

# Expression of methylation-modulated tumor-related genes in endoscopically resected early esophageal squamous neoplasia

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**Abstract.** Smoking and alcohol consumption are major risk factors for the development of esophageal squamous cell carcinoma (ESCC). Recent studies have demonstrated that smoking and alcohol consumption may be associated with altered DNA methylation in human cancer development. The aim of the present study was to evaluate methylation-modulated protein expression of tumor-related genes (TRGs) in the early stages of esophageal squamous neoplasia (ESN). ESN tissue samples (n=141) comprising 19 cases of low-grade intraepithelial neoplasia (LGIN), 70 of high-grade intraepithelial neoplasia/carcinoma *in situ* (HGIN/CIS) and 52 of invasive cancer, were endoscopically resected. The methylation-modulated protein expression of 5 TRGs [fragile histidine triad (*FHIT*), E-cadherin, MutL homolog 1 (*MLH1*)/MutS homolog 2 (*MSH2*) and cyclooxygenase-2 (*COX-2*)] as well as *p53* was examined with immunohistochemistry, and their expression was compared with patient clinicopathological characteristics. Reduced or loss of *FHIT*, E-cadherin, *MLH1/MSH2* and *COX-2* expression was detected in 26.3 (5/19), 5.3 (1/19), 0 (0/19) and 63.2% (12/19) of LGIN cases, 61.4 (43/70), 18.6 (13/70), 7.1 (5/70) and 65.7% (46/70) of HGIN/CIS cases, and 78.8 (41/52), 50.0 (26/52), 11.5 (6/52) and 59.6% (31/52) of invasive cancer cases, respectively. Reduced or absent expression of *FHIT* and E-cadherin was significantly associated with neoplastic progression (*FHIT*,  $P=0.0007$ ; E-cadherin,  $P=0.00014$ ). The mean number of TRGs (*FHIT*, E-cadherin, *MLH1/MSH2*, and *COX-2*) that exhibited reduced or absent expression in LGIN, HGIN/CIS and invasive cancer specimens was  $1.12\pm 0.61$ ,  $1.66\pm 0.93$  and  $2.09\pm 0.96$ , respectively, demonstrating a significant stepwise increment from LGIN to HGIN/CIS and then to invasive cancer ( $P<0.05$ ). *p53* overexpression was

frequently detected in ESN with head and neck carcinomas. However *p53* overexpression was not significantly associated with ESN progression. An increase in the number of the 5 TRG proteins with reduced or loss of expression in the early stages of esophageal tumorigenesis was demonstrated, and their decreased expression was observed to be associated with tumor progression. Therefore, smoking and alcohol drinking may be associated with not only carcinogenesis but also the progression of ESN.

## Introduction

In Japan, esophageal squamous cell carcinoma (ESCC) accounts for >90% of esophageal cancer cases (1). As ESCC is frequently detected at an advanced stage, it has one of the worst prognoses among digestive carcinomas (1). Therefore, early detection of malignant changes in the esophageal epithelium is the most effective way to improve the prognosis for ESCC. Detection at an early stage is gradually becoming more feasible due to advances in endoscopy, and the number of cases that can be potentially treated endoscopically is increasing (2).

A variety of genetic and epigenetic alterations are associated with esophageal carcinoma (3,4), including mutation of the *p53* gene (5,6). A number of previous studies have concluded that *p53* is the most frequently mutated gene in ESCC (7,8). The role of epigenetic changes, including aberrant DNA methylation, is particularly significant in human cancer development (9,10). An increasing number of tumor-related genes (TRGs) that are inactivated by the hypermethylation of CpG islands have been reported in esophageal cancer, including ESCC (11,12). However, the role of hypermethylation of CpG islands in early neoplastic lesions is not well understood.

In esophageal carcinomas, *FHIT*, E-cadherin and *MLH1/MSH2* have been previously demonstrated to be inactivated through hypermethylation of their promoters (13-17). These genes may be inactivated by a combination of genetic or epigenetic alterations of two alleles. However, epigenetic change is likely to be the predominant mechanism associated with the loss of *FHIT*, E-cadherin and *MLH1/MSH2* function in sporadic esophageal carcinomas (13-17).

Numerous epidemiological studies have identified tobacco smoking and alcohol consumption as the major risk factors for the development of ESCC (18,19). Increasing evidence

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indicates that smoking and alcohol induce epigenetic alterations, particularly aberrant patterns of DNA methylation. Such alterations to methylation are induced by tobacco and alcohol consumption in the development of ESCC (20,21), and may be important contributing factors in carcinogenesis (20,22).

In the present study, the clinical significance of TRG protein expression was evaluated during the early stages of esophageal carcinogenesis. Previous studies have demonstrated that the DNA methylation status of the TRGs *FHIT*, *E-cadherin*, *MLH1/MSH2* and *COX-2* can be detected by immunohistochemical analysis of tumor specimens (13-17,23). Therefore, the protein expression of these 5 TRGs and p53 was analyzed by immunostaining in 141 early esophageal neoplasia tumor specimens, comprising 19 low-grade intraepithelial neoplasia (LGIN), 70 high-grade intraepithelial neoplasia/carcinoma *in situ* (HGIN/CIS) and 52 invasive cancer tissue samples extracted during endoscopic resection.

### Patients and methods

**Patient and tissue samples.** A total of 141 esophageal squamous neoplasia (ESN) tumor specimens were obtained from 112 patients (including 102 males and 10 females) who underwent endoscopic resection at Tottori University Hospital (Yonago, Japan) between January 2004 and December 2014. The age of the patients ranged from 43 to 85 years (mean, 68.6 years). None of the patients had received preoperative radiotherapy or chemotherapy. Based on a histological examination, the 141 tumor specimens were classified as 19 cases of low-grade dysplasia, 70 of high-grade dysplasia and 52 of invasive cancer confined to the lamina propria mucosa or submucosa based on the World Health Organization classification (24) (Table I). Among the 112 patients with ESN, 98 (87.5%) had a history of smoking and 99 (88.4%) had a history of drinking. A total of 5 patients (4.4%) were neither smokers nor drinkers. All cases were analyzed anonymously, i.e., all specimens were assigned numbers without any associated personal information. Approval was obtained for the present study from the Institutional Ethics Review Board of Tottori University (grant nos. 314 and 1508A024; Yonago, Japan), which certified that the study complied with the Declaration of Helsinki and written informed consent was obtained from all patients.

**Immunohistochemical staining.** For immunohistochemical staining, paraffin-embedded, 4- $\mu$ m-thick tumor sections were first deparaffinized in xylene and rehydrated in ethanol. Subsequently, the sections were immersed in citrate buffer (0.01 M, pH 6.0) and heated in a microwave oven for 20-30 min for antigen retrieval. Endogenous peroxidase activity was blocked by incubation with 3% H<sub>2</sub>O<sub>2</sub> for 30 min at room temperature. The sections were subsequently incubated with a primary antibody overnight at 4°C. The primary antibodies used included rabbit polyclonal anti-FHIT (dilution, 1:100; clone F130; cat. no. 18163; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan), mouse monoclonal anti-p53 (dilution, 1:50; clone DO-7; cat. no. M7001; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), anti-E-cadherin (dilution, 1:50; clone HECD-1; catalog no. M106; Takara, Bio Inc., Otsu, Japan), anti-MSH2 (dilution, 1:100; clone FE11; cat. no. 33-7900; Oncogene

Table I. Clinicopathological characteristics and patient background of patients with early esophageal neoplasia.

Characteristic	n
Gender	
Male	102
Female	10
Age, mean $\pm$ SD, years	68.6 $\pm$ 8.7
Tumor size, mm	
<10	16
$\geq$ 10 to <20	51
$\geq$ 20	74
Tumor location	
Upper	62
Middle	64
Lower	15
Macroscopic tumor type	
Elevated	18
Flat	59
Depressed	64
Invasion depth	
Low-grade intraepithelial neoplasia	19
High-grade intraepithelial neoplasia/carcinoma <i>in situ</i>	70
Lamina propria invasion	27
Muscularis mucosa invasion	11
Submucosal invasion	14
Alcohol history	
Drinker	99
Non-drinker	13
Smoking history	
Current/previous smoker	98
Never smoked	14
Brinkman index <sup>a</sup> , mean $\pm$ SD	791 $\pm$ 689
History of head and neck cancer	9

A total of 141 samples were used from 112 patients. SD, standard deviation. <sup>a</sup>Brinkman index: number of cigarettes/day x years.

Research Products; EMD Millipore, Billerica, MA, USA), anti-MLH1 (dilution, 1:50; clone G168-15; cat. no. 550838; BD Pharmingen, San Diego, CA, USA) and anti-COX-2 (dilution, 1:100; clone 33/Cox-2; cat. no. 610204; BD Biosciences, Franklin Lakes, NJ, USA) antibodies. As a negative control, the primary antibody was replaced with normal rabbit IgG (cat. no. GTX35035; Gene Tex, Irvine, CA, USA) or normal mouse IgG (cat. no. NC494H; Biocare Medical, Pike Lane Concord, CA, USA) at a similar dilution. Antibody binding was detected by incubating with anti-rabbit (dilution, 1:200; cat. no. BA-1000; Vector Laboratories, Inc., Burlingame, CA, USA) or anti-mouse (dilution, 1:200; cat. no. BA-2000; Vector Laboratories, Inc., Burlingame, CA, USA) with avidin-biotin-peroxidase for 30 min at room temperature, and was then visualized using the chromogen diaminobenzidine

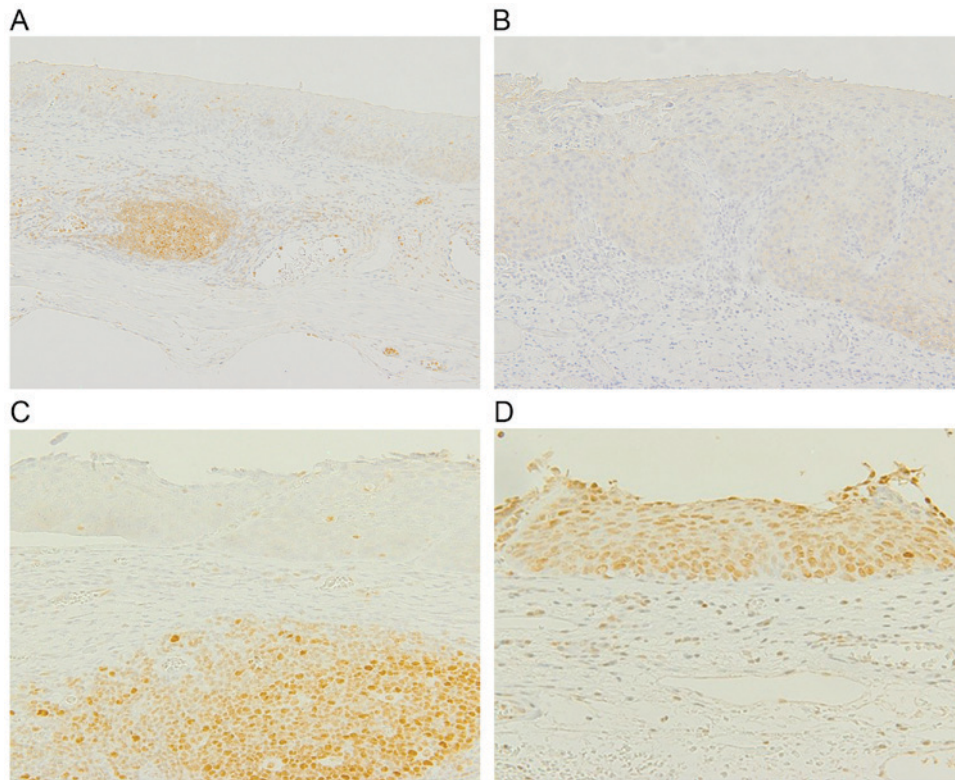


Figure 1. Representative immunostaining results of FHIT, E-cadherin, MLH1 and MSH2 in neoplastic tissue. Esophageal squamous neoplastic tissue obtained from endoscopic resection was stained with antibodies against FHIT, E-cadherin and MLH1/MSH2. (A) A HGIN/CIS lesion with reduced FHIT immunostaining, with positive staining of the adjacent germinal center and infiltrating lymphocytes. (B) A HGIN/CIS lesion with negative E-cadherin immunostaining. (C) A HGIN/CIS lesion with negative MLH1 immunostaining. (D) A HGIN/CIS lesion with positive MSH1 immunostaining. FHIT, fragile histidine triad; MLH1, MutL homolog 1; MSH1, MutS homolog 1; HGIN/CIS, high-grade intraepithelial neoplasia/carcinoma *in situ*.

tetrahydrochloride (cat. no. SK-4100; Vector Laboratories, Inc., Burlingame, CA, USA). The signal was detected as described in the Vectastain Elite avidin-biotin complex kit protocol (Vector Laboratories, Inc., Burlingame, CA, USA). Hematoxylin was used as a counterstain.

For each specimen, >5 fields were viewed under a light microscope (magnification,  $\times 100$ ) (Olympus Corporation, Tokyo, Japan). Protein expression was evaluated by two independent observers. p53 overexpression was defined as a distinct nuclear immunoreaction in >30% of the cells of the sample.

FHIT expression was graded, based on the intensity of cytoplasmic staining, as 'reduced', 'absent' or 'positive' as described previously (25). FHIT expression in normal esophageal squamous epithelia and normal esophageal glands served as positive controls. The adjacent normal squamous epithelium was used as an internal positive control. FHIT weak positive lesions showed positive reactivity at a level that was weak compared with the level in the normal epithelia, and these lesions were described as reduced.

E-cadherin, MLH1/MSH2 and COX-2 expression were classified as 'positive' or 'decreased'. Cases with staining (E-cadherin, membrane; MLH1/MSH2, nucleus; COX-2, cytoplasm) in <30% of the tumor cells or with a complete absence of staining were categorized as decreased, respectively.

**Statistical analysis.** Statistical analysis was performed using the  $\chi^2$  test with the Yates correction or the Newman-Keuls

test.  $P < 0.05$  was considered to indicate a statistically significant difference. All statistics were calculated using Stat Flex version 6.0 (Artech Co., Ltd, Osaka, Japan).

## Results

**Immunohistochemical analysis of the expression of TRGs (FHIT, E-cadherin, MLH1/MSH2 and COX-2) in ESN tumors.** Representative images of immunohistochemical staining of FHIT, E-cadherin and MLH1/MSH2 in ESN tumors is included in Fig. 1, and the results are quantified in Table II. There was a reduced or loss of FHIT, E-cadherin, MLH1/MSH2 and COX-2 expression in 26.3% (5/19), 5.3% (1/19), 0% (0/19) and 63.2% (12/19) of LGIN cases, in 61.4% (43/70), 18.6% (13/70), 7.1% (5/70) and 65.7% (46/70) of HGIN/CIS cases, and in 78.8% (41/52), 50.0% (26/52), 11.5% (6/52) and 59.6% (31/52) of invasive cancer cases, respectively. The incidence of absent or reduced FHIT or E-cadherin expression was significantly associated with neoplastic progression from LGIN to HGIN/CIS to invasive cancer (FHIT,  $P = 0.0007$ ; E-cadherin,  $P = 0.00014$ ). In the present study, absent or reduced protein expression of any of the 5 TRGs (FHIT, E-cadherin, MLH1, MSH2 and COX-2) in early ESN samples was not associated with the expression of the other TRGs, or with other clinical parameters, including size, age, gender and tumor location (data not shown).

There was an increase in the mean number of the TRG proteins with reduced or loss of expression with increasing

Table II. Analysis of FHIT, CDH1, MLH1/MSH2 and COX-2 protein expression in patients with early esophageal squamous neoplasia.

Reduced or loss of expression	LGIN, n (%)	HGIN/CIS	Invasive cancer	Total
Total samples, n	19	70	52	141
FHIT	5 (26.3) <sup>a</sup>	43 (61.4) <sup>a</sup>	41 (78.8) <sup>a</sup>	89 (63.1)
CDH1	1 (5.3) <sup>b</sup>	13 (18.6) <sup>b</sup>	26 (50.0) <sup>b</sup>	40 (28.4)
MLH1	0 (0)	5 (7.1)	5 (9.6)	10 (7.1)
MSH2	0 (0)	0 (0)	2 (3.8)	2 (1.4)
COX-2	12 (63.2)	46 (65.7)	31 (59.6)	89 (63.1)

<sup>a</sup>P=0.0007, <sup>b</sup>P=0.0001;  $\chi^2$  test with Yates' correction. A total of 1 sample with reduced and loss of MLH1 and MSH2 expression. CDH1, E-cadherin; COX-2, cyclooxygenase-2; FHIT, fragile histidine triad; MLH1, MutL homolog 1; MSH2, MutS homolog 2; LGIN, low-grade intraepithelial neoplasia; HGIN/CIS, high-grade intraepithelial neoplasia/carcinoma *in situ*.

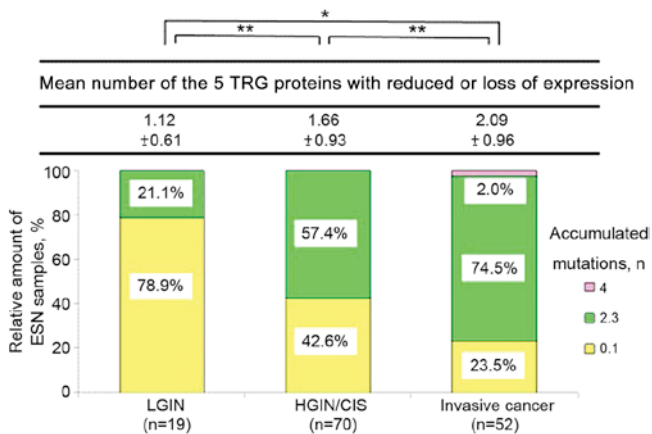


Figure 2. Number of TRGs (*FHIT*, *CDH1*, *MLH1/MSH2* and *COX-2*) with negative expression in early esophageal squamous neoplasia. The number of TRG proteins for which the expression was decreased or absent in tumors gradually increased in the progression from LGIN and HGIN/CIS to invasive cancer. \*P<0.01, \*\*P<0.05; Newman-Keuls test. Data are presented as the mean  $\pm$  standard deviation. CDH1, E-cadherin; COX-2, cyclooxygenase-2; TRG, tumor-related genes; FHIT, fragile histidine triad; MLH1, MutL homolog 1; MSH1, MutS homolog 1; LGIN, low-grade intraepithelial neoplasia; HGIN/CIS, high-grade intraepithelial neoplasia/carcinoma *in situ*.

tumor progression from LGIN (mean  $\pm$  standard deviation, 1.12 $\pm$ 0.61) to HGIN/CIS (1.66 $\pm$ 0.93) to invasive cancer (2.09 $\pm$ 0.96; Fig. 2). The presence of a trend was evaluated using the Newman-Keuls test on the three groups (LGIN, HGIN/CIS and invasive cancer), which identified a significant increase in the number of the TRG proteins with reduced or loss of expression from LGIN to invasive cancer (P<0.05).

**Immunohistochemical analysis of p53 in ESN tumors.** p53 overexpression was detected in 42.1% (8/19) of LGIN cases, in 57.1% (40/70) of HGIN/CIS cases, and in 55.8% (29/52) of invasive cancer cases (data not shown). p53 overexpression was not associated with ESN progression. In addition, p53 overexpression was not associated with absent or reduced protein expression of any of the 5 TRGs or with the number of TRGs expressed. The percentage of tumors with p53 overexpression was higher in ESN with head and neck carcinoma

(HNC; 8/9; 88.9%) compared with in ESN with other cancer (25/50; 50.0%; P=0.064).

## Discussion

The process of carcinogenesis and the early stage of tumor progression in ESCC are poorly understood. Frequent hypermethylation of CpG islands was previously observed in ESCC (3,4,26). Epigenetic changes, including hypermethylation of the CpG island associated with a TRG, may result in the transcriptional silencing of a gene, with the subsequent loss of protein expression that may contribute to tumorigenesis (27). However, the majority of previous studies on the methylation of TRGs limited to analysis in surgically resected ESCC samples and did not study the status of TRG genes in early neoplastic lesions (25,28). In the present study, IHC staining for 5 TRGs (*FHIT*, *E-cadherin*, *MLH1*, *MSH2*, and *COX-2*) was used to analyze methylation status, and it was identified that the expression of all 5 TRGs may be lost or reduced in cases of endoscopically resected early ESN. Additionally, ESN progression was indicated to be associated with the accumulation of methylation-modulated absence or reduced expression of the TRGs.

The expression of FHIT and E-cadherin appeared to be reduced or lost with the progression from LGIN to HGIN/CIS and then to invasive cancer. This finding is consistent with a previous study by the present authors (29) where the average number of the 5 TRG proteins with reduced or loss of expression in ESN showed a significant increase from LGIN to invasive cancer (P<0.05). Ishii *et al* (30) reported that the accumulation of the DNA methylation of TRGs is associated with tumor progression in ESCC. Therefore, a continuous reduction or loss in the number of the TRG proteins expressed during ESCC progression may be an important mechanism of pathogenesis, not only for initial tumor development but also for the progression of ESCC.

*MLH1* and *MSH2* are post-replication mismatch repair genes (31). *MLH1* and *MSH2* promoter hypermethylation has been reported in 3-62% (15,32) and 29-32% (16,17) of ESCCs, respectively. In a previous study (28), the loss of MLH1 or MSH2 expression was detected in 28.7% of primary ESCCs

that underwent radical esophagectomy and was significantly associated with increased malignancy as assessed by the increasing metastasis to lymph nodes, extent of invasion and decreasing level of tissue differentiation. There are only few studies in which abnormalities in the expression of *MLH1* and *MSH2* genes were investigated in the early stage of ESCC, including from endoscopically resected specimens. In the present study, lost or reduced *MLH1/MSH2* expression was detected in 0% (0/19) of LGIN cases, 7.1% (5/70) of HGIN/CIS cases, and 11.5% (6/52) of invasive cancer cases, indicating that loss of *MLH1/MSH2* gene expression is involved in the carcinogenesis of some cases of ESCC.

*COX-2* overexpression has been associated with a strong inflammatory response and gastrointestinal carcinoma development (33). However, past studies have identified a low expression of the *COX-2* gene in gastric and colorectal cancer cases, associated with gene promoter hypermethylation (34,35). Additionally, it has been reported that *COX-2* expression is regulated by promoter methylation in human esophageal cancer cell lines (36). However, in the present study, a low level of *COX-2* expression was observed in 63.2% (12/19) of LGIN cases, 65.7% (46/70) of HGIN/CIS cases, and 59.6% (31/52) of invasive cancer cases, therefore indicating no association between the loss of *COX-2* expression and tumor progression.

Tobacco smoking and alcohol consumption are two major risk factors for ESCC, and there is increasing evidence for the ability of tobacco smoke-associated carcinogens and polymorphisms of carcinogen-metabolizing genes to modulate DNA methylation in certain types of tobacco-associated cancer, including lung and upper aerodigestive tract cancer (37,38). Cigarette smoke was demonstrated to induce the promoter methylation of genes including *FHIT* and *COX-2* in ESCC (14,23,39). E-cadherin promoter methylation was previously observed to be associated with increasing number of pack-years in HNC (40). There is also evidence to support the association of alcohol use with aberrant DNA methylation patterns in several types of cancer (20). ESCC has been suggested to be associated with promoter methylation of particular genes, including *FHIT* and *MLH1* (39,41,42). In general, chronic inflammation induced by alcohol/tobacco use is considered an inducer of aberrant DNA methylation (43).

In the present study, expression of the *p53* gene was also analyzed. *p53* is a tumor-suppressor gene, and the *p53* protein is crucial in a number of processes, including growth suppression, apoptosis and DNA repair (44). Tobacco and alcohol consumption may cause *p53* mutation, one of the most frequent events in esophageal carcinogenesis (45). Point mutations in the *p53* gene typically occur at an early stage of ESCC and correlate with tumor progression (46), therefore suggesting an important role of this genetic alteration in ESCC development. However, in the present study as well as a previous study, altered *p53* expression was not significantly associated with the early stage of ESCC progression (47). From an epidemiological point of view, HNC and esophageal cancer are associated with the same carcinogens, including alcohol and tobacco (45,48). Alterations in the *p53* gene and its expression have been reported in a variety of types of epithelial tumor and premalignant lesion, including HNC and esophageal cancer (45,48). In the present study, the percentage

of tumors with *p53* overexpression was higher in ESNs with HNC compared with in ESN with other cancer.

In conclusion, methylation-modulated absent or reduced expression of 5 TRGs in the early stages of esophageal tumorigenesis was demonstrated. A loss or reduced protein expression of these TRGs was identified as associated with ESN progression. An improved understanding of the methylation modulation of TRGs will provide new insights into esophageal carcinogenesis, cancer treatment and reveal feasible targets for chemoprevention. Further investigations in this area are required to gain an improved insight into ESCC development and to support the development of novel therapeutic strategies.

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