.

Conditional expression of wt WWOX reproducibly imposed a moderate inhibition of clonogenic survival and anchorage-independent colony formation of A549, NCI-H460, and H1299 cells in vitro (Figure 1, Supplemental Figure 2, http://links.lww.com/JTO/A132). Interestingly, no suppression of clonogenic survival was observed on expression of the WWOX<sup>A132T</sup> mutant bearing a structurally disrupted SDR domain (Figure 1). This suggested that a structurally and possibly functionally preserved SDR domain is required for the tumor-suppressive activity of WWOX in NSCLC models. To further substantiate this hypothesis, we grafted A549 cells conditionally expressing WWOX (A549-WWOX) or WWOX<sup>A132T</sup> (A549-WWOX<sup>A132T</sup>) in immunodeficient NOD/SCID mice and monitored tumor growth. Feeding doxycycline to NOD/SCID mice grafted with A549-WWOX cells to induce intratumoral expression of wt WWOX significantly (p < 0.006, analysis of variance) suppressed the growth of NSCLC tumors in vivo, when compared with tumor growth in mice not fed with doxycycline (Figure 2A). In contrast, induction of WWOX<sup>A132T</sup> in vivo failed to suppress the growth of A549-WWOX<sup>A132T</sup> xenografts, when compared with mice not receiving doxycycline (Figure 2B). Transgene expression in response to doxycycline treatment was confirmed by immunohistochemical analysis of explanted A549-WWOX tumors (Figure 2C). Hence, WWOX acts as a tumor suppressor in NSCLC models in vitro and in vivo, and this activity depends on a structurally intact SDR domain.

WWOX Expression is Associated with an Improved Outcome in Patients with Early-Stage NSCLC

To explore WWOX as a modulator of NSCLC biology in patients, we studied TMA prepared from 85 NSCLC patients.
tumors obtained at surgical resection (Supplemental Table 1). An established IRS ranging from 0 to 12 was applied to classify tumors (Figure 3A) as WWOX negative (IRS <3), WWOX positive (IRS ≥3), or strongly positive (IRS >8). Patients with preserved intratumoral WWOX expression (IRS ≥3) had a significantly (p = 0.022, Log-rank Mantel-Cox test) superior outcome after surgical resection, when compared with patients suffering from NSCLC, which stained negatively for WWOX (Figure 3B). No significant association between WWOX expression and established prognostic parameters, such as tumor stage, age, and gender, was observed (data not shown). These findings lend further support to a role for WWOX as tumor suppressor in lung cancer.

**FIGURE 3.** WWOX expression is associated with an improved outcome in patients with surgically treated non-small cell lung cancer (NSCLC) cancer. A, Representative photomicrographs of immunohistochemical stainings of NSCLC with absent (IRS = 0), weak (actual immunoreactivity score [IRS] in brackets), moderate, and strong endogenous WWOX expression. B, Kaplan-Meier Plot of overall survival of a cohort of 85 patients with surgically resected NSCLC in relationship to WWOX expression status. Patients with WWOX-positive tumors (yellow line) showed a significantly improved survival (p = 0.022, Log-rank [Mantel-Cox] test), when compared with patients with WWOX-negative tumors (blue line).

**WWOX Protects Lung Cancer Cells against Apoptosis Induced by Broad Spectrum Kinase Inhibitors**

Overexpression of WWOX has been reported to induce apoptotic cell death in lung cancer models. Accordingly, a proapoptotic activity is viewed as the mechanistic basis for WWOX-imposed tumor suppression. Against this background, we devised our systems to further study the proapoptotic activity of conditionally expressed WWOX in NSCLC models. Induction of both, WWOX and WWOX at IRS 0

**FIGURE 2.** Inhibition of tumor growth by WWOX in a murine xenograft model. Growth of A549-WWOX (A) and A549-WWOX (B) xenografts in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice treated with doxycycline (+Dox, closed triangles) or vehicle (−Dox, open boxes). C, Immunohistochemical analysis of transgene expression in response to doxycycline treatment from explanted A549-WWOX tumors. IRS, immunoreactivity score; 12 = strongly positive.
physiological expression levels (Supplemental Figure 1C) failed to trigger apoptosis of NSCLC cells in vitro or in vivo (not shown). Next, we assessed whether conditional restoration of WWOX in NSCLC cells impacts on apoptosis induced by various exogenous stimuli. To exclude potential artifacts resulting from cotreatment with doxycycline, NSCLC cell lines stably expressing the rtTA-M2 transactivator, but not the WWOX expression vectors, were used as controls. Conditional expression of WWOX failed to modulate cell death in response to etoposide, paclitaxel, or UV radiation in A549 and NCI-H460 NSCLC cells (Figures 4C, D and not shown). Surprisingly, NSCLC cells expressing WWOX were significantly protected against apoptosis induced by the broad spectrum kinase inhibitor staurosporine (Figure 4A, Supplemental Figures 3A, B, http://links.lww.com/JTO/A133). This finding was in stark contrast to the proapoptotic activity of WWOX overexpression in lung cancer and additional cancer models. As all these stimuli are able to trigger several cell death mechanisms in addition to apoptosis, we next devised ABT-737, a pharmacologic BH3 mimetic with a purely proapoptotic mechanism of action in lung cancer models. In line with our previous findings, conditionally expressed WWOX had no impact on apoptosis induced by ABT-737 treatment of A549 and NCI-H460 cells in vitro (Figure 4E).

FIGURE 4. WWOX protects lung cancer cells against apoptosis induced by staurosporine and genistein. A, A549-WWOX cells (left), NCI-H460-WWOX cells (center), and NCI-H1299-WWOX cells (right) were treated for 48 hours with indicated concentrations of staurosporine in WWOX-induced (+Dox) or -uninduced (−Dox) state. Cells were permeabilized and stained with propidium iodide, and the percentage of cells with sub-G1 DNA content, corresponding to apoptotic cells, was determined by flow cytometry. B, A549-WWOX cells (left), NCI-H460-WWOX cells (center), and NCI-H1299-WWOX cells (right) were treated for 48 hours with indicated concentrations of genistein in WWOX-induced (+Dox) or -uninduced (−Dox) state. The fraction of apoptotic cells was determined as in (A). A549-WWOX and NCI-H460-WWOX cells in WWOX-induced (+Dox) or -uninduced (−Dox) state were treated for 48 hours with etoposide (12.5 µM, C) or UV-C irradiation (20 ml/cm², D). Apoptosis was determined as in (A). E, Apoptosis induction in A549-WWOX cells on 48 hours treatment with 25 or 50 µM of ABT-737 in WWOX-induced (+Dox) or -uninduced (−Dox) state. Mean values ± standard deviation from three independent experiments; ***p < 0.0002; **p = 0.0086; *p < 0.0374 (unpaired t test).
FIGURE 5. Modulation of cell signaling by the kinase inhibitors staurosporine and genistein. A, A549-rtTA (control cells) and A549-WWOX cells (upper panel) and NCI-H460-WWOX cells (lower panel) cultured in the presence (+) or absence of doxycycline (−Dox) were left untreated or were incubated for 4 hours with 250 μM staurosporine or 100 μM genistein. Cellular lysates were analyzed by immunoblotting using the indicated primary antibodies. B, A549-rtTA (control cells) and A549-WWOX cells were starved overnight in Dulbecco’s Modified Eagle Medium containing 0.5% fetal calf serum (FCS). This was followed by stimulation with Dulbecco’s Modified Eagle Medium containing 10% FCS and sequential (20 minutes) or simultaneous (4 hours) incubation with genistein (Gen, STS, 250 μM, lower panel) as indicated.
tyrosine kinase inhibitor genistein (Figure 4B, Supplemental Figures 3B, C). We then assessed clonogenic survival of NSCLC cells treated with staurosporine in relationship to conditional expression of WWOX. This assay is influenced by the antiproliferative activity of transgenic WWOX expression (Figure 1). Hence, results obtained for clonogenic long-term survival are confounded, when compared with short-term cell death assays, which are not influenced by cell proliferation. Nevertheless, we found in NCI-H460 cells that even clonogenic survival after staurosporine treatment can be enhanced by conditional expression of the putative tumor suppressor WWOX (Supplemental Figure 3D).

To further elucidate the mechanistic basis of this protection, we explored whether WWOX modulated the phosphorylation of protein kinase targets in the presence of staurosporine or genistein. Immunoblot analyses of cell lysates prepared from A549-WWOX cells, NCI-H460-WWOX cells, and the respective rTA-M2 control cells revealed that staurosporine and genistein effectively altered constitutive and serum-induced phosphorylation of extracellular-signal regulated kinase, S6, protein kinase B, or glykogen synthase kinase-3B. Although conditional expression of transgenic WWOX was preserved under these experimental conditions, it failed to significantly modulate the phosphorylation profile of these protein kinase targets (Figures 5A, B). Probing the cell lysates with an antibody reactive with phosphorylated tyrosines, pattern changes observed in response to staurosporine and genistein treatment were not consistently altered by expression of WWOX, thus arguing against a modulation of the kinase inhibitory activity of these agents by the transgene (Figure 5A).

DISCUSSION

Although a tumor-suppressive activity of WWOX was corroborated by experiments conducted in several cancer models and by gene targeted mice,14 the underlying molecular mechanism of action still is incompletely defined. Transgenic expression of WWOX at supraphysiological levels was shown to induce apoptosis on its own or to sensitize cancer cell lines to drug-induced cell death.26–28 These results have to be interpreted with caution, as such type of studies might overestimate the actual physiological role of a transgene within the respective signal transduction pathway. Moreover, mutation of the endogenous Drosophila homolog of WWOX in vivo to study its role within a nonmammalian, physiological context resulted in an unexpected outcome. Deficiency in Drosophila WWOX resulted in enhanced sensitivity to radiation-induced apoptosis, which was reduced by reintroduction of either the human or Drosophila WWOX gene.29 Using conditional expression at physiological levels (Supplemental Figure 1C) in several human NSCLC cells with known alterations of endogenous WWOX, we confirmed that wt WWOX consistently suppressed tumor growth in vitro and in vivo. In contrast, a WWOX mutant bearing a charge-relevant amino acid exchange within the SDR domain failed to suppress tumor growth. This suggested that the tumor-suppressive activity of WWOX depends on the SDR domain’s structural integrity. Neither in vitro nor in vivo, we could observe significant induction of apoptosis in response to conditional WWOX expression. To the contrary, we unexpectedly found that WWOX protected NSCLC cells to apoptosis induced by the broad-spectrum kinase inhibitors staurosporine and genistein. By comprehensive analyses of multiple protein kinase targets, we ruled out that this specific protection resulted from interference with the inhibitory activity of these agents or that these agents interfered with conditional WWOX expression. Although at this point the molecular basis of the protection by WWOX against specific apoptotic stimuli remains unclear, it is tempting to speculate that our findings may have revealed a second function of WWOX in human cancer cells, which is distinct from its role as a tumor suppressor. The latter is supported by the strictly antiproliferative activity of WWOX in our experimental models in vitro and in vivo, which depended on a structurally intact SDR domain. In contrast, WWOX protected against apoptosis induced by kinase inhibitors.

In conclusion, our models of conditional expression of WWOX at physiological levels support its role as a suppressor of tumor growth in lung cancer in vitro and in vivo. This is in keeping with the unfavorable outcome after surgical resection of NSCLC observed in those patients with tumors staining negatively for endogenous WWOX expression at immunohistochemistry. Nevertheless, from our data, it seems less likely that WWOX exerts its tumor-suppressive activity by creating a cellular context more permissive for stress-induced apoptosis. To the contrary, WWOX protected NSCLC cells against specific apoptotic stimuli as provided by broad-spectrum kinase inhibitors. This unexpected finding is reminiscent of the result of mutagenic inactivation of the Drosophila WWOX homolog in vivo, which discovered a protective role of WWOX against radiation-induced apoptosis.29 These observations point at a second activity of WWOX, which might be independent from its role as a tumor suppressor.

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