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Original Article

Prognostic value of ISG15 mRNA level in drinkers with esophageal squamous cell cancers

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Abstract: ISG15, the protein encoded by interferon (IFN)-stimulated gene 15, was the first identified ubiquitin-like protein, which could be strongly upregulated by type I interferons as a primary response to diverse microbial and cellular stress stimuli. Although the biological activities of ISG15 have yet to be fully elucidated, it is frequently over-expressed in various cancers. As the role of ISG15 in esophageal squamous cell cancer (ESCC) has not been well reported, the current study aimed to elucidate the role of ISG15 in predicting outcomes of ESCC patients. Samples were collected from 153 ESCC patients, including 54 pairs of tumor tissues and non-tumor tissues. Compared with the paired non-tumor tissues, higher expression of ISG15 mRNA were detected in ESCC tissues. The cut-off value 1.28 determined by ROC curve analysis divided the ESCC patients into high and low ISG15 mRNA expression group. High-ISG15 mRNA expression appeared with more frequency in ever-drinkers (P = 0.018). Kaplan-Meier analysis indicated that Low-ISG15 mRNA expression group had a longer cancer-specific survival (CSS) compared with High-ISG15 mRNA expression group. Multivariate analysis revealed that ISG15 mRNA (P = 0.024; hazard ratio, 2.759, 95% CI, 1.841-4.134) as well as Pathological staging (P < 0.001; hazard ratio, 1.634, 95% CI, 1.065-2.505) were independent prognostic factors. Subgroup analysis revealed that the discernibility of ISG15 mRNA level on ESCC outcomes was only pronounced in ever-drinkers (P = 0.026) not in never-drinkers (P = 0.138). ISG15 might serve as a novel prognostic biomarker in drinkers with ESCC.

Keywords: ISG15, mRNA, esophageal squamous cell cancers, ever-drinkers, prognostic factor

Introduction

With an annual global incidence of more than 456,000 cases, esophageal cancer (EC) has been ranked as the eighth most common malignancy. The mortality can be as high as 4.9%, which means more than 400,000 people are dead of EC every year [1]. Esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) are the two major histologic types. Although EAC has emerged as the major type in some Western countries [2], the world-wide incidence of ESCC is still staying at a high level. ESCC remains the predominant type of EC in China [3]. Surgery is the most important treatment of ESCC provides the possibility of completely cure. After complete surgical removal of the tumor, the five-year survival rate exceeds 95% for stage 0 disease, and is 50-80% for stage I disease, 30-40% for stage IIA disease, 10-30% for stage IIB disease, and 10-15% for stage III disease [4]. However, it's quite difficult to make an early detection, which makes the clinical prognosis of ESCC patient with advanced stage remaining poor. Therefore, the identification of the reliable markers for evaluation of treatment efficiency and survival can be helpful to define patient groups that are in need for alternative, novel therapeutic approaches.

Well-conducted epidemiological studies had proved that alcohol consumption might be a main contributor for ESCC [5-7]. Drinking can cause chronic irritation and inflammation of the esophageal mucosa, and then a series of molecular changes, which could increase the incidence of ESCC [8, 9]. A recent study had revealed that alcohol drinking was associated with decreased survival in ESCC patients [10].

However, the specific molecular targets affected by alcohol-derived carcinogens have not been thoroughly identified.

ISG15, the protein encoded by interferon (IFN)-stimulated gene 15, is an ubiquitin-like protein (Ubl) that is conjugated to intracellular target proteins upon activation by interferon-alpha and interferon-beta. Several functions have been ascribed to ISG15, including chemotactic activity towards neutrophils, direction of ligated target proteins to intermediate filaments, cell-to-cell signaling, and antiviral activity during viral infections [11-13]. As a member of Ubl family, ISG15 shows a significant sequence homology to ubiquitin. It is covalently conjugated with cellular proteins in an enzymatic pathway, which is called ISGylation. This pathway comprises of the activating E1, conjugating E2 and ligating E3 enzymes. ISGylation provides a tag that either marks the labeled protein for degradation or modulates its function [11, 13].

ISG15 can be strongly induced by type I IFNs [14, 15]. While the activation of type I IFN signaling pathways is one of the key components of the innate immune response in regulating cancer development [16, 17]. ISG15 is commonly recognized as a tumor suppressor, however, the perturbation of ISG15 regulation is correlated with enhanced tumor progression [13]. Mounting studies had indicated a possible link between ISG15 and tumorigenesis in human tumors and cell culture models. High levels of ISG15 and its conjugates were detected in many types of primary tumors, such as prostate cancer, bladder cancer and breast cancer [18-20]. However, the role of ISG15 in ESCC development has not been studied.

In this study, we measured the expression level of ISG15 mRNA in paired tumorous/non-tumorous tissues obtained from ESCC patients, and evaluated its prognostic significance in ESCC.

Materials and methods

Study population

The institutional review board approval was obtained (Sun Yat-Sen University Cancer Center Institutional Review Board, Guangzhou, Guangdong, China) before subject enrollment, and all subjects provided written informed consent. All patients who underwent esophagectomy between March 2002 and October 2008 were enrolled in this study. We excluded cases with (1) history of other cancers, (2) neoadjuvant chemotherapy or radiotherapy, (3) perioperative death, (4) cervical lymph nodes metastases and (5) incomplete surgical resection. Drinking history of patients was obtained from chart review. Patients were divided to ever-drinkers (who ever drunk) and never-drinkers (who never drunk). Histological differentiation (G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated), tumor invasion (pathological T category) and nodal status (pathological N category) were determined by pathologic examination. The TNM staging criteria was in accordance with the seventh edition of American Joint Committee on Cancer Staging Manual [21].

Quantitative real-time polymerase chain reaction (PCR) assays

Tumor tissues were taken from the potential curative tumor regions, and non-tumor tissues were procured at least 2 cm distant from tumor regions. All the specimens were confirmed to be ECSS tissues and normal esophageal tissues by pathological examination. After resection, tissues were immediately preserved in liquid nitrogen and then frozen at -80°C. The RNA of tumor and non-tumor tissues was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Each cDNA was synthesized from 1 mg of total RNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA), and stored at -80°C. We used Glyceraldehyde-3-phosphate dehy-drogenase (GAPDH 'housekeeping' gene) as an internal standard to control variability in amplification. The primers used were as follows: ISG15, forward primer 5'-GGACAAATGCGACGAACCTCT-3' and reverse primer 5'-GCCCGCTCACTTGCTG-CTT-3' [NCBI: NM_005101.3]. GAPDH, forward primer 5'-ACTTCAACAGCGACACCCACTC-3' and reverse primer 5'-TACCAGGAAATGAGCTTGAC-AAAG-3' [NCBI: NM_001256799.1]. Kyse410 ESCC cell cline was used as an internal control (calibrator) to adjust the variations of ISG15 mRNA expression in different real-time PCR assays. Gene analysis was carried out by PCR using following PCR mixture: 0.12 mL of cDNA, 5 mL of 2 × Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 0.25 mL of 20 mmol forward primer, 0.25 mL of reverse primer and 4.38 mL of distilled water. Real-time

Table 1. Clinicopathologic characteristics of the all patients enrolled (n = 153)

Characteristics	Number of patients or mean ± SD (median)	Percent
Age (years)	58.4 ± 9.2 (59.0)	
Sex		
Men	114	74.5
Women	39	25.5
Smoking status		
Yes	97	63.4
No	56	36.6
Drinking status		
Yes	59	38.6
No	94	61.4
Tumor location		
Upper	30	19.6
Middle	88	57.5
Lower	35	22.9
Histological differentiation		
Grade 1	36	23.5
Grade 2	76	49.7
Grade 3	41	26.8
pT category		
T1b	5	3.3
T2	30	19.6
T3	112	73.2
T4a	6	3.9
pN category		
N0	80	52.3
N1	40	26.1
N2	25	16.3
N3	8	5.2
Pathological staging		
I	8	5.2
II	77	50.3
III	68	44.4

pN, pathological N; pT, pathological T; SD, standard deviation.

PCR was done in Light Cycler 480 (Roche Applied Science, Penzberg, Germany). Thermal cycling profile was carried out as following: 95°C for 10 min, then 40 cycles of amplification (95°C for 10 s, 60°C for 20 s and 72°C for 30 s) and followed by 40°C for 30 s. Assays were performed in triplicates, and retested was performed if any sample with a coefficient of variance greater than 10%. ISG15 mRNA expression level in tumor and non-tumor tissues were calculated as $2^{-\Delta\Delta Ct\,(sample)}$. ΔΔCt (sample) = ΔCt (calibrator)-ΔCt (sample), and ΔCt (calibrator) of ISG15 mRNA = Ct (calibrator) of ISG15-Ct (calibrator) of GAPDH; ΔCt (sample) = Ct (sample) of ISG15-Ct (sample) of GAPDH. The difference of Ct value was equal to 2^n-fold difference.

Statistical analysis

All statistical analyses were performed by SPSS version 16.0 (SPSS Inc., Chicago IL, USA). Chi-square test was employed to compare the Categorical data, while paired two-tailed t-test to compare the differences between paired tumorous/non-tumorous tissues. Receiver operating characteristic (ROC) curve was generated to select the optimal cut-off value, as well as to analyze the highest combined sensitivity and specificity with respect to predict cancer-specific survival (CSS) of ESCC patients after esophagectomy. CSS was calculated from the time of surgery to either the time of death from ESCC or last follow up (December 31, 2013). Kaplan-Meier analyses along with log-rank test were performed to test the prognostic value of ISG15 mRNA as a predictor for surgical outcomes. Univariate and multivariate Cox proportional-hazards models were used to evaluate various parameters associated with hazard ratio (HR). Multivariate analysis included potential prognostic factors with a *P*-value less than 0.10 and constructed with the forward stepwise method. *P*-value less than 0.05 was considered as a statistical significance difference.

Results

Clinical and pathological data

A total of 153 patients were enrolled, including 54 patients with paired tumorous and non-tumorous tissues. There were 114 male and 39 female patients, with a mean age of 58.4 years. The baseline clinicopathologic characteristics of the study population are presented in **Table 1**. The median follow-up of the whole patients was 50.0 months (range: 4-109 months). Eighty-five patients died at the end of follow-up, including 3 patients who died from non-cancer diseases, such as cerebral infarction (n = 1), respiratory failure (n = 1) and traffic accident (n = 1).

ISG15 mRNA expressions in tumorous/non-tumorous tissues

Compared with non-tumorous tissues, ISG15 was significantly upregulated in tumorous tis-

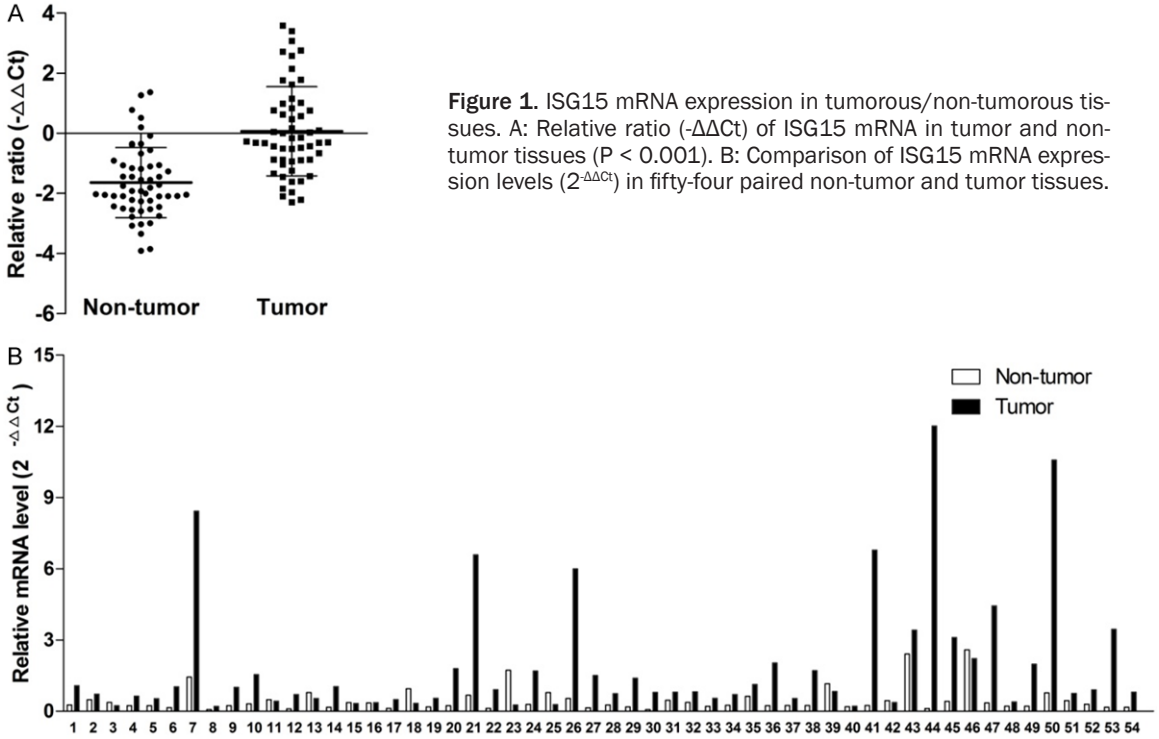

Figure 1. ISG15 mRNA expression in tumorous/non-tumorous tissues. A: Relative ratio (-ΔΔCt) of ISG15 mRNA in tumor and non-tumor tissues ($P < 0.001$). B: Comparison of ISG15 mRNA expression levels ($2^{-\Delta\Delta Ct}$) in fifty-four paired non-tumor and tumor tissues.

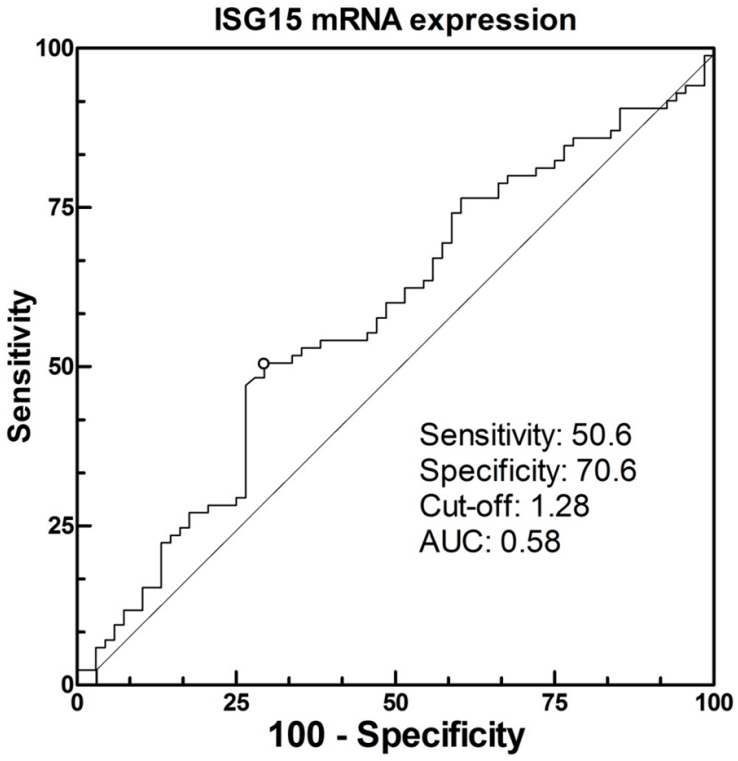

Figure 2. ROC curve using ISG15 mRNA expression level. The optimal cut-off value was 1.28 with a sensitivity of 50.6% and a specificity of 70.6%. AUC, Area under the ROC curve.

sues ($P < 0.001$, **Figure 1A**). The expression of ISG15 mRNA was up-regulated in 36/54 (66.7%) tumorous tissues (defined as a 2-fold higher of ISG15 expression in non-tumor counterparts), and the hightest tumor/non-tumorou (T/N) ratio was up to 101.1-fold (**Figure 1B**). In these 36 patients with ISG15 mRNA up-regulated, 17 patients (47.2%) were ever-drinkers, whereas there were only 7 ever-drinkers (38.9%) for the other 18 patients. Although more ever-drinkers were found in the former group, the difference of the drinking status in these two groups was not significant ($P = 0.387$).

Association of ISG15 mRNA expressions with clinicopathologic features in ESCC patients

The cut-off value determined by ROC curve analysis (**Figure 2**) was 1.28, which provided the threshold value for ISG15 mRNA expression to distinguish cancer-specific survivors from nonsurvivors with the highest combined sensitivity and specificity. According to this threshold, 63 ESCC patients (41.2%) with

Table 2. Correlations between ISG15 expression and clinicopathologic features

Characteristic	Number of patients	ISG15 expression		*P*-value*
		Low level (%)	High level (%)	
Age				
≤ 59 years	86	52 (60.5)	34 (39.5)	0.381
> 59 years	67	38 (56.7)	29 (43.3)	
Sex				
Men	114	64 (56.1)	50 (43.9)	0.168
Women	39	26 (66.7)	13 (33.3)	
Smoking status				
Yes	97	55 (56.7)	42 (43.3)	0.298
No	56	35 (62.5)	21 (37.5)	
Drinking status				
Yes	59	28 (47.5)	31 (52.5)	0.018
No	94	62 (66.0)	32 (34.0)	
Histological differentiation				
Grade 1	36	23 (63.9)	13 (36.1)	0.306
Grade 2/3	117	67 (57.3)	50 (42.7)	
pT category				
T1b/T2	35	22 (62.9)	13 (37.1)	0.363
T3/T4a	118	68 (57.6)	50 (42.4)	
pN category				
N0	80	51 (63.7)	29 (36.3)	0.129
N1/N2/N3	73	39 (53.4)	34 (46.6)	
Pathological staging				
I/II	85	53 (62.4)	32 (37.6)	0.204
III	68	37 (54.4)	31 (45.6)	

*Chi-square test.

ISG15 mRNA expression higher than 1.28 were defined as high-level expression group, whlie the other 90 ESCC patients (58.8%) were defined as low-level expression group. Chi-square test was used to analysis the association between expression pattern of ISG15 mRNA and clinicopathologic features in ESCC patients. We only found that high expression of ISG15 mRNA was significantly correlated with drinking status (P = 0.018), while no significant association was detected between ISG15 expression and age, sex, smoking status, histological differentiation, pT category, pN category and pathological staging (**Table 2**).

Association of ISG15 mRNA expressions with survival in ESCC patients

According to the Kaplan-Meier analysis, high-level expression of ISG15 mRNA was significantly associated with poorer CSS of ESCC patients (log-rank = 7.271, *P* = 0.007). The 5-year CSS was 54.4% in the low-level expression group, but only 34.9% in high-level expression group (**Figure 3A**). Pathological T category, pathological N category and pathological staging were also detected as significant prognostic factors of CSS by univariate survival analyses. When it came to the multivariate survival analysis, only pathological staging and ISG15 mRNA were found to be independent prognostic factors in ESCC patients (**Table 3**).

When subclassified by drinking status, the difference was significant in ever-drinkers (Log Rank = 4.960, P = 0.026, **Figure 3C**) but not in never-drinkers (Log Rank = 2.197, P = 0.138, **Figure 3B**). Moreover, multivariate survival analysis also revealed ISG15 mRNA as an independent prognostic factors of CSS in ever-drinkers (HR: 2.212, 95% CI: 1.102-4.441, P = 0.026, **Table 3**).

Discussion

In our study, we found that higher expression of ISG15 mRNA was detected in ESCC tissues compared with the paired non-tumor tissues. In addition, both univariate and multivariate analysis revealed ISG15 mRNA expression along with pathological staging are associated with shorter CSS, suggesting that ISG15 is a potential prognostic marker for worse CSS in ESCC patients. To our knowledge, this is the first study to investigate the role of ISG15 in ESCC development.

ISG15 is the first identified ubiquitin-like protein [15]. The expression of ISG15 is mainly induced by type I IFNs or other stimuli, such as exposure to viruses and lipopolysaccharide. ISG15 is traditionally considered as a cytokine modulating immune responses and participates in regulating signal transduction pathways, ubiquitination, antiviral responses [11]. As increased ISG15 had been observed in multiple human cancers, more and more studies

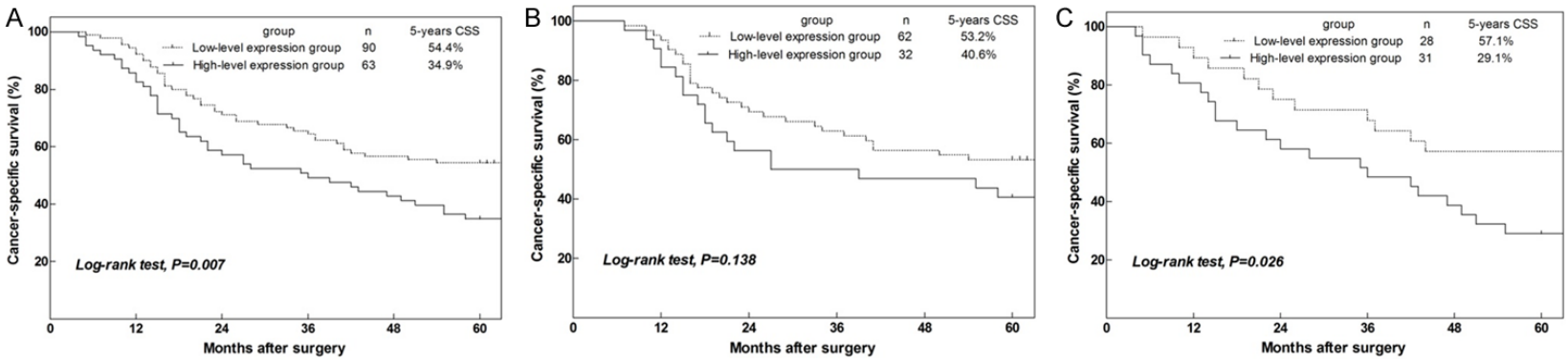

Figure 3. Kaplan-Meier survival analysis in ESCC patients. A: Cancer-specific survival (CSS) curve for whole cohort of patients according to ISG15 mRNA expression (log-rank = 7.271, P = 0.007). B: CSS curve for never-drinkers according to ISG15 mRNA expression (Log Rank = 2.197, P = 0.138). C: CSS curve for ever-drinkers according to ISG15 mRNA expression (Log Rank = 4.960, P = 0.026).

Table 3. Univariate and multivariate analysis of prognostic variables for cancer-specific survival

	Entire				Drinkers			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Characteristics	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (≤ 59/> 59 years)	1.069 (0.697-1.639)	0.760			0.906 (0.453-1.813)	0.781		
Sex (men/women)	0.91 (0.556-1.491)	0.709			2.532 (0.339-18.926)	0.365		
Smoking status (No/Yes)	1.228 (0.781-1.931)	0.374			1.258 (0.301-5.255)	0.753		
Drinking status (No/Yes)	1.220 (0.793-1.876)	0.366			-	-		
Histological differentiation (grade 1/2/3)	1.334 (0.980-1.816)	0.067			1.391 (0.877-2.205)	0.161		
pT category (T1b/T2/T3/T4a)	1.626 (1.046-2.529)	0.031			1.587 (0.692-3.636)	0.275		
pN category (N0/N1/N2/N3)	1.725 (1.405-2.119)	0.000			1.706 (1.261-2.309)	0.001		
Pathological staging (I/II/III)	2.823 (1.889-4.219)	0.000	1.634 (1.065-2.505)	0.000	3.086 (1.633-5.831)	0.001	3.267 (1.696-6.292)	0.000
ISG15 mRNA (low/high expression)	1.777 (1.160-2.721)	0.008	2.759 (1.841-4.134)	0.024	2.134 (1.073-4.242)	0.031	2.212 (1.102-4.441)	0.026

Cox's proportional hazards regression analysis (forward stepwise). CI, confidence interval; HR, hazard risk; ISG15 Interferon-stimulated gene 15; pN, pathological N; pT, pathological T.

focuses on the role of ISG15 playing in tumorigenesis. ISG15 was commonly recognized as a tumor suppressor, as it participated in host defense and stress response pathways [13]. Multiple human cancers were found to be associated with the dysregulated expression and chromosomal alterations of ISG15 pathway genes [22, 23]. However, recent studies had revealed dysregulated overexpression of ISG15 was positively correlated with some malignancies. ISG15 may act as a component of host tumor immunity, which could activate natural killer (NK) cells proliferation, or induce IFNγ and then contribute to a proinflammatory response, thus the killing of both tumor and basement membrane cells could be enhanced, facilitating invasive growth and tumor progression [13]. Increased expression of ISG15 was found in prostate cancer (PC), and knockdown of ISG15 expression resulted in marked reduction of PC cell numbers [18]. This implyed ISG15 is a promoter of PC. This expression pattern of ISG15 was also observed in bladder cancer, breast cancer and hepatocellular cancer [19, 20, 24]. Consistent with previous studies, ISG15 was over expressed in ESCC in our study.

Interestingly, in our study, we found that the expression of ISG15 mRNA in ESCC was associated with drinking status. In subgroup analysis, we divided patients into ever-drinkers and never-drinkers. Both univariate and multivariate analysis demonstrated ISG15 expression is the independent prognostic marker for worse CSS only in ever-drinkers, but not in never-drinkers. Alcohol is a well known cancer-causing agent, which had been listed as group carcinogens by the International Agency for Research on Cancer (IARC). The relationship between drinking and ESCC had been confirmed by countless well-conducted studies. More important than alcohol itself, acetaldehyde, the first metabolite of ethanol oxidation, plays a central role in tumorigenesis [25-27]. As a tumor-suppressor gene, the primary function of p53 is to maintain human genetic stability and DNA repair capacity [28]. Mutation of p53 can be induced by acetaldehyde, which can result in a high accumulation of p53 protein [29, 30]. While, as a target gene of p53, the level of ISG15 mRNA can be increased by p53 [31, 32]. This might explain the result we observed that the ESCC patients were associated with higher ISG15 expression, especially for ever-drinkers. These findings might have clinical importance in identifying ESCC patients at high risk with cancer-specific mortality. Adjuvant treatment might be helpful as to the drinkers with high level of ISG15 mRNA expression. It is noteworthy for us to carry out cytological and zoological experiments in our further study to clarify the potential mechanism of ISG15 in tumorigenesis and metastasis in ECSS.

One limitation is that we only divided the patients into ever-drinkers and never-drinkers, Sample size limited us to further divide ever-drinkers into subgroup according to amount of alcohol consumption. As increasing amount of alcohol consumption can be associated with increased risk of cancers and poor CCS [10, 33], we will make a further study with enlarged sample in future.

In conclusion, the expression of ISG15 is an independent prognostic factor in ESCC. High expression of ISG15 mRNA in ESCC patient may indicate a poor outcome, especially in patients who ever drunk. This study implied a possibility that there may be an association between ISG15 mRNA expression and drinking status in ECSS patients. It is necessary for exploring the pathogenesis of tumorigenesis and progression in ECSS patients who ever drunk in future study, which could further found an individual treatment or prevention to these specific groups of patients.

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Disclosure of conflict of interest

None.

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