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Association between Decreased WWOX Protein Expression and Thyroid Cancer Development

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Abstract

Context—Chromosomal fragile sites are often related to cancer development. The WW domain-containing oxidoreductase gene (WWOX) spans the second most common chromosomal fragile site (FRA16D) and encodes an important proapoptotic protein.

Objective—To verify our hypothesis that underexpression of WWOX could contribute to malignant transformation of the thyroid cells.

Method—We compared WWOX expression among follicular adenomas (FAs) and differentiated thyroid carcinomas [follicular thyroid carcinomas (FTCs) and papillary thyroid carcinomas (PTCs)] in 53 thyroid tumors resected from patients submitted to total thyroidectomy.

Design—Multiple fields of tumor areas of FAs, FTCs, and PTCs as well as normal thyroid tissue were stained with WWOX antiserum, and classified by the extent of staining (percentage of cells staining) and staining intensity.

Main outcome—PTCs showed a significantly decreased expression of WWOX when compared to FAs and FTCs. Further, using a unique model of comparison in patients in whom FAs and PTCs were concomitantly present, we detected the same result (i.e., no expression in PTCs).

Conclusion—We conclude that WWOX underexpression is an important step that might increase the vulnerability to the carcinogenesis process in PTCs.

Introduction

Chromosomal fragile sites are often hot spots for translocation, deletion, gene amplification, and the integration of oncogenic viruses. These chromosomal regions frequently exhibit DNA strand breaks, often following exposure to chemicals that delay DNA replication (1). Shortly after the discovery of common fragile sites, it was observed that the cytogenetic locations of these fragile sites frequently coincided with the cytogenetic locations nonrandomly altered in specific cancers. Thus, it is possible that the recombinogenicity of fragile regions predisposed them to chromosome re-arrangements during cancer development (1).

Of the common chromosomal fragile site loci, FRA3B and FRA16D are the most frequently expressed (2). Studies on these two fragile site loci have provided compelling evidence that these regions are indeed prone to DNA instability in cancer cells (3).

The WW domain-containing oxidoreductase gene (*WWOX*) encoding a protein with two WW domains and a short-chain dehydrogenase (adh) domain is a proapoptotic tumor suppressor gene that was identified in chromosome 16q23.3–24 (4,5). It was independently cloned by a second group (6), who named the gene *FOR* (fragile-site FRA16D oxidoreductase) and reported several alternative splice forms (*FORI–FORIII*) with unique 3' sequences. The *WWOX* spans a genomic region greater than 1Mb in size where the second most common chromosomal fragile site, FRA16D, is present (7).

Recently it has been demonstrated that loss of both alleles of the *WWOX* gene results in osteosarcomas in some early postnatal mice, whereas loss of one allele significantly increases the incidence of spontaneous and chemically induced tumors, suggesting that haploinsufficiency of *WWOX* itself is cancer predisposing (8). Indeed, reduced or even loss of expression of *WWOX* was found in various human neoplasias, including breast, ovarian, hepatic, gastric, pancreatic, esophageal, lung, and hematopoietic malignancies. In addition, it has also been demonstrated that in breast cancer this lower expression was associated with markers of bad prognosis (9–11).

Recent data have shown that *WWOX* is preferentially highly expressed in secretory epithelial cells of reproductive, endocrine, and exocrine organs, as well as in ductal epithelial cells from specific segments of the urinary system (12). Interestingly, significant *WWOX* protein expression was demonstrated in various cell types of neural origin, including neurons, ependymal cells, and astrocytes; however, no expression of *WWOX* was detected in adipose, connective, and lymphoid tissues, myelinated structures, and blood vessels (12).

We have previously demonstrated a reduction in the expression of *WWOX* in 50% of the primary human oral squamous cell carcinomas (OSCCs) compared with normal mucosa, changes that were associated with a novel mutation of the *WWOX* (13). These results show that the *WWOX* is frequently altered in OSCCs and contributes to the carcinogenesis processes in oral cancer.

Recently, Sbrana *et al.* (14) have demonstrated an association between two chromosomal fragile sites, FRA3D and FRA16D, in lymphocytes from thyroid papillary cancer-affected patients from the Belarus area, 14 years after the Chernobyl accident.

The phylogenetic association of the thyroid gland and the gastrointestinal tract is evident in several functions, including the capacity of the salivary and gastric glands to concentrate iodine in their secretions. Further, the salivary glands contain enzymes that are capable of iodinating tyrosine in the presence of hydrogen peroxide (15). Given that the thyroid originates from the epithelium of the pharyngeal floor and that *WWOX* is involved in OSCC induction, we here hypothesize that underexpression of *WWOX*, a proapoptotic protein, could contribute to malignant transformation in thyroid tumorigenesis. In this report we sought to compare *WWOX* expression among follicular adenomas (FAs) and differentiated thyroid carcinomas [follicular thyroid carcinomas (FTCs) and papillary thyroid carcinomas (PTCs)] in 53 tumors of patients submitted to total thyroidectomy.

Materials and Methods

Samples

We studied tumor samples of 46 patients who underwent total thyroidectomy after the diagnosis of PTC or follicular tumor, by fine needle aspiration biopsies, guided by ultrasound. All these patients had normal thyroid function tests (thyroid-stimulating hormone and free thyroxine). The material had been fixed in 10% formalin and embedded in paraffin (ranging from one to three blocks per case). Surgical specimen confirmed 15 FAs, 11 FTCs, and 20 PTCs cases. In addition, two FA specimens showed concomitant PTC (microcarcinomas) and five PTC specimens also harbored FA. Taken together, we analyzed 53 tumors, taken out of 46 patients, with the final diagnosis of 22 PTCs, 11 FTCs, and 20 FAs. Some patients also presented with colloid nodular goiter. This study was approved by the local ethics committee.

WWOX immunohistochemistry

Tissue sections were stained with *WWOX* antiserum. Briefly, 4 μ paraffin-embedded sections were dewaxed in xylene and hydrated with graded ethanol. Endogenous peroxidase activity was blocked with 3% H_2O_2 in water for 10 minutes. Heat-induced epitope retrieval was performed with 1mM EDTA buffer, pH 8.0, for 30 minutes in a steamer at 96°C. Primary polyclonal rabbit anti-*WWOX* antiserum (140 μ g/mL) was used at a 1:100 dilution (in bovine serum albumin 0.5%) for 18 hours at 4°C. This was followed by incubation with the labeled streptavidin-biotin (LSAB) Kit (DakoCytomation California, Carpinteria, CA). Peroxidase activity was developed with 3,3, diaminobenzidine (DAB) (Sigma, St. Louis, MO) with timed monitoring using a positive control sample. The sections were then counterstained with hematoxylin, dehydrated, and mounted. The positive control was assessed in each slide, using the expression of normal thyroid tissue within the same surgical specimen (internal control). The negative control was done in all batches of slides, omitting the primary antibody (*WWOX*), to exclude nonspecific staining. Only one pathologist examined multiple fields of the lesions and the normal thyroid, and considered positive the cytoplasmic staining in the cells. Tissue sections were scored for extent of staining

(percentage of cells staining) and staining intensity (0, absence; 1+, weakly positive; 2+, moderately positive; 3+, strongly positive). Fisher's Exact Test was used for statistical analyses.

Results

All thyroid tumors were analyzed for the extent and intensity of staining. Normal thyroid tissue always expressed WWOX, showing strong positive cytoplasmic staining for WWOX in the cuboidal follicular cells. Weak staining was observed in thyroid samples representative of inactive glandular activity that displayed mostly flat follicular cells as shown by colloid goiter also found in our samples (Fig. 1J).

In thyroid tumors, immunostaining for WWOX was observed in 100% of FTCs, 95% of FAs, and 36% of PTCs. Analysis of Figure 2 reveals that the maximum intensity (3+) was demonstrated in 82% of FTCs and 20% of FAs and in none of PTCs. Moderate intensity (2+) was seen in 9% of FTCs, 55% of FAs, and 9% of PTCs. Among the PTCs, 6 out of 22 were classified as follicular variants of PTC and one of them had WWOX moderate expression.

Absence of expression was detected in none of FTCs, 5% of FAs, and 64% of PTCs. The difference in staining amongst the three different types of tissues was statistically significant and is clearly shown in Figure 1.

Concerning the size of the tumors and WWOX expression in PTC, we noted no expression in seven out of seven (100%) microcarcinomas (≤ 1 cm) and no or weak expression (+) in 80% of the other tumors (greater than 1 cm). As far as invasiveness is concerned in FTCs, 3 out of 11 (27%) tumors were frankly invasive and all of them showed intense expression as shown in Figure 1L.

In benign tumors (FAs), the majority of them (75%) showed intense or moderate (2+) expression, resembling FTC pattern (Fig. 1C).

Seven out of the 46 patients had more than one lesion (FA and PTC) present concomitantly in the same surgical specimen (15%). Interestingly, the same immunostaining pattern was observed as PTCs presented no (5/7) or weak intensity, while all FAs showed moderate (5/7) or strong intensity.

Discussion

Differentiated thyroid carcinomas are the most common endocrine cancers and the fastest rising incident (3% per year increase in the actual rate) in the US, mainly due to PTC (16). Indeed, recent data using the National Cancer Institute's Surveillance Epidemiology and End Results (SEER) program demonstrate that the incidence of thyroid cancer in the last two decades has substantially increased whereas mortality remained stable (www.seer.cancer.gov).

The main genetic defects in FTCs are mutations in *RAS* and rearrangements of *PAX8/PPAR γ* . In PTCs, gene mutations and rearrangements that result in activation of the mitogenic-activated protein kinase (MAPK) are responsible for the majority of cases. The chimeric genes *RET/PTCs* result in the production of proteins with constitutive tyrosine kinase activity contributing to the development of PTC. The frequency of these rearrangements is higher in children than in adults. The most common genetic defect in PTC is the T1799A mutation of the *BRAF* isoform of *RAF* gene. The protein product BRAF V600E has increased basal kinase activity and triggers the RAF-MEK-MAPK signaling pathway (17). To date, the role of tumor suppressor genes in PTC has not been well established.

Chromosomal fragile sites are chromosome regions prone to DNA instability frequently observed in cancer. The most active fragile site, FRA3B, at 3p14, lies within the tumor suppressor gene *FHIT* that performs its tumor-suppressor function by proapoptotic activity that involves the cytoplasmatic caspase-8-dependent apoptosis pathway. Recently, a correlation among aberrant *FHIT* and *p53* expression, low rate of apoptosis, and thyroid malignancy has been shown. Concomitant aberration of *FHIT* gene and *p53* could be responsible for development of highly malignant types of thyroid cancer and may be considered as a prognostic marker for these tumors (18). The second most common chromosomal fragile site, FRA16D, at 16q23, has been linked to the tumor-suppressor gene *WWOX*, which is involved in stress and apoptotic responses and also regulates the activation of both TP53 and JNK1 (14). High frequency of *WWOX* alternations in different cancers is likely associated with sensitivity of FRA16D.

In this study, we analyzed *WWOX* expression in the most common thyroid tumor types by investigating 53 surgical specimens of 46 patients. Further, taking advantage of the coexistence of more than one type of tumors in a single patient, we studied the expression of *WWOX* in these different tumors when concomitantly present.

We found *WWOX* expression in normal thyroid tissue in agreement with the findings of Nunez *et al.* (12), showing strong positive cytoplasmic staining in the cuboidal follicular cells and weak staining in follicular cells representative of inactive glandular activity (flat follicular cells). It is worth noting that in all instances, normal and tumor, *WWOX* staining was exclusively cytoplasmic as previously shown in breast and ovarian tumors (7).

In agreement with our hypothesis, we demonstrated that 91% of PTC areas displayed extremely reduced or even absent expression of *WWOX*, an important proapoptotic protein. This was true irrespective of the size of the tumors (microcarcinomas or tumors bigger than 1cm) and the presence of multi-centricity or lymphonodal metastasis. It is worth noting that one out of the two PTCs with moderate *WWOX* expression was classified as a follicular variant, and the other, a 0.6 cm microcarcinoma, was found in association with an FTC.

On the contrary, 82% of FTCs showed intense expression of *WWOX*, while the absence of expression was not observed in this type of cancer. The pattern was similar when sorting the tumors by their grade of invasiveness. Additionally, FA, the benign counterpart of FTC, also expressed *WWOX* with high or moderate intensity in 75% of the samples.

It is remarkable that we found the same results comparing PTCs (absence or weak expression) with FAs (moderate or intense expression) when concomitantly present in the same patient. These findings suggest a different molecular pathway between the two most common thyroid cancer types, the follicular and papillary lineages.

The importance of genes present in chromosome fragile sites was previously studied by Zou *et al.* (19) in a series of 57 thyroid tumor specimens. These authors detected truncated FHIT transcripts in 3 of 8 (38%) benign adenomas, 9 of 40 (23%) papillary carcinomas, and 2 of 5 (40%) anaplastic carcinomas. In addition, they also found no molecular alterations of the *FHIT* gene in FTCs. Taken together with our results, this indicates that an additional tumor suppressor gene is involved in the tumorigenesis of FTCs.

We suggest that WWOX underexpression in PTC arouses increased proliferation, which then leads to further genomic instability, with the superimposition of another genetic/epigenetic event, which would provide the follicular epithelial cell with a clonal advantage, contributing to carcinogenesis process in PTC. Since WWOX mutations are very rare, the mechanism of inactivation of this gene might involve epigenetic modifications and/or loss of heterozygosity (LOH) (8). As we learn more about the fragile sites and the functions of genes encompassing them, further insight into their roles in the initiation and progression of thyroid carcinogenesis will be established.

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FIG. 1.

Representative hematoxylin and eosin staining (**A–F**) and WWOX immunostaining (**G–L**) of thyroid lesions: (**A, G**) papillary thyroid carcinoma (PTC) showing no expression (10×); (**B, H**) PTC (upper half) and normal thyroid tissue immunostaining comparison (20×); (**C, I**) follicular adenoma showing intense expression (10× and 20×, respectively); (**D, J**) colloid nodule showing the same expression as normal thyroid (10× and 20×, respectively); (**E, K**) follicular carcinoma showing intense expression (4× and 10×, respectively); (**F, L**) widely invasive follicular carcinoma with neoplastic embolus with intense expression (20×). Note that **H, I**, and **K** allow comparison between tumor and normal thyroid tissue, present in the same area.

FIG. 2.

Graphical representation of WWOX expression in the different thyroid lesions analyzed. Fisher Exact Test = 0.000 (comparing papillary thyroid carcinomas [PTCs] with follicular thyroid carcinomas [FTCs] and follicular adenomas [FAs]).