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Original Article

Expression and significance of CDX2, FXR, and TGR5 in esophageal cancer

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Abstract: This study explored the expression and significance of three critical morphogenesis genes in normal esophagus, reflux esophagitis (RE), Barrett's esophagus (BE), esophageal adenocarcinoma (EA), and esophageal squamous cell carcinoma (ESCC). Esophageal tissue samples and tissue microarrays were used. CDX2, FXR, and TGR5 protein expression were measured by immunohistochemistry in normal esophageal, RE, BE, EA, and ESCC tissues. All 3 proteins had markedly changed expression during the progression of EA. The expressions of CDX2 and FXR were positively correlated in EA. In addition, TGR5 expression was positively correlated with CDX2 in RE and BE. The expressions of CDX2 and FXR were also positively correlated in ESCC. Although CDX2, FXR, and TGR5 were upregulated in ESCC, these factors might not be markers for the prognosis of ESCC. These results suggested that CDX2, FXR, and TGR5 might play different roles in EA and ESCC. They may represent novel therapeutic targets for patients with these cancers.

Keywords: Esophageal squamous cell carcinoma, CDX2, FXR, TGR5, BE

Introduction

Esophageal cancer is a common malignant tumor worldwide [1]. The two main types of esophageal cancer are esophageal adenocarcinoma (EA) and esophageal squamous cell carcinoma (ESCC). Barrett's esophagus (BE) is a precancerous lesion before esophageal adenocarcinoma characterized by intestinal columnar epithelium replacing normal esophageal squamous epithelium [2]. Previous studies indicated that BE could be derived from reflux esophagitis caused by chronic stimulation of gastroesophageal reflux, including saliva, bile acid, pepsin, mucus, trypsin, and pancreatic enzymes [3]. In addition, bile acids have been identified as an independent risk factor for intestinal transformation at the esophagogastric junction [4]. However, the mechanism of bile acid-induced intestinal metaplasia is still unclear.

Takeda G protein-coupled receptor 5 (TGR5) and farnesoid X receptor (FXR) are two novel receptors of bile acid. TGR5, a G-protein-coupled receptor, plays a significant role in maintaining bile acid-related energy and metabolic balance [5-7]. A previous study suggested that the expression level of TGR5 in normal and BE tissues was significantly lower than that in ESCC tissues. Additionally, bile acid-induced NOX5-S is regulated by activation of TGR5 [8]. FXR is a nuclear receptor of bile acid. As reported, FXR was very upregulated in BE compared

to normal esophagus, RE, and EA. Moreover, FXR plays a crucial role in the regulation of apoptosis in BE-derived cell lines [9]. Another study showed that bile acid-stimulated FXR could increase the immune response in BE by recruiting immune cells, which may be an important molecular mechanism involved in esophageal neoplastic progression [10]. Caudal type homeobox 2 (CDX2) is an intestinal-related transcription factor that plays a major role in intestinal differentiation and development [11]. Many studies have confirmed that CDX2 promotes intestinal metaplasia in gastric and esophageal mucosa [12-14]. Our previous research demonstrated that bile acid could induce the activation of downstream KLF4, HNF4α, and cadherin 17 in gastric cells by activating CDX2 [15]. Similarly, it has been proven that this molecule can induce the transformation of esophageal mucosal cells into intestinal cells [16, 17].

Although studies have revealed that the expression of CDX2, FXR, and TGR5 change markedly during the progression of BE and EA, the specific molecular mechanism remains unclear. Furthermore, the relationship between CDX2 and these two bile acid receptors in EA, esophageal precancerous lesions, and ESCC is still unknown.

Here, we detected the expression levels of CDX2, FXR, and TGR5 in normal esophageal mucosa, BE, and ESCC tissues, and further analyzed their correlation in tumor tissues to explore their role in malignant transformation of esophageal cells.

Materials and methods

Patients and samples

A total of 197 patient esophageal tissues (83 cases with normal squamous mucosa, 43 cases with reflux esophagitis and 71 cases with BE) were collected from Xijing Hospital of Digestive Diseases, Xi'an No. 1 Hospital, Xianyang Central Hospital, The First Affiliated Hospital of Zhengzhou University and Yanan University Affiliated Hospital between 2012 and 2018 (121 male [61.4%], 76 female [38.6%]). All esophageal tissues were formalin-fixed and paraffin-embedded. This study was approved by the ethics committees of each participating hospital and was conducted according to the Declaration of Helsinki (KY20202102-F-1). Written informed consent was obtained from all patients in the study.

Tissue microarray

Paraffin-embedded consecutive esophageal tissue slides (ES803, ES8011a, Alenabio, China) contained 22 cases of RE and 70 cases of EA. The median age of the 22 patients with RE was 57 years old (range 38-72 years), and 13 of these patients were male (59.1%). The median age of the 70 EA patients was 60 years old (range 33-75 years), and 54 of them were male (85.8%). Paraffin-embedded consecutive ESCC tissue slides (HEsoS180Su05) were purchased from Outdo Biotech (Shanghai, China) and included paired ESCC and paracancerous specimens from 75 individuals with follow-up data. Of these ESCC patients, 57 were male (76%). The median age was 64 years (range 48-82 years).

Immunohistochemistry (IHC)

CDX2 (ab76541, Abcam), FXR (PP-A9033A-00, R&D) and TGR5 antibodies (ab72608, Abcam) were used to examine the expression of these proteins in EA, RE, and ESCC tissues. IHC staining was performed according to standard procedure.

The results of IHC staining were scored independently by two observers according to the intensity of staining and the proportion of positive cells. Percentage of positive cells: 0 (<1%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). Staining intensity: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The final score was the product of the proportion of positive cells and the staining intensity, and the result was divided into four grades (negative: 0, weak: 1-4, moderate: 5-8, strong: 9-12). When analyzing the correlation between these three molecules and the clinical features of ESCC, we categorized the combined score as low expression (score: 1-6) or high expression (score: 7-12).

Statistical analysis

All data were analyzed with SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA). The Kruskal-Wallis H test was performed to compare the differences between multiple groups. Pearson correlation analysis was used to deter-

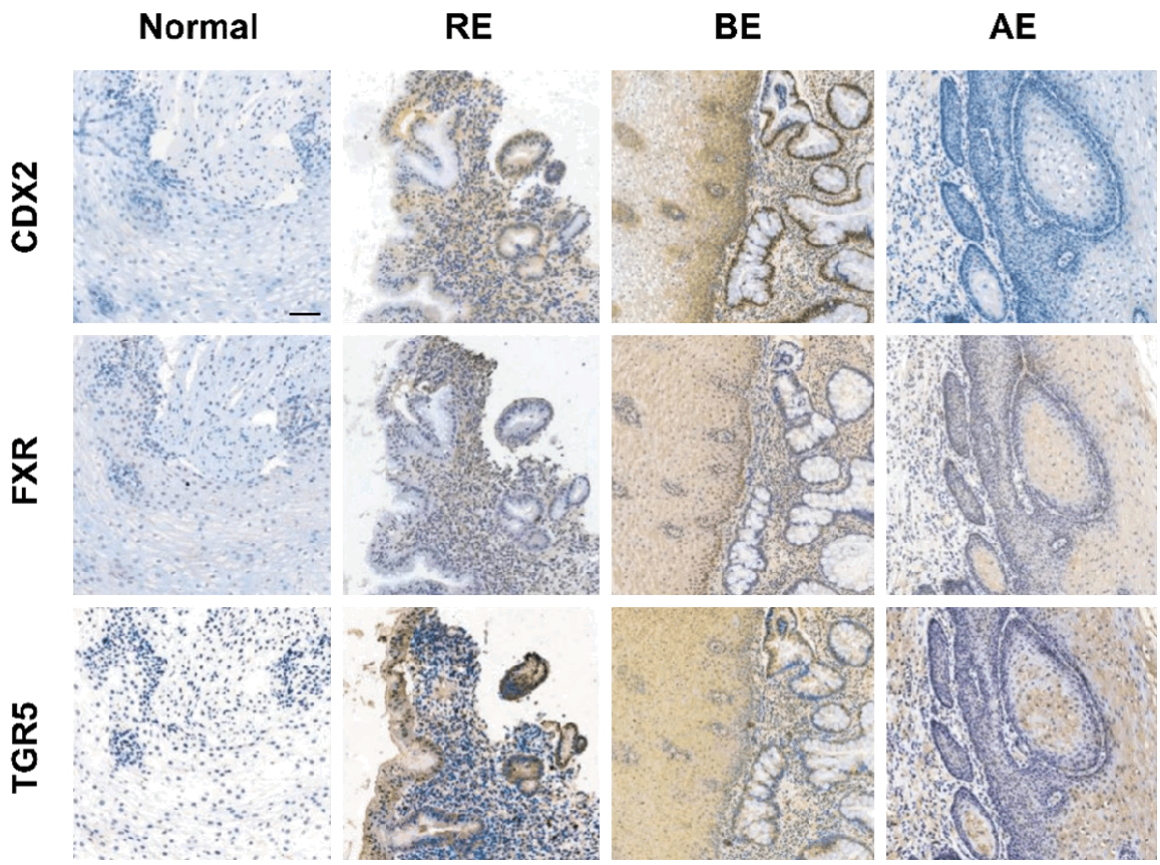

Figure 1. Expression of CDX2, FXR, and TGR5 in normal esophageal mucosa, RE, BE, and EA tissues. RE: Reflux esophagitis; BE: Barrett's esophagus; EA: Esophageal adenocarcinoma. Scale bars: 100 μm.

mine the correlation between CDX2 and FXR or TGR5. Comparisons between two groups were performed using an unpaired Student's t test. Kaplan-Meier analysis was used to calculate the survival curves of each group. P<0.05 was considered significant.

Results

Expression of CDX2, FXR, and TGR5 in EA and esophageal precancerous lesions

The expression levels of CDX2, FXR, and TGR5 in EA and esophageal precancerous lesions were investigated by IHC in three esophageal tissue microarrays and patient esophageal tissues collected from 5 different hospitals. CDX2 and FXR expression were mainly located in the nucleus in all positive tissues, while TGR5 expression was located in both the cytoplasm and cell membrane (**Figure 1**). We found that 79.7% of RE showed weak CDX2 staining, which was significantly higher than that of normal mucosa (P<0.01) and EA (P<0.01). CDX2 presented weak to moderate staining in 83.1% of BE tissues but high staining in the other three groups (P<0.01 for all). A total of 44.9% of EA and 62.8% of normal mucosas showed weak CDX2 staining (P=0.237) (**Table 1**). A total of 21.3% of RE, 46.3% of BE and 6.0% of EA showed moderate to strong FXR staining, which was higher than that of normal mucosa (0.0%) (P<0.01 for all). Similarly, BE showed the highest FXR expression level compared to the other groups (P<0.01 for all). Although their expression levels were slightly different, there was no statistical significance between EA and RE (P=0.279) (**Table 1**). TGR5 showed moderate to strong staining in 63.1% of BE but weak staining in normal mucosa (32.5%) (P<0.01), RE (38.1%) (P<0.01), and EA (19.7%) (P<0.01), while there was no significant difference among normal mucosa, RE, and EA (P=0.688, P=0.661, P=0.425 for every two groups) (**Table 1**).

Table 1. CDX2, FXR, and TGR5 expression in normal esophagus, RE, BE, and EA

	CDX2 expression level					
	-	+	++	+++	H	*P*-values
Normal esophagus (%)	26/78 (33.3)	49/78 (62.8)	3/78 (3.8)	0/78 (0.0)	90.42	
RE (%)	4/64 (6.3)	51/64 (79.7)	6/64 (9.4)	3/64 (4.7)	147.91	$P^{\#}<0.01$
BE (%)	0/59 (0.0)	24/59 (40.7)	25/59 (42.4)	10/59 (16.9)	216.74	$P^{\#}<0.01$, $P^{*}<0.01$
EA (%)	27/69 (39.1)	31/69 (44.9)	8/69 (11.6)	3/69 (4.3)	105.47	$P^{\#}=0.237$, $P^{*}<0.01$, $P^{+}<0.01$
	FXR expression level					
Normal esophagus (%)	57/77 (74.0)	20/77 (26.0)	0/77 (0.0)	0/77 (0.0)	65.63	
RE (%)	9/47 (19.1)	28/47 (59.6)	8/47 (17.0)	2/47 (4.3)	143.48	$P^{\#}<0.01$
BE (%)	2/67 (3.0)	34/67 (50.7)	17/67 (25.4)	14/67 (20.9)	192.22	$P^{\#}<0.01$, $P^{*}<0.01$
EA (%)	12/66 (18.2)	50/66 (75.8)	4/66 (6.0)	0/66 (0.0)	128.45	$P^{\#}<0.01$, $P^{*}=0.279$, $P^{+}<0.01$
	TGR5 expression level					
Normal esophagus (%)	6/80 (7.5)	48/80 (60.0)	20/80 (25.0)	6/80 (7.5)	122.36	
RE (%)	5/63 (7.9)	34/63 (54.0)	16/63 (25.4)	8/63 (12.7)	127.65	$P^{\#}=0.688$
BE (%)	1/65 (1.5)	23/65 (35.4)	16/65 (24.6)	25/65 (38.5)	186.87	$P^{\#}<0.01$, $P^{*}<0.01$
EA (%)	6/66 (9.1)	47/66 (71.2)	10/66 (15.2)	3/66 (4.5)	116.64	$P^{\#}=0.661$, $P^{*}=0.425$, $P^{+}<0.01$

$P^{\#}$: compared with normal esophagus; P^{*}: compared with RE; P^{+}: compared with BE.

Association of CDX2, FXR, and TGR5 in EA and esophageal precancerous lesions

The correlation of CDX2 with FXR and TGR5 in the normal esophagus, RE, BE, and EA was investigated by Pearson correlation analysis. Our results indicated that the expressions of CDX2 and FXR were positively correlated in EA (r=0.2726, P=0.0268). There was no obvious correlation between CDX2 and FXR in the normal esophagus (r=0.0829, P=0.5050), RE (r=0.0629, P=0.6848), or BE (r=0.0212, P=0.8804) (**Figure 2A**). TGR5 was positively correlated with CDX2 in RE (r=0.3541, P=0.0184) and BE (r=0.3065, P=0.0256), while there was no correlation between them in the normal esophagus (r=0.0678, P=0.5856) or EA (r=0.1407, P=0.2600) (**Figure 2B**).

Expression of CDX2, FXR, and TGR5 in ESCC and para-cancer (PC) tissues

Figure 3 shows representative staining of CDX2, FXR, and TGR5 in normal esophageal, ESCC, and PC tissues. Moderate to strong staining of CDX2 was observed in 31.5% of PC tissues, which was higher than that of normal esophagus (3.8%) (P<0.01). The percentage of ESCC with moderate to strong CDX2 staining (62.9%) was higher than that of normal esophageal (P<0.01) and PC (P<0.01), which represented a gradual increase in CDX2 in normal esophageal, PC and ESCC tissues (**Table 2**). Similarly, **Table 2** also shows gradual increases in FXR and TGR5 levels in these three types of tissues, and all pairwise comparisons of the tissue groups were significant (P<0.01 for all).

Association of CDX2, FXR, and TGR5 in ESCC and PC tissues

Using Pearson correlation analysis, the correlation of CDX2 with FXR and TGR5 in ESCC and PC tissues was investigated separately. Results indicated that the expression levels of CDX2 and FXR were positively correlated in ESCC (r=0.2616, P=0.0070). However, CDX2 and FXR showed no correlation in normal esophageal PC tissues (r=0.1596, P=0.1937) (**Figure 4A**). In addition, there was no correlation between CDX2 and TGR5 in either PC (r=-0.0360, P=0.7709) or ESCC tissues (r=0.0848, P=0.3900) (**Figure 4B**).

Correlation of CDX2, FXR, and TGR5 expression with clinicopathologic characteristics of ESCC

As clinicopathologic data and prognoses were given only in ESCC tissue slides, we next inves-

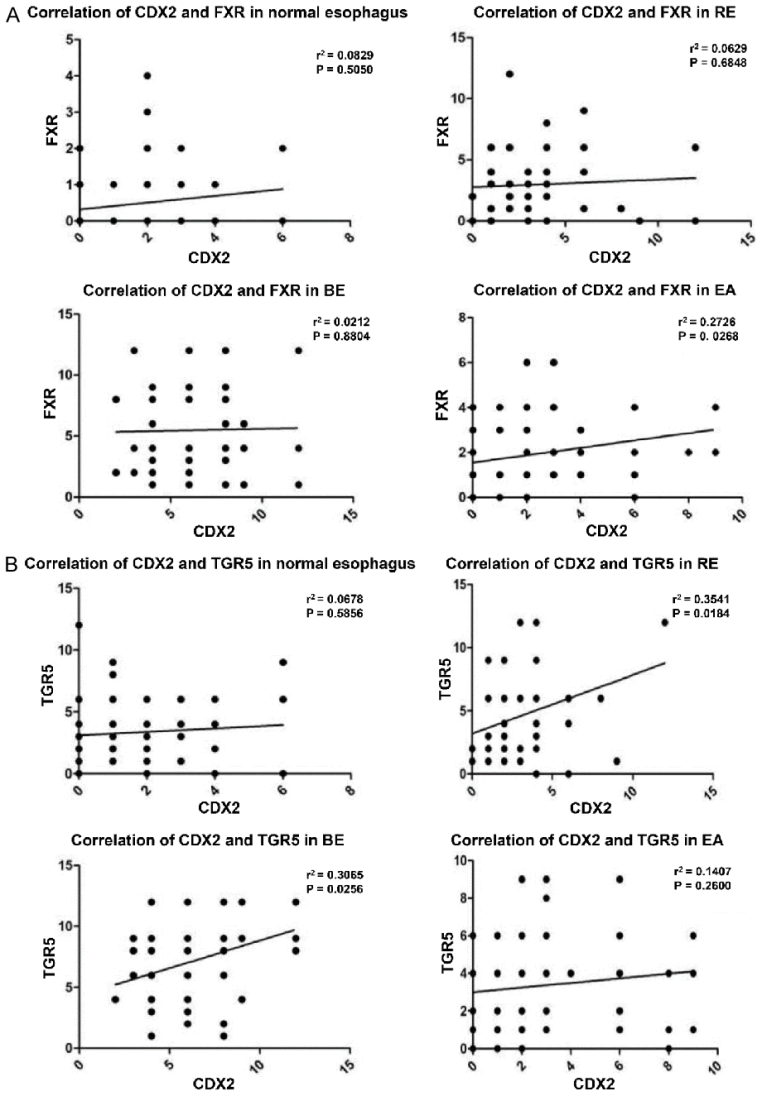

Figure 2. Correlation of CDX2 with FXR and TGR5 in normal esophageal mucosa, RE, BE, and EA. A. The correlation of CDX2 with FXR in normal esophageal mucosa, RE, BE, and EA. B. The correlation of CDX2 with TGR5 in normal esophageal mucosa, RE, BE, and EA.

tigated the association of CDX2, FXR, and TGR5 expression with the clinicopathologic characteristics of ESCC patients. The relative levels as well as the age, sex, tumor size, invasion depth and lymph node metastasis stage of these patients were analyzed. All patients were divided into two groups according to the staining score: 1-6 as the low expression group and 7-12 as the high expression group. As shown in **Table 3**, the expression of CDX2 in ESCC had no correlation with the age, tumor size, invasion depth, or lymph node metastasis stage of ESCC patients (P>0.05). However. CDX2 was shown to be closely correlated with patient sex (P< 0.05). Additionally, there was no correlation of FXR expression with the age, sex, tumor size, invasion depth, or lymph node metastasis stage of ESCC patients (P>0.05) (**Table 3**). High TGR5 expression was present in 86.7% of cases with larger tumor sizes (≥5 cm) but only in 44.4% of cases with smaller tumor sizes (<5 cm), indicating that the protein might be associated with tumor size in ESCC patients (P<0.05). However, none of the other clinicopathologic characteristics were significantly associated with TGR5 (P>0.05) (**Table 3**). Moreover, survival analysis showed that there was no correlation between the survival time of patients with esophageal cancer and the expression levels of CDX2, FXR, or TGR5 (P=0.5573, P=0.0598, P= 0.2875) (**Figure 5A**-**C**).

Discussion

Gastroesophageal reflux with Barrett esophagus (GERD with BE) is an important risk factor for adenocarcinoma (EA) [18-20]. Additionally, it has been reported that RE, BE, and dysplasia may progress to esophageal cancer [21]. However, the molecular mechanism underlying this progression is still not fully understood. According to a previous study, the expression of TGR5 in BE and EA was significantly increased [8]. Another study indicated that TGR5 expression was much higher in high-grade dysplasia and EA tissues than in BE tissues, but there was no statistically significant difference between low-grade dysplasia and BE mucosa [22]. In addition, as a nuclear receptor of bile acid, FXR can be used to distinguish whether BE has progressed to dysplasia or EA [23]. CDX2 is an intestinal-specific transcription factor, and its abnormal expression in gastric mucosal cells can promote the occurrence of IM [24, 25]. Moreover, our previous research

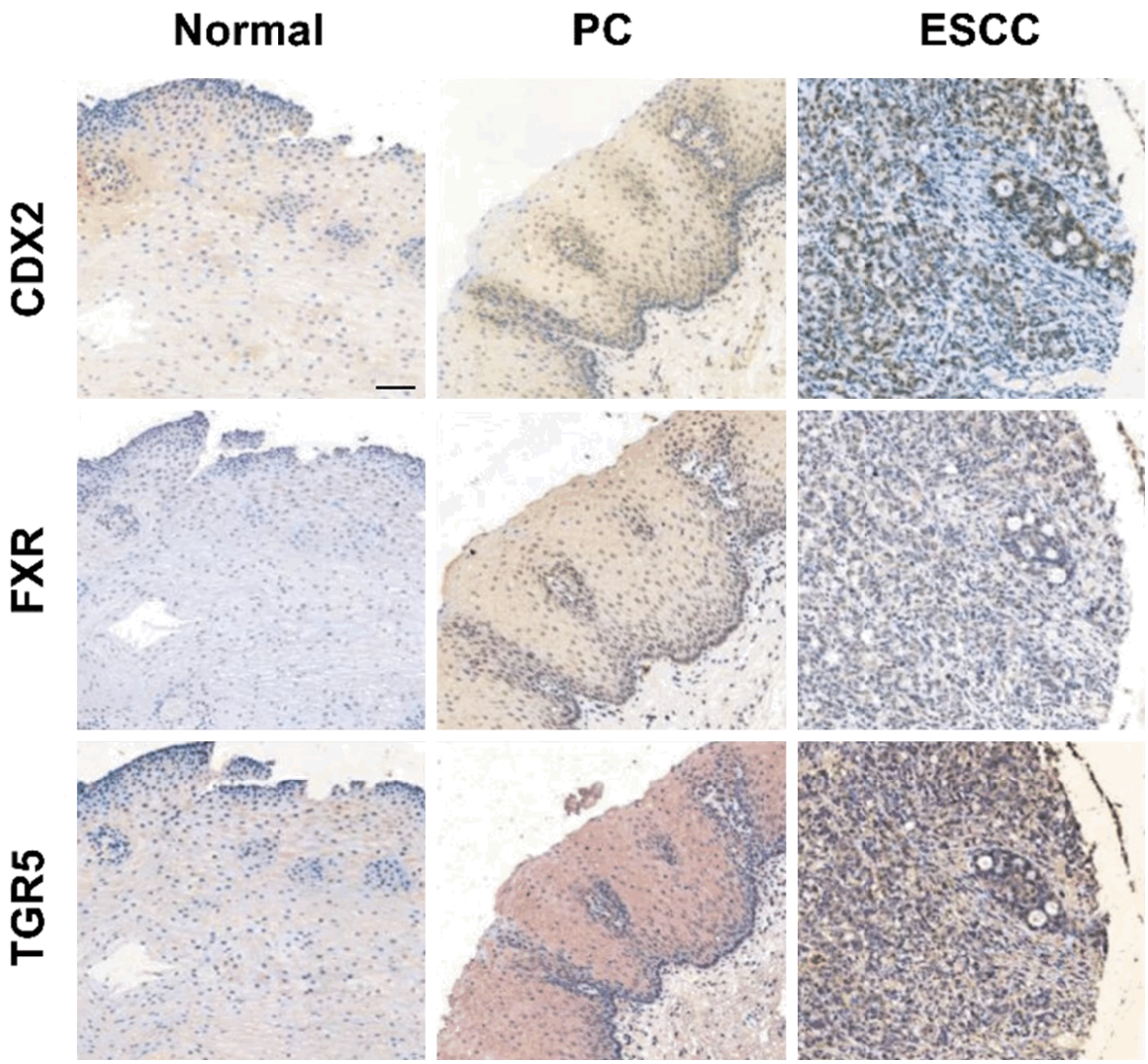

Figure 3. Expression level of CDX2, FXR and TGR5 in normal esophageal mucosa, PC, and ESCC tissues. PC: paracancer; ESCC: esophageal squamous cell carcinoma. Scale bars: 100 μm.

Table 2. CDX2, FXR and TGR5 expression in normal esophagus, PC, and ESCC

	CDX2 expression level					
	-	+	++	+++	H	*P*-values
Normal esophagus (%)	26/78 (33.3)	49/78 (62.8)	3/78 (3.8)	0/78 (0.0)	53.08	
PC (%)	2/73 (2.7)	48/73 (65.8)	19/73 (26.0)	4/73 (5.5)	134.71	$P^{\#}<0.01$
ESCC (%)	0/105 (0.0)	39/105 (37.1)	40/105 (38.1)	26/105 (24.8)	180.32	$P^{\#}<0.01$, $P^{*}<0.01$
	FXR expression level					
Normal esophagus (%)	57/77 (74.0)	20/77 (26.0)	0/77 (0.0)	0/77 (0.0)	45.16	
PC (%)	3/69 (4.3)	38/69 (55.1)	19/69 (27.5)	9/69 (13.1)	133.44	$P^{\#}<0.01$
ESCC (%)	0/105 (0.0)	22/105 (21.0)	33/105 (31.4)	50/105 (47.6)	180.39	$P^{\#}<0.01$, $P^{*}<0.01$
	TGR5 expression level					
Normal esophagus (%)	6/80 (7.5)	48/80 (60.0)	20/80 (25.0)	6/80 (7.5)	76.31	
PC (%)	1/69 (1.4)	36/69 (52.2)	21/69 (30.4)	11/69 (16.0)	112.19	$P^{\#}<0.01$
ESCC (%)	0/105 (0.0)	18/105 (17.1)	30/105 (28.6)	57/105 (54.3)	176.56	$P^{\#}<0.01$, $P^{*}<0.01$

$P^{\#}$: compared with normal esophagus; P^{*}: compared with PC.

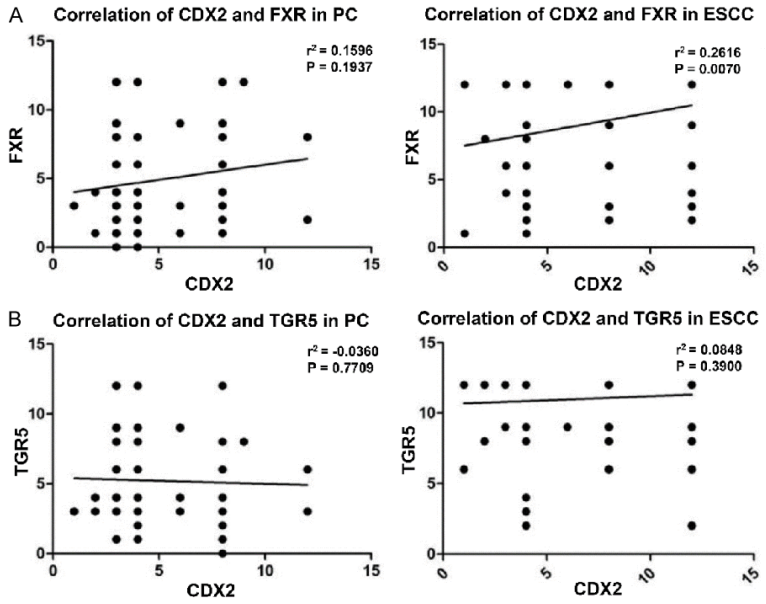

Figure 4. Correlation of CDX2 with FXR and TGR5 in PC and ESCC. A. The correlation of CDX2 with FXR in PC and ESCC. B. The correlation of CDX2 with TGR5 in PC and ESCC.

demonstrated that the enhancement of miR-92a-1-5p induced by bile acid could activate CDX2 through the FOXD1/NF-κB pathway, which further promotes the development of intestinal metaplasia in gastric cells [26]. In brief, the expression of these three critical genes might participate in the progression of intestinal phenotype conversion of bile acid reflux esophagitis.

In the present study, we examined the expression levels of CDX2, FXR, and TGR5 in the normal esophagus, RE, BE, EA, and ESCC using IHC in patients from different hospitals. Our results showed that CDX2 was higher in BA tissues than in normal esophageal and EA tissues, which was consistent with the results of a previous study showing that its expression was progressively upregulated in human gastric intestinal metaplasia, dysplasia, and cancer [27]. This result reminds us that the progression of EA and gastric cancer may share a similar molecular pathway, which might help us to further understand the regulatory mechanism of abnormal CDX2 expression in gastric intestinal metaplasia and BE. In addition, we found that FXR was mainly expressed in BE, which was also consistent with the results of published research that suggested that FXR was significantly overexpressed in BE compared with normal mucosa, esophagitis and EA [22]. In this study, the expression of TGR5 was significantly upregulated in BE, while most normal mucosa and EA tissues showed weaker staining of TGR5, which was inconsistent with other researchers' results [8]. This contradiction might be due to the different patients, tissue microarrays, and antibodies used in this study. Interestingly, our results indicated that the expression patterns of FXR and TGR5 in esophageal tissues coincided with CDX2 to some extent, which suggested that these three genes might be closely related to the progression of EA.

To investigate the correlation of CDX2, FXR, and TGR5 expression in esophageal neoplastic progression, we analyzed the correlation between every two molecules using Pearson correlation analysis in the normal esophagus, RE, BE, and EA. Our results suggested that the expression of CDX2 and FXR was positively correlated in EA. TGR5 expression was positively correlated with CDX2 in RE and BE.

Although many studies have demonstrated that bile acid reflux is a leading cause of EA, no relevant study has revealed the relationship between ESCC and bile acid reflux [28-30]. Moreover, the underlying mechanism of ESCC needs to be further investigated. In this study, we demonstrated that there were gradual increases in CDX2, FXR, and TGR5 levels in normal esophageal, PC and ESCC tissues. Additionally, the expressions of CDX2 and FXR were positively correlated in ESCC. These results indicated that CDX2, FXR, and TGR5 might all be involved in the progression of ESCC. However, survival analysis showed that there was no significant correlation between the survival time of patients and the expression levels of CDX2, FXR, and TGR5, which means that all three molecules might not be markers for the prognosis of ESCC patients.

In conclusion, our study indicated that the expression of CDX2, FXR, and TGR5 changed

Table 3. Association of CDX2, FXR, and TGR5 with clinicopathologic characteristics of ESCC

Clinicopathologic feature	No. of cases	%	CDX2		*P* value
			Low expression	High expression	
Age (years)					
≥60	51	69.9%	16	35	
<60	22	30.1%	6	16	0.7261
Gender					
Male	55	75.3%	20	35	
Female	18	24.7%	2	16	0.0427
Tumor size					
≥5 cm	29	50.9%	5	24	
<5 cm	28	49.1%	10	18	0.1133
Invasion depth					
T1, 2	14	20.0%	3	11	
T3, 4	56	80.0%	18	38	0.4339
Lymph node metastasis					
N0, 1	59	83.1%	19	40	
N2, 3	12	16.9%	3	9	0.6228
Clinicopathologic features	No. of cases	%	FXR		*P* value
			Low expression	High expression	
Age (years)					
≥60	50	69.4%	18	32	
<60	22	30.6%	6	16	0.5907
Gender					
Male	54	75.0 %	17	37	
Female	18	25.0%	7	11	0.5758
Tumor size					
≥5 cm	29	51.8%	8	21	
<5 cm	27	48.2%	6	21	0.7612
Invasion depth					
T1, 2	13	18.8%	3	10	
T3, 4	56	81.2%	20	36	0.5204
Lymph node metastasis					
N0, 1	58	82.9%	20	38	
N2, 3	12	17.1%	4	8	1.0000
Clinicopathologic feature	No. of cases	%	TGR5		*P* value
			Low expression	High expression	
Age (years)					
≥60	50	69.4%	16	34	
<60	22	30.6%	9	13	0.5920
Gender					
Male	54	75.0 %	19	35	
Female	18	25.0%	6	12	1.0000
Tumor size					
≥5 cm	29	51.8%	4	26	
<5 cm	27	48.2%	15	12	0.0016
Invasion depth					
T1, 2	13	18.8%	3	10	
T3, 4	56	81.2%	22	34	0.3488
Lymph node metastasis					
N0, 1	58	82.9%	20	38	
N2, 3	12	17.1%	5	7	0.7437

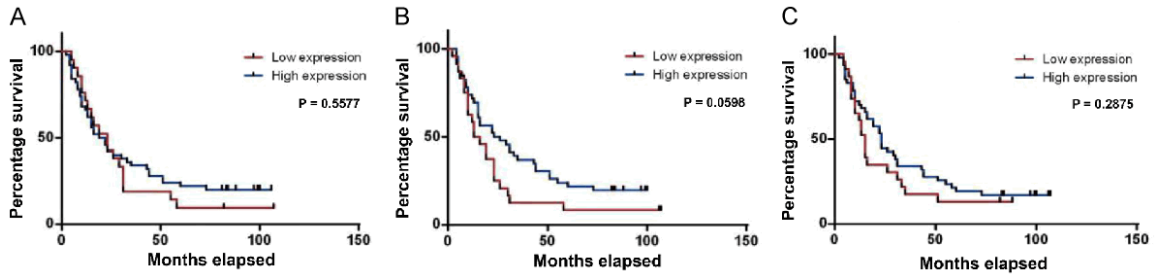

Figure 5. Correlation between the expression of CDX2, FXR, or TGR5 and survival time of patients with esophageal cancer. A. Survival curves of CDX2 high expression group and CDX2 low expression group. B. Survival curves of FXR high expression group and FXR low expression group. C. Survival curves of TGR5 high expression group and TGR5 low expression group.

markedly during the progression of EA. The expressions of CDX2 and FXR were positively correlated in EA. TGR5 expression was positively correlated with CDX2 in RE and BE. Moreover, the expressions of CDX2 and FXR were also positively correlated in ESCC. Although CDX2, FXR, and TGR5 were upregulated in ESCC, they might play different roles in EA and ESCC. Therefore, bile acid receptors FXR and TGR5 play an important role in mediating the transformation of esophageal epithelial cells to intestinal type cells, which further suggests that inhibition of gastroesophageal reflux may be the to preventing the occurrence of intestinal metaplasia and even esophageal cancer.

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Disclosure of conflict of interest

None.

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