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Original Article

Methylation and mRNA expression of WWOX and smad4 gene in gastric cardia adenocarcinoma

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Abstract: Methylation status at the promoter regions of WWOX and Smad4 gene and their expression in gastric cardia adenocarcinoma (GCA) were investigated. And their relationship with the development of GCA and the possible mechanism were also discussed. The methylation status of WWOX and Smad4 gene and their mRNA expression levels in the GCA and the corresponding normal mucosal tissues were investigated by nested methylation specific PCR (MSP) and RT-PCR, respectively. Methylation rate of WWOX gene in the GCA and para-carcinoma tissues was 26.1% and 4.2%, respectively, with significant difference ($P < 0.05$); methylation rate of WWOX gene in the GCA patients of stage III/IV (43.3%) was higher than that in the patients of stage I/II (12.8%), with significant difference ($P = 0.004$). The relative expression level of WWOX mRNA in the GCA and para-carcinoma tissues was 0.77±0.16 and 0.89±0.12, respectively, with significant difference ($P < 0.05$); the relative expression level of Smad4 mRNA in the GCA and the para-carcinoma tissues was 0.75±0.17 and 0.88±0.14, respectively, showing significant difference ($P < 0.05$). WWOX gene methylation probably contributes to pathogenesis of GCA and promotes progression of GCA.

Keywords: GCA, methylation, WWOX, Smad4, gene, RT-PCR

Introduction

Epidemiological surveys show that incidence and mortality of gastric cancer are on the decline, but the incidence of gastric cardia adenocarcinoma (GCA) is on the rise [1]. Therefore, the etiological study on GCA cannot be ignored. WWOX is a newfound tumor suppressor gene, and its downregulated expression increases the susceptibility of cells to cancer cells. Its abnormal expression is common in breast cancer, ovarian cancer, lung cancer, multiple myeloma and esophageal squamous cell carcinoma but rarely reported in GCA. Smads are important signaling intermediates and regulators in TGF-β signal transduction system, which can directly transmit the TGF-β signal from the cell membrane into the nucleus. Disorder of TGF-β signal transduction pathway is inevitably caused by the inactivation of Smad4, and the inhibitory effect on cell proliferation is lost, leading to cancer. In this research, we investigated the methylation status in the promoter regions of WWOX and Smad4 gene, and their expression in GCA to reveal the relationship between the methylation of WWOX and Smad4 gene and the development of GCA as well as the possible mechanism, providing a significant candidate indicator for the early detection and treatment of GCA.

Materials and methods

Materials

Main reagents: Proteinase K (Merck), hydroquinone, sodium bisulfite (Sigma), Wizard DNA purification kit (Promega), Trizol (Invitrogen, USA). All primers were synthesized by Beijing SBS Genetech.

Subjects and sample sources: One hundred and sixty GCA and 160 corresponding para-carcinoma tissues were all taken from the GCA patients who underwent surgical treatment in the Affiliated Hospital of Hebei University of Engineering from May 2008 to December 2013 (**Table 1**). There were 112 males and 48 females, aged 37 to 83 years (61.8±8.7 years), including 66 cases ≤ 60 years and 94 cases ≥

Table 1. Clinical and pathological characteristics of GCA patients

Groups	n (%)
Gender	
Male	112 (70.0)
Female	48 (30.0)
Mean age in years (SD)	
Lymph node status	
Negative	68 (42.5)
Positive	92 (57.5)
Pathological differentiation	
Well	41 (25.6)
Moderate	54 (33.8)
Poor	65 (40.6)
Differential degree	
I+II	70 (43.8)
III+IV	90 (56.2)

60 years. All patients had not been treated by chemotherapy or radiotherapy before surgery. HE staining verified that the tumor samples were mostly tumor tissues and the para-carcinoma tissues were free from tumor cell infiltration. The primary tumor tissues were sampled from each GCA patient and some of the samples were collected from the para-carcinoma mucosal tissues (5 cm from tumor). All collected samples were immediately stored in liquid nitrogen. The samples were transferred from liquid nitrogen to a -80°C refrigerator for tissue DNA and RNA extraction. There were 90 cases of stage I/II and 70 cases of stage III/IV according to UICC TNM staging system; they were divided into three groups according to pathological stage, well differentiated group (41 cases), moderately differentiated group (54 cases) and poor differentiated group (65 cases); there were 92 cases with regional lymph node metastasis and 68 without regional lymph node metastasis. The protocol was approved by the Ethics Committee of the Affiliated Hospital of Hebei University of Engineering, and informed consent was obtained from all patients.

Methods

Nested methylation specific PCR (MSP): Proper amount of sample DNA was purified by Wizard DNA purification kit according to instructions. Treated by sodium bisulfite, C of DNA was transformed into U unless the CpG island was methylated. The corresponding primers designed based on this principle (**Table 2**) were used to determine whether the genes were methylated or not. Reaction conditions: predenaturation at 95°C for 10 min, denaturation at 94°C for 45 s, annealing at 60.5°C (methylated primer) or 55.2°C (unmethylated primer) for 50 s, extension at 72°C for 45 s, 40 circles; final extension at 72°C for 10 min. After 2% agarose gel electrophoresis was performed on the amplified products, the result was subjected to image analysis by the UV gel imaging and analysis system. The genomic DNA treated by methylase (Sss I, New England BioLabs) was used as the positive control of MSP and subjected to PCR. Sterilized double-distilled water was used as the negative control in place of the DNA template and subjected to PCR. 10% of the samples were selected for repeated trials as the quality control of the MSP test.

RT-PCR test: The total RNA was extracted according to the instructions of Trizol (Invitrogen), and transformed into cDNA by reverse transcription according to the instructions of the RT-PCR kit (Reverse Transcription System A3500, Promega). Synthesized primers of WWOX and Smad4 are show in **Table 3**. Reaction conditions: preheating of RNA at 70°C for 10 min, 25°C for 10 s, 42°C for 60 min, 99°C for 5 min, 4°C for 5 min, 40 circles. 2% agarose gel electrophoresis was performed on the PCR products, with GAPDH as the internal reference.

Results determination and statisticals

The methylated amplified fragments of expected lengths were generated by the amplification of the DNA which was treated by Sss I methylase using the methylation specific primer (M), but nothing was generated in the blank control trial, indicating that the primers and reagents used were appropriate and the results were plausible. Three different results of the methylation state of WWOX and Smad4 gene in the GCA tissues may appear: firstly, the objective band was generated by the methylation specific primer (M), while no band was generated by the unmethylated specific primer (U), as was specified as methylation; secondly, the objective band was generated by the unmethylation specific primer (U), while no band was generated by the methylated specific primer (M), as was specified as unmethylation; thirdly, the objective bands were generated by both primers, as

Table 2. WWOX, Smad4 gene primer in MSP

Gene	CpGStatus	Primer sequence (5'-3')	Annealing Temperatur (°C)	Product Size (bp)
WWOX	Unmethylated	TATGGGTGTTGTTTTTTA	56	347
		CAATCTCCACAATATCACAAC		
	Methylated	TATGGGCGTCGTTTTTTAGTC	51	347
		CAATCTCCGCAATATCGCGAC		
Smad4	Unmethylated	TTTGTAATGAGATGTTAATTTTTTTGGT	55.7	274
		CAACTTATCAAAAAACCACTAAACATACA		
	Methylated	GTAACGAGATGTTAATTTTTTCGGC	49	269
		ACTTATCGAAAAACCACTAAACATACG		

Table 3. WWOX, Smad4 gene Primers used in RT-PCR

Gene	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
GAPDH	5'CGGGAAGCTTGTGATCAATGG 3'	342	60
	5'GGCAGTGATGGCATGGACTG 3'		
WWOX	5'GAGCAAACACATCTGACCTAC 3'	258	58
	5'CAAAATGAGCATCCCCTCC 3'		
Smad4	5'ATCTGAGTCTAATGCTACC3'	452	58
	5'CGTATCCATCAACAGTAAC3'		

Table 4. Frequency of WWOX, Smad4 methylation in GCA and GCA

Gene	Methylation ratio		χ^2	P
	GCA	Corresponding nonmalignant		
WWOX	26.1%	4.2%	9.598	0.002*
Smad4	2.9%	8.3%	0.783	0.376*

*: *P* value of GCA against corresponding nonmalignant.

was specified as hemimethylation, recorded in methylation.

Determination of the relative content of the PCR amplified products: After 1.5% agarose gel electrophoresis was performed on the PCR amplified products (6 μL), the result was photographed by the gel imaging and analysis system; the grey value was measured by Gel Pro Analysizer 3.1; the 1OD value of WWOX and Smad4 gene was standardized using the 1OD value of the internal reference GAPDH, and the relative content was obtained and analyzed. The formula is given below:

Relative expression of mRNA = (1OD value of WWOX/Smad4)/(1OD value of GAPDH)

Statistics

The data were processed with SPSS 13.0 software. The intergroup compare of rate was performed by Chi-square test or corrected Chi-square test. The intergroup compare of the relative expression was conducted by t test or variance analysis. $P < 0.05$ indicated significant difference.

Results

*Methylation of WWOX and Smad4 gene in the GCA samples and the corresponding para-carcinoma tissues (**Table 4**)*

WWOX gene methylation rate of the tumor tissues and the corresponding para-carcinoma tissues among the 160 GCA patients was 26.1% and 4.2%, respectively, showing significant difference ($P < 0.05$) (**Figure 1**).

Smad4 gene methylation rate of the tumor tissues and the corresponding para-carcinoma tissues among the 160 GCA patients was 2.9% and 8.3%, respectively, showing no significant difference (**Figure 2**).

Correlation between methylation of the two genes and clinical data

Relations between WWOX gene and clinical pathological data: No significant difference was observed between WWOX gene methylation in the GCA tissues and clinical data such as gender, age, pathological grading, presence of lymph node metastasis, etc. ($P > 0.05$). The statistics based on TNM staging showed that WWOX gene methylation rate among the GCA patients of stage III/IV (43.3%) was higher than that among the patients of stage I/II (12.8%), with significant difference ($P = 0.004$).

Relations between Smad4 gene and clinical pathological data: No significant difference was observed between Smad4 gene methylation in

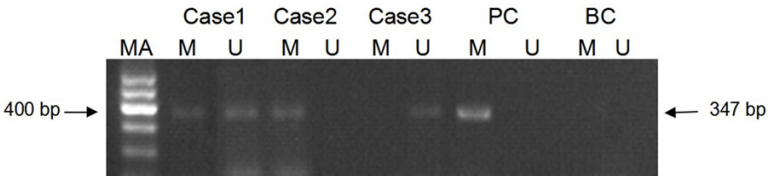

Figure 1. Methylation analysis of WWOX gene in GCA tissues. Case1: Hemi-Methylated; Case2: Methylated; Case3: Unmethylated; PC: Positive control; BC: Blank control; M: Methylated; U: Unmethylated; MA: Marker.

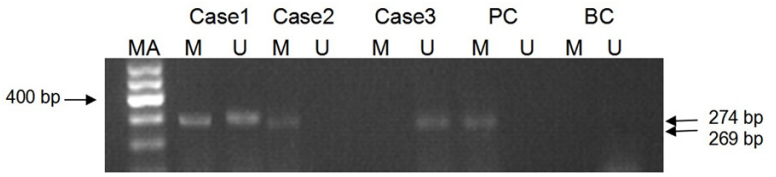

Figure 2. Methylation analysis of Smad4 gene in GCA tissues. Case1: Hemi-Methylated; Case2: Methylated; Case3: Unmethylated; PC: Positive control; BC: Blank control; M: Methylated; U: Unmethylated; MA: Marker.

Table 5. The expression of WWOX, Smad4 mRNA in adjacent carcinoma andGCA

Group	N	WWOX	Smad4
Adjacent cancer	44	0.89±0.12	0.88±0.14
Cancer	15	0.77±0.16	0.75±0.17

the GCA tissues and clinical data such as gender, age, pathological grading, TNM staging, presence of lymph node metastasis, etc. ($P > 0.05$).

*MRNA expression of WWOX and Smad4 gene in the GCA and the para-carcinoma tissues (**Table 5**)*

The relative expression level of WWOX mRNA in the GCA and the para-carcinoma tissues was 0.77±0.16 and 0.89±0.12, respectively, with significant difference ($P < 0.05$) (**Figure 3**); the relative expression level of Smad4 mRNA in the GCA and the para-carcinoma tissues was 0.75±0.17 and 0.88±0.14, respectively, showing significant difference ($P < 0.05$) (**Figure 4**).

Relationship between WWOX gene methylation and mRNA expression

The relative mRNA expression level of WWOX gene in the methylation positive GCA tissues (0.73±0.15) was lower than that in the methylation negative GCA tissues (0.76±0.17), but the difference was not significant ($P = 0.13$).

No correlation was found between the relative expression levels of WWOX and Smad4 gene.

Discussion

Oncogenesis is a multi-step, multi-stage complex process involving multiple genetic alterations, mainly concerning oncogene activation and anti-oncogene inactivation and eventually leading to abnormal gene expression. Abnormal gene expression usually contains genetic alterations such as mutations, deletions, overexpression, etc., and epigenetic alterations including DNA methylation, histone acetylation, etc. In recent years, DNA methylation has become a hot topic in the research area of tumor pathogenesis and treatment.

WW domain containing oxidoreductase (WWOX) gene is a newfound candidate tumor suppressor gene, situated at the chromosomal fragile site FRA16D and encoding a 414-amino acid protein of 46 kDa. The WWOX protein contains two N-terminal WW domains (residues 18-47, 59-88) and a short-chain dehydrogenation reductase site at the C-terminal, and its essential function is signal transmission and information transduction.

WWOX protein is a pro-apoptotic protein engaging in multiple signal transduction pathways to inhibit oncogenesis. Chang et al. [2, 3] found that mitochondrial WWOX probably engaged in the molecular pathway of apoptosis triggered by p53 in the mitochondrion, increasing the susceptibility of the tumor cell to tumor necrosis factor (TNF) cytotoxin; meanwhile, Aqeilan et al. [4, 5] found that WWOX could enhance the pro-apoptotic effect of P73. However, the gene silencing and down-regulated expression caused by genetic and epigenetic alterations of the WWOX gene lessen its pro-apoptotic effect, promoting tumor formation and progression. During the process, epigenetic alterations, especially the methylation of the promoter region of the gene, may play a significant role.

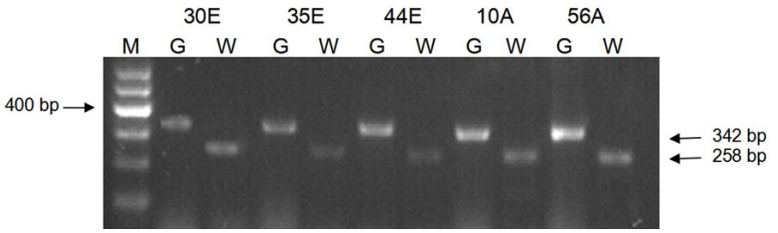

Figure 3. mRNA analysis of WWOX gene and GAPDH. M: Marker; A: Adjacent non-cancerous tissue; E: GCA tissue; G: GAPDH; W: WWOX.

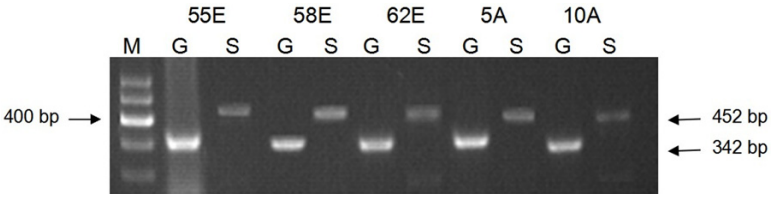

Figure 4. mRNA analysis of Smad4 gene and GAPDH. M: Marker; A: Adjacent non-cancerous tissue; E: GCA tissue; G: GAPDH; S: Smad4.

DonatiV et al. [6] detected methylation at the WWOX promoter in the study on non-small cell lung cancer, and found a close relationship between WWOX gene methylation and its negative expression in lung cancer tissues, with WWOX protein lost in 62% of the lung cancer cells due to the gene methylation. Qin HR et al. [7] found a significantly lower WWOX mRNA and protein expression in the prostate cancer cell lines (LNCaP, DU145, PC-3) due to the DNA methylation in the WWOX regulatory domain than that in the non-tumorous prostate cell (PWR-1E), and found a significant increase in WWOX mRNA and protein expression in the prostate cancer cell lines treated with the DNA methylation inhibitor AZA, especially in DU145. In a study on WWOX gene methylation and breast cancer, Wang X et al. [8] found that methylation rate of the promoter region of WWOX gene was 55% in breast cancer. But no methylation was found in the promoter region of WWOX gene in the normal para-carcinoma tissues. Moreover, WWOX gene methylation was closely correlated with the down-regulated mRNA expression in tumor tissues.

In this research, we found a significantly higher methylation rate of WWOX gene in the GCA tissues of the 160 cases (26.1%) compared to the normal para-carcinoma tissues (4.2%), indicating a probable relationship between WWOX gene methylation and GCA development possibly with the hypermethylation of the WWOX gene as a molecular biological event in the early stages of the GCA progression. Meanwhile, in this study we found methylation in the promoter region of WWOX gene was independent of gender, age, tumor pathological grading, presence of lymph node metastasis, etc. ($P > 0.05$), but was correlated with tumor pathological staging ($P < 0.05$). And methylation rate of the WWOX gene promoter region among the GCA patients of stage III/IV (43.3%) was higher compared to the patients of stage I/II (12.8%), indicating possible participation of methylation of the WWOX gene promoter region in the GCA progression. The results of the study also showed that the relative expression of the mRNA in the tumor tissues was significantly lower than that in the para-carcinoma tissues, but the relative expression level of the mRNA in the methylation positive GCA tissues was not significantly lower than that in the methylation negative GCA tissues. The possible reasons are as follows: Hemimethylation might contribute little to the down-regulated expression of the gene; other genetic alterations mediating down-regulated expression of the WWOX gene might exist in GCA besides methylation. Except methylated tumor cells in the investigated tumor tissues, unmethylated tumor cells and abundant interstitial cells with normal expression of WWOX gene coexisted, which obscured the impacts of methylation on expression. No correlation was found in this study between the relative expression of the mRNA and clinical data, indicating that the WWOX deletion might occur at the early stages of the GCA progression. The concrete mechanism of the abnormal methylation in the promoter region of the WWOX gene causing malfunction to WWOX mRNA transcription remains to be further studied.

The TGFβ-Smads signal transduction pathway displays various biological activities, playing an important role in regulating cellular growth, differentiation, apoptosis, adhesion, the synthesis and deposition of extracellular matrix, embryogenesis and tissue repair, inflammatory responses and fibrosis. It plays a significant

role in inhibiting oncogenesis predominantly through regulating intranuclear CKI (cyclin dependent kinase inhibitor) expression, preventing cell cycle running, inhibiting cellular malignant transformation, which requires the Smad family in the cytoplasm to serve as a linking bridge. The Smad4 gene and the encoded protein play a central role in the TGFβ-Smads pathway as a tumor suppressor gene and the only Co-Smad in the pathway, respectively. Once Smad4 expression is negative or down-regulated, the whole TGF-β signal transduction pathway will be destroyed, thereby unable to inhibit cell proliferation and losing the potential ability to suppress oncogenesis.

The abnormal expression of Smad4 causes malfunction in TGF-β signal transduction pathway and eventually leads to oncogenesis, which is most commonly found in pancreatic cancer [9], with a mutation rate of 50%. It is secondly common in digestive system cancers such as colon cancer, gastric cancer, bile duct cancer, liver cancer, etc. And it is also found in ovarian cancer, acute myeloid leukemia, lung cancer, head and neck squamous cell carcinoma, etc. [10]. Xu et al. [11] verified that the haploid deletion on the mouse Smad4 gene could promote the development of gastric polyps and gastric cancer. Takagi et al. [12] found missense mutation of the Smad4 gene and its abnormal expression were caused by gene deletion in the study on primary colorectal cancer. Koyama et al. [13] found the loss of heterozygosity of the Smad4 gene in 78% of the patients in the study on primary colorectal cancer, with mutation and insertion in several cases. Furthermore, researches abroad showed that mutation was not the primary cause of Smad4 gene inactivation. Therefore, it is estimated that hypermethylation in the promoter region of the gene functions as the second blow leading to the abnormal expression of Smad4. Onwuebusi et al. [14] conducted methylation analysis on the promoter sequence of Smad4 in the study about esophageal adenocarcinoma, and found a significant correlation between the abnormal methylation of the promoter sequence and the negative expression of the Smad4 protein. It was estimated that epigenetic modifications silenced the gene, inhibited the gene expression and hindered TGF-β1/Smad4 signal transduction. However, there are few reports about methylation of Smad4 gene both at home and abroad. Methylation of Smad4 gene remains to be further studied.

This trial was designed and carried out in an effort to investigate the role of methylation of Smad4 gene in the pathogenesis and development of GCA. The results showed that the Smad4 gene methylation rates in the tumor tissues and the corresponding para-carcinoma tissues among the 160 GCA patients were 2.9% and 8.3%, respectively, without significant difference ($P > 0.05$), probably indicating no correlation between methylation of Smad4 gene and the pathogenesis of GCA. In this study, the relative expression of Smad4 mRNA in the GCA tissues was significantly lower than that in the para-carcinoma tissues; however, no correlation was found between the relative mRNA expression and clinical data as well as between methylation of Smad4 and the down-regulated mRNA expression, probably indicating a certain relationship between the down-regulated expression of the Smad4 mRNA and genetic alterations such as the gene deletion. KiKD et al. [15] detected the down-regulated expression of Smad4 mRNA in tumor tissues in the study on the expression and mutation of TGFβ-Smads in human cervical carcinoma; Leng A et al. [16] found a significantly lower expression of Smad4 in tumor tissues than that in para-carcinoma tissues, a significantly lower expression of Smad4 in the poor differentiated group than that in the well and the moderately differentiated groups, and a lower expression in the group with lymph node metastasis than that in the group without lymph node metastasis. All of the findings were consistent with the results of this study.

Hsu LJ et al. [17] found a TGF-β1 signal transduction pathway independent of TβRII, and hyaluronidase could enhance WWOX gene activity. The TGF-β1/Hyal-2 complex is formed through the combination of TGF-β1 and the hyaluronidase Hyal-2 on the membrane surface, which not only induces the intracellular aggregation of WWOX and Smad4, but also accelerates the combination of Hyal-2 and WWOX protein. Subsequently, the triplet formed by the Hyal-2/WWOX complex and Smad4 enters the nucleus, engaging in the regulation process of the Smads protein on the transcriptional activity of the promoter region through Smad4. It is suggested that WWOX probably engages in the TGFβ/Smads signal transduction and WWOX plays a role in inhibiting oncogenesis in coordination with Smad4. However, the more concrete mechanism of the interaction between WWOX and Smad4 and their correlation remain to be further investigated.

Tumor pathogenesis and development are an extremely complicated process involving multiple genes, predominantly concerning oncogenes activation and anti-oncogenes inactivation. The results of this study indicated that the hypermethylation of WWOX gene in tumor tissues was correlated with the pathogenesis and development of GCA. However, the correlation between the methylation of Smad4 gene and the development of GCA remains uncertain.

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Disclosure of conflict of interest

None.

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