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E-cadherin Promoter Methylation Comparison and Correlation with the Pathological Features of the Squamous Cell Carcinoma of Esophagus in the High Risk Region

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Abstract

E-cadherin is among tumor suppressor genes which mostly subjects to the down-regulation in squamous cell carcinoma of esophagus (SCCE). The gene is tightly associated with the tumor invasion and metastasis in multiple human cancers, especially SCCE. CpG islands' methylation in the promoter region of *E-cadherin* is among the mechanisms that have been suggested for the *E-cadherin* silencing, however, little is known regarding (SCCE) in the high risk region of the world. To establish a correlation between *E-Cadherin* promoter methylation with the pathological features of SCCE, as well as history, and demographic features we undertook the present investigation. Following to the surgical resection tissue samples of the 44 patients with SCCE was used for *E-cadherin* promoter methylation examination. Analysis was done using methylation specific polymerase chain reaction (MS-PCR). Furthermore, for evaluating *E-daherin* expression levels, reverse transcription PCR (RT-PCR) was applied. Results have indicated that twenty four out of the forty four tumor DNA samples (54.5%) were aberrantly methylated. In contrast, all normal tissues were found to be unmethylated. In addition a significant association was found between methylation status of *E-Cadherin* promoter with type I and II of the tumor differentiation ($p = 0.024$), stages T2 and T3 of tumors ($p = 0.026$), as well as lymph node invasion ($p = 0.004$). *E-cadherin* tumor suppressor gene subjects to epigenetic silencing through aberrant promoter CpG islands methylation; a mechanism which is most commonly contributed with the other important tumor suppressor genes in the SCCE carcinogenesis in the world's highest risk region for SCCE.

Keywords: *E-Cadherin*, DNA methylation**,** SCCE (Squamous Cell Carcinoma of the Esophagus).

Introduction

The locus q22 on chromosome 16 encodes *E-cadherin*; a transmembrane glycoprotein that mediates inter-cellular epithelial cells' adhesion as well as signaling in conjunction with the cytoplasmic catenin [1]. *E-cadherin* mediates homophilic, Ca^{2+}-dependent intercellular adhesion in the epithelial tissues. The proper action and expression of this protein is essential

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for the maintenance of the normal tissue architecture [2]. E-cadherin is highly expressed in the normal epithelial tissues. Interestingly the expression of E-cadherin could be seen in the undifferentiated embryonic stem cells as well as the well-differentiated cancer cells, but, a large reduction of its expression could be observed in the undifferentiated cancer cells [3], which may indicate an important role for this protein in the differentiation.

Loss of *E-cadherin* expression either as a result of genetic or epigenetic alterations correlates with the invasion and metastasis in a variety of human tumors [4]. While loss of expression and hypermethylation of *E-cadherin* could frequently be observed in most tumors, mutations are less common and were observed in a small number of specific tumor subtypes [4]. While downregulation of *E-cadherin* is a common event in the SCCE and associates with the increased invasive and metastatic potential, however, mutations in the *E-cadherin* have rarely been reported [5] which may indicate the role of other genetic alterations in the *E-cadherin* suppression.

Esophageal cancer ranks among the top 10 most frequent cancers which is characterized by poor prognosis and the 5-years survival rate less than 10%. Despite many efforts, the mechanism underlying development of esophageal cancer is not well understood [6]. Iran is located in the so-called Asian esophageal cancer belt where reports indicate the highest incidence for the squamous cell carcinoma of esophagus (SCCE) from certain regions of this country. There were many efforts in the recent years to unravel the molecular etiology of SCCE in this region of the world which have also resulted in the achievement of suitable molecular markers [7-9]. However, further efforts are required for achieving a deeper insight and a clearer picture regarding the molecular etiology of SCCE in this region of the world.

Late diagnosis is a major problem associated with SCCE. The disease could be diagnosed most often in the advanced stages of SCCE. In addition, the high invasive and metastatic potential of SCCE results in the low curative resection and a high frequency of relapse. To develop effective strategies for prediction, diagnosis, treatment, and follow-up of SCCE, the availability of appropriate molecular markers is of the prime importance. While recent reports from Iran [7, 8, 10] indicate attempts for obtaining suitable markers for the early diagnosis of SCCE, nevertheless, additional efforts are required. Herein, we report the aberrant methylation of *E-cadherin* promoter in SCCE patients. We correlate association of this molecular marker with the pathological features of the SCCE, as well as history, and demographic data such as grade of tumor differentiation, stages of tumors, in addition to lymph node invasion with the methylation status of the *E-Cadherin* promoter.

Materials and Methods

Patients

Forty-four SCCE patients whom were diagnosed for SCCE during 2006–2008 and underwent surgery were included in the present study. Among these patients, 29 were male (72%) and 15 were female (28%) with the age range 41–87 years (mean age ±SD: 60.8± 13.9 years). Patients were admitted in the two hospitals in Tehran: the Madaen Hospital and the Cancer Institute of Iran, Imam Khomeini Hospital Complex. None of patients had received irradiation or chemotherapy pre-operation. Tumor stage was assessed according to a modified TNM system [11, 12] and histology reports were obtained from pathology department. Questionnaires were prepared with regard to patients' history, demographic status, as well as other information by the trained interviewers. The study was approved by the ethical committee of the Imam Khomeini hospital and Tumor Bank [10, 13]; one of the two hospitals referred as source of tissue acquisition. The histopathological features of the patients' tumors are presented in table 2. Over half of the patients' tumors (26 out of 44) or 59% were in the stage II of SCCE. Moreover, the histology reports showed the following percentages regarding the frequency for T3, N1 and M1 according to TNM system: 63%, 47.7%, and 9% respectively.

Sample collection, DNA extraction, and bisulfite treatment

Surgically respected tissue samples: 44 tumor and 19 non-tumor adjacent normal tissues were collected in the liquid nitrogen, transported to the lab, and stored at -70°C until DNA extraction [10]. Genomic DNA was isolated from tissues using phenol/chloroform method and treated with sodium metabisulfate using EZDNA Methylation-GoldTM Kit (ZymoResearch Co., CA, USA) according to the manufacturer instruction.

Methylation specific polymerase chain reaction (PCR)

Methylation specific PCR (MSP) was carried out using specific primers for TPEF (transmembrane protein containing epidermal growth factor and follistatin domains), as well as methylated and unmethylated *E-Cadherin* promoter (Table 1). All amplifications were carried out in 12.5 µL reaction volume containing 1 mM dNTPs "(Fermentase Co., Burlington, Ontario,

Table 1. Primers and the target genes used in the present study

Oligo name	Primer Sequences 5'--> 3'	Product Length (bp)	Ref.
TPEF	TTAGTGGATGATTTTTTTGTTTTG (sense) AACAACAACAATAACAATAA (antisense)	120	[10]
Ecad _M*	TTAGGTTAGAGGGTTATCGCGT (sense) TAACTAAAAATTCACCTACCGAC (antisense)	112	[35, 36]
Ecad_U*	TAATTTTAGGTTAGAGGGTTATTG (sense) CACAACCAATCAACAACACA (antisense)	120	[35, 36]
β-actin	TCACCAACTGGGACGACATG (sense) ACCGGAGTCCATCACGATG (antisense)	242	[35]
Ecad_RT	CCTTCCTCCCAATACATCTCCC (sense) TCTCCGCTCCTTCTTCATC (antisense)	432	[5]

*M: Methylated-specific primers; *U: Unmethylated-specific primers

Canada)", 0.2 µM of each specific oligonucleotide primers, 100 ng modified DNA, and 1 U/µL Taq polymerase "(Hot start,Qiagen,Valencia, CA, USA)". After 15 min initial denaturation at 95°C, the first amplification was carried out for 35 cycles of 95°C denaturation and annealing at 53°C and 50°C for TPEF, Meth and UnMeth *E-cadherin* primers, respectively. Elongation was carried out at 72 °C for 30 s, followed by the final extension at 72 °C for 10 min in a PeQLab, 96 universal gradient, UK thermal cycler. PCR products were run in 1.5% agarose gel Fisher "(Manufacturer**;** Fisher Scientific, Brand**;** Fisher BioReagents,UK)". DNA from the peripheral blood lymphocytes of healthy individuals was treated with SssI methyltransferase "(New England Biolabs,Inc., Beverly,MA,USA)", subjected to Bisulfite modification, and was used as positive control. Negative controls (unmethylated normal DNA and no DNA) were also included in each PCR reaction set.

Total RNA Extraction and reverse transcription-PCR analysis

Total RNA was isolated from fresh frozen tissue samples using the QIAamp DNA Mini Kit and RNeasy Mini Kit (QIAGEN,Hilden,Germany) according to the manufacturer's protocol and 2µg of which was reverse transcribed for 1 h at 37°C in 20 µl using 4 µl reverse transcriptase buffer, 1 µl of 100 pmol dT_{15} primer, 2 µl of 0.1 M 1,4-dithiothreitole, 1 µl of RNAsin (Invitrogen, Carlsbad, USA) and 200 U of MMLV-reverse transcriptase (Intron, Korea). 2 µg of the cDNA was used in 25 µl PCR-reaction in a PeQLab 96 universal gradient UK thermal cycler for 35 cycles and the final extension at 72°C for 10 min. The annealing temperatures were 53 and 50°C for *β-Actin* and *E-cadherin*, respectively. PCR products were run in 1.5% agarose gel containing ethidium bromide and visualized using UV light. *β-Actin* (Table 1) was used as an internal standard of loadings.

Statistical analyses

The association between demographic features in addition to pathology data with promoter methylation status of the *E-cadherin* was evaluated using *t*-test. Statistical analysis was done using SPSS software, version 11, and P value < 0.05 was considered as statistically significant.

Results

During 2006 to 2008 forty four patients were admitted to Madaen and Imam Khomeini Hospitals for cancer treatment and underwent surgical resection for SCCE. The demographic features as well as pathology data of the patients are summarized in Table 2. Among these features, a significant association was found between grades I and II of tumor differentiation (p= 0.024), stages T2 and T3 of tumors (p= 0.026), as well as lymph node invasion (p= 0.004) with methylation of E-Cadherin promoter (Table 2).

***E-cadherin* promoter methylation pattern in the high risk region for SCCE**

DNA extracted from tumor biopsies and the normal tissues were analyzed for *E-Cadherin* promoter methylation using methylation specific PCR (MSP). Following to bisulfite treatment, DNA samples were checked for the proper C to U conversion using primers for the unmethylated *TPEF* promoter which differentiates unmethylated cytosines in CpG islands. All DNA samples confirmed to be properly bisulfite modified. Samples were then analyzed for methylation of *E-Cadherin* promoter applying primers specific for methylated cytosines. Twenty four out of 44 tumor DNA samples (54.5%) were found to be aberrantly

Table 2. Personal, demographic, and histopathological features associated with the esophageal cancer and methylation at the *E-cadherin* gene promoter of patients.

	Total	Methylated E-cadherin (N=24)%	Unmethylated E-cadherin (N=20)%	p
Age (mean±SD), years		62.5.±12.2	60.5±13.5	>0.05
Age				
<45y	5	2(804)	3(15)	0.64
>45y	39	22(91.6)	17(85)	
Sex				
Males, %	29	17(70.8)	12(60)	0.50
Female, %	15	7(29.2)	8(40)	
Race				
Fars	13	9(37.5)	4(20)	0.3
Azari	31	15(62.5)	16(80)	
Smoking				
Yes	18	10(41.7)	8(40)	1.0
No	26	14(58.3)	12(60)	
Tumor size(cm)				
<4.9	19	13(54.1)	5(25)	0.1
>5	25	11(45.9)	14(75)	
Grade Differentiation				
I	10	2(8.4)	8(40)	0.024
II	26	17(70.8)	9(45)	GII vs. GI
III	5	4(16.6)	1(5)	
Unknown	3	1(4.2)	2(10)	
Tumor T stage				
T1	1	0(0)	1(5)	
T2	10	4(16.6)	6(30)	0.026
T3	28	18(75)	10(50)	
T4	1	1(4.2)	0(0)	
Unknown	4	1(4.2)	3(15)	
Lymph node invasion				
N0	13	3(12.5)	10(50)	0.004
N1	21	16(66.6)	5(25)	N1 vs. N0
NX	10	5(20.9)	5(25)	
Metastasis				
M0	12	4(16.7)	8(40)	0. 26
M1	4	3(12.5)	1(5)	
MX	28	17(70.8)	11(55)	
Tumor Necrosis Present				
YES	15	5(20.8)	10(50)	
NO	21	14(58.4)	7(35)	0.08
Unknown	8	5(20.8)	3(15)	

methylated. In contrast, all normal tissues were found to be unmethylated (Fig.1). Such high percent of methylation of cytosine nucleotides in promoter of *E-Cadherin* further support important role of tumor suppressor genes inactivation in SCCE tumorigenesis. Furthermore these results indicates that the pattern of *E-cadherin* promoter methylation in this high risk region for SCCE resembles the same estimated median pattern for the other regions of the world (54.5 % in this study versus 58% worldwide), which further indicates applicability of *E-cadherin* promoter methylation analysis as a suitable molecular marker in combination with other markers such as P53, APC, β-tropomyosin [7-9]. To further validate specificity of our experimental procedure and the authenticity of the present MSP we used SssI methyl transferase treated DNA, which could only be amplified using methylation-specific primers.

Correlating tumor differentiation with the methylation status of *E-cadherin* promoter, 8.4% of tumors were in stage I (2 out of 10), 70.8% of tumors stage II (17 out of 26), and 16.6% of tumors stage III (4 out of 5) were found to be methylated. While a larger sample size would be required for establishing a significant association between prognosis of SCCE and *E-cadherin* promoter methylation; our results indicate a significant correlation between SCCE and *E-Cadherin* promoter methylation regardless of the tumor stage, while a strong correlation could be stated for the stage II

Teble 3. Percentage of the E-cadherin methylation in the SCCE patients among populations from different regions of the world.

E-cad meth%	Type of sample	Population	Ref.
61%MSPCR	Tissue	Japan	[19]
83%RT-PCR			
58.8%	Tissue	Japan	[20]
9%	Blood	Japan	[21]
43%	Tissue	Korea	[22]
84%	Tissue	USA	[5]
66%	Tissue (EAC)	USA	[28]
12%	(nonmalignant tissue) EAC		
66 %	Tissue	China	[23]
59.6%	Tissue	China	[24]
66.6%	cell lines	China	[18]
80%	Tissue		
68.5%	Tissue	China	[37]
85%	invasive SCC	China	[25]
50%	SCC in situ		
44%	actinic keratosis		
22%	non-neoplastic skin		
65%	biopsy of NPC (nasopharngeal carcinoma)	China	[38]
100%	cell line		
35%	blood		
(94%)=44%full meth+50% partial meth	breast cancer	Iran	[39]
50%	gastric adenocarcinoma	Iran	[40]

Table 4. Mechanisms that have been suggested for the inactivation of *E-cadherin* in sporadic human tumors [4].

Tumor type	Frequency of mutation(%)	Frequency of Hypermethylation (%)	Frequency of LOH* (%)
Diffuse gastric cancer	41	75	24
Other gastric cancer	0	49	10
Lobular breast cancer	32	77	81
Other breast cancer	0	44	38
Colorectal	0	46	13
Bladder	3	43	14
Leukaemia	-	48	2
Oesophageal	0	82	68
Hepatocellular carcinoma	0	41	46
Synovial sarcoma	24	-	-
Thyroid	4	43	7
Uterine	4	28	15
Oral SCC	0	36	-
Prostate	0	54	38

* Loss of heterozygosity

of SCCE and methylated promote of *E-cadherin*.

E-cadherin is down regulated in SCCE

Using RT-PCR; *E-cadherin* expression was analyzed in 44 tumor and 19 normal tissues. Results indicate loss or decreased levels of *E-cadherin* transcription in 24 out of 44 (54.5%) tumor biopsies. Normal tissue was used as positive control and β-actin as an internal loading control (Table 2). Observing the same percentage (54.5%) of expression in this analysis similar to what we have already obtained in promoter methylation study indicates that down-regulation of *E-cadherin* takes place through promoter hypermethylation rather than other means of *E-cadherin* inactivation such as mutations in the high risk region for ESCC. This observation is also in accordance with the former studies that have shown that in the majority of the epithelial derived tumors, mutation in the *E-cadherin* plays no significant role [14].

Decreased E-cadherin expression was significantly associated with the following features: grades I and II of tumor differentiation, stages T2 and T3 of the tumors, as well as lymph node invasion ($p<0.05$).

Discussion

Through hemophilic adhesion, *E-cadherin* plays important role in the maintenance of the cell to cell contacts in the epithelial cell layer and its integrity. Down regulation or loss of *E-cadherin* is a common

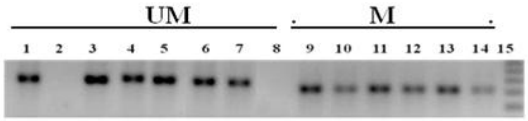

Fig. 1. E-cadherin promoter is methylated in SCCE. Modified DNA samples were subjected to methylation-specific PCR using primers specific for unmethylated TPEF promoter and methylated E-cadherin promoter. All samples were positive for the unmethylated TPEF; indicating proper DNA modification was done. Image shows an ethidium bromide stained agarose gel for MSP signal of the methylated and unmethylated E-cadherin promoter.
Lane 1: positive control for UM (Unmethylated); Lane 2: negative control for UM (Unmethylated). lanes 3-7: five of tissue samples displaying unmethylated E-cadherin(1, 2, 4, 8, 10) promoter. Lane 8: negative control for Unmethylated. Lane 9: positive control for Methylated. Lanes10-14: five tissue samples displaying methylated for E-cadherin (3, 24, 27, 40, 42). Lane 15: molecular size marker (123 bp ladder). UM indicates unmethylation E-cadherin promoter and M indicates methylation E-cadherin promoter.

Fig. 2A

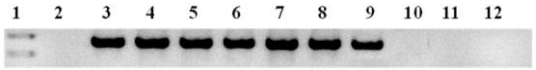

Fig. 2B

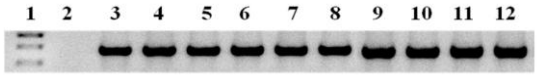

Fig. 2A. Assessment of E-cadherin expression in SCCE. RT-PCR was done using primers specific for E-cadherin and β-actin both for the normal and cancer tissues. A- Lane 1: molecular size marker (123 bp ladder), Lane 2: negative control for E-cadherin expression; Lane 3: positive control for E-cadherin expression; Lanes 4–9: E-cadherin expression for three Unmethylated tissue samples (samples number 1, 4, 10), and normal tissues (samples number 3 and 24); lanes 10-12: E-cadherin expression for three Meth tissue samples (3,24, 27).
Fig. 2B. Lane 1: molecular size marker (123 bp ladder), Lane 2: negative control for β-actin expression; Lane 3: positive control for β-actin expression; Lanes 4–8: β-actin expression for a few UnMeth samples (samples number 1, 4, 10), and normal tissues (samples number 3 and 24); lanes 10-12: β-actin expression for three Meth tissue samples (3,24, 27).
Normal tissue was used as a positive control and β-actin as an internal loading control.

feature in many types of tumors. Mutations in the *E-cadherin* are associated with the familial gastric cancer and metastasis of the sporadic tumors [15]. Although high frequencies of the *E-cadherin* mutations were found in the two specific subtypes of cancers, for the majority of epithelial derived tumors, mutation in *E-cadherin* does not play significant role [14]. *E-cadherin* mutations were first reported in the two loosely adherent gastric carcinoma cell lines. While *E-cadherin* mutations were found in gynecologic cancers, diffuse type of gastric carcinomas, and infiltrative lobular breast cancer [16], nevertheless, as table 4 indicates *E-cadherin* mutations are not the key determinant for many other types of cancers. Therefore alternative mechanisms for *E-cadherin* inactivation should be involved, and in particular, the promoter hypermethylation (Table 4). Accumulating evidences suggest that *E-cadherin* promoter methylation is associated with the reduced *E-cadherin* expression, progression of the disease to malignancy, as well as metastasis [17]. Hypermethylation of *E-cadherin* could be observed in a wide range of human tumors [4] including SCCE in which it plays essential role in the process of carcinogenesis (Table 4).

The present study has shown that methylation of *E-cadherin* promoter takes place in more than 50% of SCCE cases. Formerly, a wide range of frequencies from 9 to 84%, with a median of 58% [5, 18-27] have been reported for *E-cadherin* promoter methylation in SCCE fairly similar to our finding. Such high rate of *E-cadherin* promoter hypermethylation indicates the important role of this tumor suppressor gene in esophageal carcinogenesis and a preferred target for any approach in the therapy of the disease. In accordance with concept other reports have also suggested that *E-cadherin* is methylated in 84% (26 out of 31 cases) of adenocarcinoma of esophagus specimens and in the 80% (16 out of 20) SCCE samples as well as 4 out of 6 SCCE cell lines subjected to the analysis [5, 18, 19]. Results published by Brock and colleagues show that 66% (27 out of 41) of *E-cadherin* promoter is methylated in esophageal adenocarcinoma (EAC) as well as 12% of the adjacent nonmalignant tissue [28]. Furthermore Takeno *et al.* have suggested that *E-cadherin* promoter methylation is a frequent genetic alteration in SCCE (88%; 16 out of 20) [19]. They also showed that treatment of carcinoma cells null for *E-cadherin* expression with demethylating agent: 5-aza-2'-deoxycytidine, restores *E-cadherin* expression [18] (Table 3). These data and figures indicate the essential role of *E- cadherin* and the promoter methylation in both types of esophageal cancers. Also a rational similarity could be established between three geographically separate and genetically distinct populations: Japanese with 58.8% [20], Chinese with 59.6% [24] and Persians with 58.8% [28] of the *E-cadherin* promoter methylation.

For further verifying the importance of the *E-cadherin* inactivation in SCCE carcinogenesis we also

focused and gathered data and works that have been done in the other parts of the world and compared with our results regarding *E-cadherin* promoter methylation status in the esophageal cancer. Unless one report from Japan that has presented data for *E-cadherin* promoter methylation using blood DNA samples as part of their report [28]; all the other reports including the same study [28] have done their analysis applying tissue specimens and have shown 61% of methylation which indicates high level of *E-cadherin* promoter methylation. For example in Korean SCCE patients the frequency of *E-cadherin* methylation was 43% [21] and for American patients with esophageal adenocarcinoma it was estimated to be 66.6% [28]. Regarding China; reports from different parts of this country indicate wide range of *E-cadherin* promoter methylation (59.6%, 66.6%, 68.5%, and 80% [23], as well as 50% [25]) in SCCE tissue specimens (table 3)). In addition former study by our colleagues on Persian patients indicates an abnormal *E-cadherin* methylation in 50% of patients with gastric adenocarcinoma [29] and 50% partial methylation in patients with breast cancer [27] (Table 3).

The high incidence of *E-Cadherin* inactivation and down-regulation through promoter methylation in SCCE as studied so far in many populations and ethnicities strongly upholds its application as an early diagnostic marker in combination with a set of other markers for esophageal cancer worldwide. Application of a combination of markers is required from that sense that there are many reports that suggest *E-cadherin* involvement in the other tissues' tumorigenesis. For instance, *E-cadherin* is a prime target for epigenetic silencing in the ultraviolet induced squamous skin cancer or in many other forms of human cancers such as oral squamous cell carcinoma, non-neoplastic gastric epithelial tumors, and cervical cancer cell lines in addition to tumors [18, 30, 31]. It appears that increased *E-cadherin* promoter methylation is associated with the advanced stages of the skin squamous cell carcinogenesis [30, 32, 33]. Aberrant methylation of the 5'CpG islands of the *E-cadherin* gene associates with the reduced levels of *E-cadherin* expression in the SCCE cancer. The absence of such aberrant methylation in normal esophageal tissues specifies that the mechanism is tumor-specific and supports previous conclusions that a positive correlation exists between *E-cadherin* aberrant methylation and its role as a biomarker and esophageal cancer risk factor.

Our results shows a significant association between methylation of *E-Cadherin* promoter with grades I and II of tumor differentiation ($p = 0.024$), stages T2 and T3 of tumors ($p = 0.026$), as well as Lymph node invasion ($p = 0.004$). These data suggest that epigenetic silencing via aberrant methylation of the *E-cadherin* promoter is the critical mechanism for the inactivation of this gene in esophageal cancer [10, 34].

Conclusion

Promoter hypermethylation is among the mechanisms for the silencing, down regulation, and inactivation of the genes. *E-cadherin* is particular in that sense that its promoter hypermethylation is the major mechanism of its silencing and inactivation in both types of esophageal cancer. This mechanism could exclusively be observed in the progression of the SCCE toward invasion and metastasis. Adding the importance of E-cadherin in the esophageal carcinogenesis to its promoter methylation as a major mechanism of its silencing, subjects *E-cadherin* as a preferred marker for SCCE detection and drug design for therapy of the SCCE.

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