Published in final edited form as:
Cancer Lett. 2010 March 1; 289(1): 40–45. doi:10.1016/j.canlet.2009.07.017.

Reduction of the most common fragile site tumor suppressor proteins in cervical cancer

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Abstract

FHIT and *WWOX* are tumor suppressor genes that span the common fragile sites FRA3B and FRA16D, respectively. To analyze possible synergisms among these genes in cervical cancer progression, we considered 159 cervical intraepithelial neoplasias, and 58 invasive squamous cell carcinomas of the uterine cervix. FHIT and WWOX proteins were examined by immunohistochemistry and their expression was inversely correlated with precancerous versus invasive lesions. Statistics among biological markers indicated a strong association between FHIT and WWOX. Protein expression of these two genes was also absent or reduced in cancer cell lines. Thus, WWOX may be considered as a novel important genetic marker in cervical cancer and the association between the altered expression of FHIT and WWOX may be a critical event in the progression of this neoplasia.

Keywords

Cervical neoplasia; immunohistochemistry; tumor suppressors; fragile sites

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Conflict of interest
None declared.

Introduction

Since the introduction of cytological screening in the 1940s, the incidence of cervical carcinoma has declined in many developed countries [1]. Considerable efforts have been made to classify preneoplastic and neoplastic lesions [2], and an association between HPV infection, especially high risk HPV types 16 and 18, and the development of precancerous lesions has been proposed [3-6]. Nevertheless, prospective studies of cervical neoplasia suggest that HPV infection alone is not responsible for tumor development and additional genetic or epigenetic changes in tumor cells are required for tumorigenesis [7-9].

The fragile histidine triad (*FHIT*) gene is a candidate tumor suppressor gene located at chromosome 3p14.2 and encompassing the FRA3B common fragile site [10]. Frequent allelic losses and homozygous deletions, as well as loss of heterozygosity in microsatellites located in *FHIT*, have been observed in several types of tumor, particularly those resulting from exposure to environmental carcinogenesis such as lung, kidney, esophageal, head and neck, stomach and cervical cancer [11]. Aberrant protein expression and inactivation of the FHIT gene have been identified in a variety of tumors, including lung [12], breast [13,14], bladder [15], esophagus [16], and colon [17]. In cervical cancer, *FHIT* gene analysis showed a high frequency of inactivation of both alleles and aberrant mRNA transcripts [18,19] while the FRA3B fragile site is also a candidate region for HPV 16 integration [20], suggesting that alterations and inactivation of the FHIT gene contribute and accelerate cervical carcinogenesis. Immunohistochemical studies showed down-regulation of FHIT in microinvasive and invasive cervical carcinomas and an aberrant expression has been reported as a poor prognosis factor independent of the human papilloma virus (HPV) [21-23].

Bednarek et al. described the gene *WWOX* when observing two WW domains at the NH2 terminus and a short-chain dehydrogenase/reductase (SDR) central domain (24). WWOX spans the second most active common fragile site in the human genome (FRA16D) at chromosome region 16q23.2 [25,26]. It has been reported that WWOX may have a role in regulating estradiol-ER interaction while the mouse homologue of the WWOX protein has been defined as an apoptogenic protein and a partner of p53 in cell death [24,27]. It was recently observed that *WWOX* altered expression is due not only to loss of heterozygosity and homozygous deletions but also to epigenetic modifications such as promoter hypermethylation [28]. The *WWOX* gene is altered at the genomic and expression level in several types of tumors, including breast [14,29-30] ovarian [31], prostate [32], hepatocellular [33], pancreatic [34], esophageal [35], small cell lung [36] and gastric cancer [37]. Because no studies correlating FHIT and WWOX protein expression and cervical cancer progression have yet been reported, in the present study we investigated whether these genes might have a pathogenetic role in preinvasive and invasive primary cervical cancer and in cervical carcinoma cell lines.

Materials and Methods

Tissue specimens

From December 1998 to November 2007 we selected cervical tissues from archival paraffin blocks originating from 217 women, 159 of which were classified as precancerous lesions with 109 cases of cervical intraepithelial neoplasia (CIN) 1, 15 cases of CIN 2 and 35 cases of CIN 3. The 58 invasive squamous cell carcinoma, graded according to the WHO histopathological classification, were classified as follows: 20 cases were well-differentiated (G1), 29 cases were moderately differentiated (G2), and 9 cases were poorly differentiated. In order to conform, all cases of invasive carcinoma were chosen at stage Ib of the FIGO classification (International Federation of Gynaecology and Obstetrics). The mean age of patients was 40.58 (SD, ± 13.38 ; range, 22-81 years) from the date of biopsy. Informed consent was obtained from each subject after the purpose and nature of the study had been explained.

Immunohistochemistry

Sections 5 μm thick were cut onto silanized glass slides and air-dried overnight at room temperature. Sections were dewaxed in xylene and rehydrated through graded alcohol. Incubating the slides for 10 min in 3% hydrogen peroxide quenched endogenous peroxidase activity. Sections for microwave antigen retrieval pre-treatment were immersed in citrate buffer (Zymed, San Francisco, CA, USA). They were irradiated twice in a microwave oven (800W) at full power for 4 min and then left to cool for 15 min in the hot buffer at room temperature. FHIT primary antibody (Zymed, San Francisco, CA, USA) was diluted 1:200 in PBS and incubated overnight at 4°C. WWOX expression was performed using a polyclonal rabbit anti-glutathione-Stransferase (anti-GST)-WWOX antibody with dilution 1: 4000 [14].

Sections were reacted with biotinylated anti-rabbit antibody and streptavidin-biotin-peroxidase (Histostain-SP Kit, Zymed Laboratories, San Francisco, CA). Diaminobenzidine was used as chromogene substrate. Finally, sections were washed in distilled water and weakly counterstained with Harry's modified hematoxylin. The primary antibodies were omitted and replaced with preimmune serum in the negative control. Keratinocyte ME 180 cell lines were used for FHIT positive control; breast cancer was used as positive control to perform WWOX protein expression.

Interpretation of immunoreactivity

The degree of all antibodies expression was evaluated semiquantitatively by measuring both intensity and extent of staining: −, 0; +, weak with <10% positive cells; ++, >10% but <50% strongly positive cells; and +++, >50% strongly positive cells. Negative immunoreactivity was scored − or +, and positive immunostaining as ++ or +++. For FHIT and WWOX a distinct brown cytoplasmic staining was scored as positive. Slides were scored independently by three pathologists (E.G., A.V., R.M.); the discordant cases were reviewed and reassigned scores based on consensus of opinion.

Cell lines

All carcinoma cell lines HT-3, SiHa, CaSki, C33a, HeLa, and HCT 116 were obtained from the American Type Culture Collection and maintained in the recommended media.

Protein isolation and Western blotting

Cells were collected and resuspended in lysis buffer (50 mM TRIS pH 7.5, 150 mM NaCl, 10%, 0.4% Nonidet P40, glycerol, protease inhibitors), and incubated in ice for 20 min. Samples were then centrifuged for 20 min at 13000 rpm. The supernatants were discharged, pellets dried and resuspended in 1X Sample buffer (2% SDS, 10% glycerol, 60 mM Tris pH 6.8).

For Western blotting, 50 μg of proteins were fractionated on SDS-polyacrylamide gel and transferred by electrophoresis on nitrocellulose membrane (Bio-Rad Laboratories, Melville, NY). Membranes were blotted overnight with rabbit polyclonal anti Wwox antibody (a gift from Kay Huebner, Ohio State University), rabbit polyclonal anti Fhit antibody (Zymed Laboratories, Carlsbad, CA, U.S.A.), and rabbit polyclonal anti actin antibody (Sigma-Aldrich U.S.A.). Antibodies bound to membrane-immobilized proteins were visualized by enhanced chemiluminescence using the ECL^{TM} Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA).

Statistics

Statistical analyses were performed using the SPSS® computer program package (SPSS for Windows, version 11.5). Frequency tables were analysed using the Chi-square test and Spearman's rank (*r*) correlation coefficient. The statistical significance was set at $P < 0.05$.

Results

Immunohistochemical analysis of protein expression

The intensity of FHIT and WWOX expression was judged on the basis of immunoreactivity of precancerous and invasive lesions. Results are shown in Table I.

Strong and moderate FHIT immunostaining was detected in CIN1 and, when available on the same biopsy section, in normal epithelium (Figure 1 case 1). Low or undetectable immunoreactivity was found in 46 out of 109 cases (42.2%) with CIN 1 and 37 out of 50 (74%) cases with CIN 2-3 (Figure 1 case 2, 3). Thirty-five out of 58 (60.3%) ISCC cases proved negative (Figure 1, cases 4). Observing normal ectocervical squamous epithelium, we detected WWOX immunoreactivity in squamous epithelium and basal layer (Figure 1 case 1). Low or undetectable expression was found in 47 out of 109 (43.1%) with CIN 1 and 25 out of 50 (50%) with CIN 2-3 (Figure 1 case 2, 3). Forty out of 58 (69%) ISCCs were found to be negative (Figure 1 case 4).

Expression of specific proteins and histological grading of ISCCs

FHIT and WWOX protein expression correlated inversely with precancerous versus invasive lesions (P=0.001; P=0.006, respectively) (Table I). Moreover, a significant relationship was observed between the histopathological grading of ISCCs and low or undetectable immunoreactivity FHIT protein expression (p=0.016) (Table II).

Statistical analysis of biological markers showed a strong correlation between FHIT and WWOX [r=0.298; (P<0.0001)].

Protein expression in cervical cancer cell lines

In order to see whether the clinical outcome had support in human cancer cells *in vitro*, five cervical cancer cell lines, HeLa, CaSki, HT-3, SiHa, and C33a, were tested for Fhit and Wwox expression by Western blot in comparison with the control cell line HTC 116 that is positive for the two proteins (Figure 2). As a result, both proteins were absent or under-expressed in all five cervical cell lines when compared with HTC 116. Only in CaSki cell line Wwox is present in a certain amount that is however 2 or 3-fold less than control. Thus, Western analysis for these two tumor suppressor proteins in cervical cancer cells *in vitro* confirms the immunoreactivity data described above.

Discussion

Aberrant *FHIT* transcript was detected in cervical carcinoma cell lines and primary carcinomas [38], and correlated with significant loss of FHIT protein by immunohistochemistry [39]. Literature reported a loss of FHIT expression in 33-76% of cervical cancers, whereas high levels of FHIT expression were found in normal cervical epithelium but less frequently in squamous intraepithelial lesions [39-41] and a correlation between tumor progression, reduced FHIT expression and poor prognosis had been previously detected [22,23]. In a previous study, we found an absence of FHIT protein in 65.2% of ISCCs and 57.1% of non invasive squamous cell carcinomas (NISCCs), observing that the FHIT protein was strongly expressed in dysplastic koilocytosis tissue but was reduced or actually disappeared in areas of invasive carcinoma [42]. Confirming previous data, in the present study we observed a significant

association between FHIT low expression and precancerous lesions. Indeed, when observing cases from CIN1 to CIN2/3, we detected an increase of 31.8% with loss of or undetectable protein expression, confirming that alteration of FHIT could occur at the stage of CIN1, thus becoming an important molecular event in invasive cervical cancer progression. Finally, we observed a strong correlation between loss or undetectable FHIT protein expression and poor histologic differentiation, suggesting that FHIT absence or down-regulation correlates with an aggressive biological behavior of cervical cancer.

In vitro and *in vivo* strategy demonstrated that WWOX is a candidate tumor suppressor gene [43]. Among its functions, WWOX suppresses the transactivation of p73, AP2 γ and ErbB-4 via its WW domain containing Yes-Associated Protein (YAP) and, more recently, an important role for the WWOX/c-jun functional interaction was observed [27,44-46]. The authors observed some similarity between WWOX and FHIT gene functions. For example, WWOX and FHIT are downregulated after exposure to environmental carcinogens involved in cellular stress and cooordinately inactivated in human cancer [47,48,14]. Correlated expression and association with failure of apoptosis in lymphocytes was observed in thyroid cancer [49] and, on examining the relationship between WWOX and FHIT methylation status in breast, lung and bladder cancer, it was observed that expression of both genes is reduced or lost coordinately with promoter region methylation [50]. Immunohistochemistry studies reported frequent loss of WWOX protein expression with a range of 63-84%. In non-small cell lung cancer, Donati et al. observed negative or weak staining intensity in 84% of squamous cell carcinomas showing a relatively high extent of staining but a very low intensity [36]. We found a reduction or absence of WWOX protein expression of 64.3% and 53%, respectively, in preinvasive and invasive cervical cancer. Interestingly, we also found abnormal WWOX protein immunoreaction in the early stage of cervical cancer, sometimes only in HPV infection and condylomatous lesions, suggesting a series of hypotheses. *WWOX* and *FHIT* genes are the most common fragile sites in human genome. The location of *WWOX* in fragile site and its inactivation pattern are similar to that of the *FHIT* gene, and so perhaps their involvement in cervical carcinogenesis is not surprising. Indeed, coordinated loss of WWOX and FHIT immunoreaction, confirmed also *in vitro* by western blot analysis in five cervical carcinoma cell lines, was detected in 30.8% of precancerous lesions and 48.3% of ISCCs, suggesting an involvement of both genes in the same molecular pathway in a subgroup of lesions. A possible interaction between HPV and *FHIT* was previously reported [51], although not, so far, between *WWOX* and HPV infection in cervical cancer.

In breast cancer, an association between WWOX immunoreactivity and a steroid hormone was reported when observing a reduction of protein expression prevalently in premenopausal women, while severely reduced WWOX staining was found in normal tissue only in postmenopausal women [14]. No relationship was found between WWOX protein expression and patient age: nevertheless, the cervical epithelium is subjected to cyclic hormonal influence at a fertile age and the immunoreactivity was performed only on biopsies with no normal counterpart.

In conclusion, in the present study, observing that the Wwox protein is reduced in high rates of cervical cancer, we speculate that alterations of *Wwox* gene may contribute to cervical tumorigenesis. Moreover, the risk of cervical cancer is associated with HPV infection, and, because an interaction is assumed between the oncovirus and *FHIT/FRA3*B, further investigation will be necessary into the possible relationship between *WWOX/FRA16D* and HPV infection in cervical tumorigenesis.

Acknowledgments

Work was supported by a Sidney Kimmel Cancer Research Foundation grant (to N.Z.) and National Cancer Institute grants (to C.M.C.). We thank Dr. Kay Huebner for supplying the Wwox antiserum and for her critical review of the manuscript and Drs Rami Aqeilan, Eugenio Gaudio, and Flavia Pichiorri for helpful advice and technical assistance.

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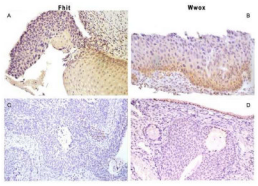

Figure 1.

Illustration of representative immunohistochemistry in normal epithelium, cervical preneoplastic and invasive lesions.

Case 1 (magnification, ×10). Fhit and Wwox positive immunoreactivity in normal ectocervical squamous epithelium.

Case 2 (magnification, ×20). Low or undetectable immunoreactivity of Fhit and Wwox in high grade cervical intraepithelial neoplasia (CIN 3) lesion.

Case 3 (magnification, ×10). A case with Low or undetectable immunoreactivity of Fhit and Wwox protein expression observed in high grade cervical intraepithelial neoplasia (CIN 3) lesion. Note Wwox positive expression in normal columnar epithelial cells.

Case 4 (magnification, ×10). In invasive squamous cell carcinoma, Fhit and Wwox protein expression show negative immunoractivity.

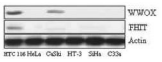

Figure 2.
Western blot showing the reduced protein expression *in vitro* of Fhit and Wwox in five cervical carcinoma cell lines, in comparison with a carcinoma cell line positive for both proteins. Actin is shown to demonstrate the same amount of proteins loaded on the gel.

Table I

FHIT and WWOX protein expression in histopathological lesions of uterine cervix

	FHIT				WWOX				
	Negative −/+		Positive ++/+++		Negative −/+		Positive ++/+++		Tot
CIN 1	46	42.2%	63	57.8%	47	43.1%	62	56.9%	109
CIN 2/3	37	74.0%	13	26.0%	25	50.0%	25	50.0%	50
ISCCs	35	60.3%	23	39.7%	40	69.0%	18	31.0%	58
Tot	118		99		112		105		217

Histopathological lesion *vs* FHIT: χ^2=15.106, p=0.001; *vs* WWOX: χ^2=10.194, p=0.006. CIN1 and CIN2/3 vs FHIT χ^2=13.89, p=0.00019. CIN2/3 and ISCCs vs FHIT χ^2=2.25 p=0.133. CIN1 and CIN2/3 vs WWOX χ^2=0.65, p=0.418. CIN2/3 and ISCCs vs WWOX χ^2=4.03, p=0.044.

Table II

FHIT and WWOX protein expression and ISCC grading

	FHIT				WWOX				
Grade ISCCs	**Negative −/+**		**Positive ++/+++**		**Negative −/+**		**Positive ++/+++**		**Tot**
G1	7	35.0%	13	65.0%	13	65.0%	7	35.0%	20
G2	21	72.4%	8	27.6%	20	69.0%	9	31.0%	29
G3	7	77.8%	2	22.2%	7	77.8%	2	22.2%	9
Tot	35		23		40		18		58

Grade *vs* FHIT: χ^2=8.277, p=0.016; *vs* WWOX: χ^2=0.473, p=0.789. G1 and G2/3 vs FHIT: χ^2=8.19, p=0.004. G1 and G2/3 vs WWOX: χ^2=0.22 , p=0.635.