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Snail family proteins in cervical squamous carcinoma: Expression and significance

Abstract

Purpose: Epithelial-mesenchymal transition (EMT) is crucial for tumor progression and metastasis. Snail family members, including Snail, Slug and Smuc, are the transcription factors that repress E-cadherin expression and induce epithelial-mesenchymal transition in some tumor tissues. In this study, the expression of snail family proteins in cervical squamous cancers was evaluated.

Methods: A series of 144 samples, comprising 28 cases of normal cervical tissues and 116 cases of squamous cell carcinoma (SCC), were used for analysis. The expression of Snail, Slug, Smuc, E-cadherin and vimentin was assessed in the tissues by immunohistochemistry and was statistically analyzed by SPSS13.0.

Results: The increase in nuclear expression of snail and smuc was associated with down-regulation of E-cadherin and up-regulation of vimentin. The nuclear expression of Snail and Smuc was positively associated with lymph node metastasis of the SCC, and the nuclear expression of Snail was also positively related with histological differentiation. In contrast, tumor size, histological differentiation, lymph node metastasis and stages of the SCC were not associated with the expression of Slug, cytoplasmic Smuc or cytoplasm levels of Snail.

Conclusion: Snail and Smuc proteins, but not Slug, may contribute to the onset of EMT in SCC. Inhibiting the expression of Snail and Smuc might be a potential therapeutic target for the treatment of metastasis and invasion of cervical carcinomas.

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Cervical cancer, the second most common malignant neoplasm in women, is one of the leading causes of cancer-related deaths for females [1]. Metastasis and recurrence are the most important risk factors for patients with early-stage cervical cancer after surgery. Epithelial-mesenchymal transition (EMT) is characterized by loss of cell adhesion and increase in cell motility, both of which are closely associated with metastasis and invasion in primary tumors [2]. The hallmarks of EMT are the loss of the cell adhesion molecule E-cadherin and the acquisition of the mesenchymal marker, vimentin [3,4]. Transcription factors, including Twist, SIP1, Snail family members and others, have been identified as critical regulators of EMT during embryogenesis, tumor progression and metastasis [5-8].

Persistent infection with high-risk human papillomavirus is closely associated with cervical cancer. HPV E7 was shown to mediate EMT in cervical cancer [9]. Recent research has shown that the up-regulation of Snail was related to the expression patterns of EMT-associated proteins, such as vimentin and E-cadherin, in SiHa cells [9]. It has previously been reported that the combination of a decrease in E-cadherin and an increase in vimentin is involved in nodal metastasis and is closely associated with histologic differentiation and poor prognosis in cervical squamous cell cancer [10]. Hence, EMT may represent an important molecular alteration during the progression and metastasis in carcinoma of the cervix.

Snail and Slug are associated with metastasis and poor outcome in several cancers, such as hilar cholangiocarcinoma, esophageal squamous cell carcinoma and oral squamous cell carcinoma [11-13]. The increase expression of Snail and Slug is also correlated with higher histological grades and reduced relapse-free survival in patients with breast carcinoma [14]. It has been reported that the increased expression of Snail and Slug in ovarian cancer, gastric carcinomas and urothelial carcinoma can lead to reduced E-cadherin expression and the onset of EMT [15-17]. Smuc is expressed in skin melanotic melanoma, lung epidermoid carcinoma and germ cell tumors [18]. As a transcriptional repressor implicated in carcinogenesis and embryogenesis, Smuc might be a potent target for oncological treatments. To date, there have been few reports on the role of Snail family members in the invasion and metastasis of cervical cancer. In this study, the subcellular localization and expression of the Snail family proteins were analyzed in squamous carcinoma of the cervix (SCC), and the associations among these proteins were also determined in patients with EMT.

Materials and Methods

Patients and Specimens

Neoplastic specimens were collected from 116 patients diagnosed with cervical squamous cell carcinoma from 2010 to 2012. The tissue samples were obtained from the Department of Pathology, Anhui Provincial Hospital, which is affiliated with Anhui Medical University, Hefei, China. The SCC disease stage of each patient was determined based on the International Federation of Gynecology and Obstetrics (FIGO) classification. None of the patients had received radiotherapy or adjuvant chemotherapy. The clinicopathological features of the SCC patients are listed in Table 1. In addition, 28 normal cervical tissues from patients who underwent hysterectomy for reasons other than neoplasia of the cervix or endometrium were used as a control group. Our study was approved by the ethics review board of the hospital. Written informed consent was obtained from each patient.

Paraffin-embedded tissue blocks were cut into 1 to 2 μ m thick serial sections. One section was stained with hematoxylin and eosin (H&E) for standard histopathological diagnosis, and the remaining serial sections were processed for immunohistochemical (IHC) processing. Each H&E-stained and IHC-processed slide was reviewed to verify the histopathological diagnosis.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized in xylene for 15 min, fixed in 100% ethanol for 5 minutes, and then rehydrated in a graded ethanol series (90%, 80% and 70%). Endogenous peroxide activity was quenched by incubation with 3% hydrogen peroxide in methanol for 10 min. The sections were washed twice with PBS and incubated with the anti-E-cadherin, vimentin, Snail, Slug and Smuc primary antibodies (dilutions 1:100, 1:400, 1:200, 1:100 and 1:200) overnight at 4°C. They were rinsed twice with PBS and incubated with the corresponding secondary antibody for 30 min at 37°C. The sections then were washed with PBS and incubated for 1 min with high-sensitivity substrate (DAB), washed with tap water, and then counterstained with hematoxylin. Finally, the slides were viewed under a light microscope. PBS was used instead of primary antibody as a negative control and lung cancer tissues were used as positive controls.

The Snail antibody was purchased from Sigma-Aldrich (AV33314, Sigma-Aldrich, USA), the Slug antibody was obtained from Abcam (ab38551, Abcam, Cambridge, UK) and the Smuc antibody was obtained from Abnova (A38821, Abnova, Taiwan, China). E-cadherin and vimentin antibodies

TABLE 1. Patient clinical and pathological characteristics

Parameter	number	%
Age (24-68 years)		
≤45	67	57.8
>45	49	42.2
Tumor size (scc)		
≤4cm	52	44.8
>4cm	64	55.2
Lymph node invasion (scc)		
Negative	55	47.4
Positive	61	52.6
Differentiation grads of tumor cell		
High	9	7.8
Moderate	52	44.8
Low	55	47.4
FIGO stage		
I	82	70.7
II	34	29.3
E-cad		
Negative expression	28	24.1
Low expression	56	48.3
High expression	32	27.6
Vimentin		
Negative expression	29	25.0
Low expression	46	39.7
High expression	41	35.3

were purchased from Zymed Laboratories (San Diego, USA). The secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, USA).

The immunostaining results were reviewed and scored independently by two pathologists who were blinded to the clinical data and to the other immunohistochemical results. The immunohistochemical staining was scored based on both the intensity of staining and the proportion of positively-stained cancer cells. The proportion of positive cells ranged from 0 to 100%, and the intensity of staining was evaluated based on staining intensity and distribution using the immunoreactive score [19, 20]: immunoreactive score = intensity score × proportion score. The intensity score was defined as 0, negative; 1, weak; 2, moderate; or 3, strong, and the proportion score was defined as 0, negative; 1, <10%; 2, 11-50%; 3, 51-80%; or 4, >80% positive cells. The total score ranged from 0 to 12. The samples were divided into three levels on the basis of

the final immunoreactivity score: negative immunoreactivity (-) was defined as a total score of 0, low immunoreactivity (+) was defined as a total score of 1-4, and high immunoreactivity (++) was defined as a total score >4 [19, 20].

Statistical Analysis

All statistical analysis was performed using SPSS 13.0 software (Chicago, IL, USA). The χ^2 test or Fisher's exact test were used to compare the levels of Snail family protein, E-cadherin and vimentin expression among patients with different clinical characteristics. The χ^2 test for a linear trend was fit to identify the correlations among Snail family protein, vimentin and E-cadherin expression levels. For all tests, $P < 0.05$ was considered statistically significant.

Results

Patient clinicopathologic characteristics

Tumor specimens were collected from 116 patients diagnosed with cervical squamous cell carcinoma. The main clinical characteristics of the patients are detailed in Table 1.

Nuclear and cytoplasm expression of Snail in cervical tissues and its association with EMT

The nuclear and cytoplasm expression of Snail was determined in normal cervical and SCC tissues. The results showed that Snail was barely observed both in the cytoplasm and nucleus of the normal cervical epithelial cells (Fig. 1). Among the 116 SCC samples, 24 cases (20.7%) showed high nuclear expression of Snail, 59 cases (50.9%) with low nuclear expression of Snail; and 39 cases (33.6%) exhibited high cytoplasmic expression of Snail, 49 cases (42.2%) with low cytoplasmic expression of Snail (Fig. 1 and Table 2). These data suggest that both the nuclear and the cytoplasm expression of Snail were increased in SCC tissue compared to normal tissue.

As shown in Table 3, 16.4% (19/116) of the SCC specimens with negative E-cadherin expression and high vimentin expression, and 32.8% (38/116) of the SCC specimens with low E-cadherin and vimentin expression were considered as occurrence of EMT in cervical cancer. In contrast, 13.8% (16/116) of SCC specimens with high E-cadherin expression and negative vimentin expression were considered non-occurrence of EMT. Furthermore, the SCC tissues were divided into three groups: Group I (19 cases): SCC tissues with negative expression of E-cadherin and high expression of vimentin (considered as occurrence of EMT and labelled SCC1 in the Figures); Group II (38 cases): SCC tissues with low expression of E-

FIGURE 1. Immunohistochemistry of Snail, E-cadherin and vimentin in cervical tissues. Snail (upper panel), aberrant E-cadherin (middle panel) and vimentin (lower panel) was shown in serial sections (400× magnification). SCC1 is indicated as cancer tissues with significant EMT (high expression of vimentin and low expression of E-cadherin); SCC2 is indicated as cancer tissues without or weak EMT (low expression of vimentin and high expression of E-cadherin).

FIGURE 2. Scatter diagram of IHC staining score for the nuclear and cytoplasm expression of Snail in cervical carcinoma tissue with different expression levels of E-cadherin and vimentin. “-” : negative expression; “+” : low expression; “++” : high expression.

TABLE 2. The relationship between snail family protein expression levels in tumors and clinical variables

Variables	# of patients	Snail nuclear expression Level				Snail cytoplasm expression Level				Slug nuclear expression Level			
		N	L	H	P	N	L	H	P	N	L	H	P
Age (years)					.229				.214				.798
≤45	67	20	30	17		20	25	22		35	26	6	
>45	49	13	29	7		8	24	17		28	18	3	
Tumor size					.698				.312				.958
≤4cm	52	16	27	9		11	26	15		29	19	4	
>4cm	64	17	32	15		17	23	24		34	25	5	
Differentiation grads					0.034				0.351				0.103
Well	9	3	6	0		2	3	4		4	5	0	
Moderate	52	15	31	6		17	20	15		31	20	1	
Poor	55	15	22	18		9	26	20		28	19	8	
Lymph node metastasis					0.007				0.893				0.130
Negative	55	23	21	11		14	22	19		28	25	2	
Positive	61	10	38	13		14	27	20		35	19	7	
FIGO stage					.383				.618				.832
I	82	22	45	15		19	37	26		46	30	6	
II	34	11	14	9		9	12	13		17	14	3	

TABLE 2 (continued)

Variables	Slug cytoplasm expression Level				Smuc nuclear expression Level				Smuc cytoplasm expression Level			
	N	L	H	P	N	L	H	P	N	L	H	P
Age (years)				.388				.492				.883
≤45	12	18	37		4	34	29		21	30	16	
>45	8	19	22		6	23	20		17	22	10	
Tumor size				.655				.170				.405
≤4cm	10	18	24		7	22	23		18	20	14	
>4cm	10	19	35		3	35	26		20	32	12	
Differentiation grads				0.098				0.593				0.640
Well	2	1	6		2	4	3		3	5	1	
Moderate	7	13	32		3	27	22		16	26	10	
Poor	11	23	21		5	26	24		19	21	15	
Lymph node metastasis				0.389				0.042				0.327
Negative	7	17	31		7	31	17		19	27	9	
Positive	13	20	28		3	26	32		19	25	17	
FIGO stage				.235				.323				.938
I	11	27	44		9	38	35		27	36	19	
II	9	10	15		1	19	14		11	16	7	

NOTE. Bold values signify P < 0.05.

Abbreviations: N, negative; L, low expression; H, high expression.

TABLE 3. The correlation between immunostaining of E-cadherin and Vimentin and snail family

Variable	# of patients	E-cadherin expression level				Snail nuclear expression level				Snail cytoplasm expression level			
		N	L	H	P	N	L	H	P	N	L	H	P
E-cadherin staining									<.001				.312
Negative	28					4	9	15		8	18	2	
Low expression	56					12	38	6		9	21	26	
High expression	32					17	12	3		11	10	11	
Vimentin staining					0.002				<.001				
Negative	29	5	8	16		20	9	0		10	14	5	.348
Low expression	46	4	38	4		6	36	4		10	13	23	
High expression	41	19	10	12		7	14	20		8	22	11	

TABLE 3. (continued)

Variable	Slug nuclear expression level				Slug cytoplasm expression level				Smuc nuclear expression level				Smuc cytoplasm expression level			
	N	L	H	P	N	L	H	P	N	L	H	P	N	L	H	P
E-cadherin staining				.623				.592				.002				0.922
Negative	16	12	0		0	8	20		0	5	23		8	13	7	
Low expression	25	26	5		16	22	18		3	42	11		20	25	11	
High expression	22	6	4		4	7	21		7	10	15		10	14	8	
Vimentin staining				.920				.371				.003				0.866
Negative	15	13	1		12	7	10		8	9	12		12	10	7	
Low expression	25	17	4		2	12	32		0	35	11		11	26	9	
High expression	23	14	4		6	18	17		2	13	26		15	16	10	

NOTE. Bold values signify $P < 0.05$.

Abbreviations: N, :negative; L, low expression; H, high expression.

cadherin and low expression of vimentin (considered as an incomplete EMT); Group III (16 cases): SCC tissues with high expression of E-cadherin and negative expression of vimentin (considered as non-occurrence of EMT and labelled as SCC2 in the Figures).

Statistical analysis of Snail expression in the cytoplasm and nucleus was performed to show the p -value. There was significant difference in Snail nuclear expression between SCC tissues with and without the occurrence of EMT ($p < 0.05$); while there was no significant difference in Snail cytoplasm expression of the SCC tissues with and without the occurrence of EMT ($p > 0.05$). A significant difference in Snail nucleus expression was seen among Groups I, II and III (all p -values < 0.05) (Fig. 2), suggesting that an increase in nuclear expression of Snail might contribute to the onset of EMT in cervical cancers.

Nuclear and cytoplasm expression of Slug in cervical tissues and its association with EMT

The nuclear and cytoplasmic expression of Slug was measured in normal cervical and SCC tissues. These results demonstrated low nuclear and cytoplasmic expression of Slug in the normal cervical epithelium (Fig. 3). Among the 116 SCC samples, 7.8% (9/116) and 50.9% (59/116) of SCC samples showed high expression of Slug in the nucleus and the cytoplasm, respectively. In addition, 37.9% (44/116) and 31.9% (37/116) of SCC samples exhibited low nuclear and cytoplasmic expression of Slug, respectively (Fig. 3 and Table 2).

Statistical analysis of Slug expression in the cytoplasm and nucleus was performed to show the p -value. There was no significant difference in Slug nuclear expression of the SCC tissues with and without the occurrence of EMT ($p > 0.05$), and there was also no significant difference in Slug cytoplasm ex-

pression of the SCC tissues with and without the occurrence of EMT ($p>0.05$). In addition, there was no significant differences in the nucleus and cytoplasm expression of Slug among Groups I, II and III (all p -values >0.05) (Fig. 4).

Nuclear and cytoplasm expression of Smuc in cervical tissues and its association with EMT

The nuclear and cytoplasmic expression of Smuc in normal cervical and SCC tissues was measured. The results indicated low nuclear expression and negative cytoplasmic expression of Smuc in normal cervical epithelium (Fig. 5): 42.2% (49/116) and 22.4% (26/116) of SCC samples had high nuclear and cytoplasmic expression of Smuc, respectively, and 57 (49.1%) cases and 52 (44.8%) cases were detected low nuclear and cytoplasmic expression of Smuc respectively (Fig. 5 and Table 2).

There was statistically significant difference in Smuc nuclear expression of the SCC tissues with and without the occurrence of EMT ($p<0.05$); while there was no significant difference in Smuc cytoplasm expression in the SCC tissues with and without the occurrence of EMT ($p>0.05$). There was also a significant difference in Smuc nucleus expression among Groups I, II and III (all p -values <0.05) (Fig. 6), indicating the observed increase in nuclear expression of Smuc may contribute to the onset of EMT in cervical cancers.

Relationship among Snail, Slug and Smuc expression levels in the tumors and the patients' clinical variables

As shown in Table 2, there was no difference in expression of Snail, Slug and Smuc expression for the ages, tumor sizes or clinical stages of the patients with SCC; however, histological grade and nodal metastasis were significantly correlated with Snail nuclear expression ($p=0.034$ and $p=0.007$, respectively) (Table 2). In addition, nuclear Smuc was significantly associated with nodal metastasis ($p=0.042$).

Association among the expression of E-cadherin, vimentin and Snail family proteins

The association among Snail family proteins and E-cadherin and vimentin expression are summarized in Table 3. Nuclear Snail and Smuc expression levels were inversely associated with E-cadherin expression ($p<0.001$ and $p=0.002$, respectively), whereas the nuclear expression levels of Snail and Smuc were directly associated with vimentin expression ($p<0.001$ and $p=0.003$, respectively). Cytoplasmic expression of Snail and Smuc, together with the expression of Slug in the nucleus and the cytoplasm, had no association with E-cadherin expression

($p=0.312$, $p=0.623$ and $p=0.592$, respectively) and vimentin expression ($p=0.348$, $p=0.920$ and $p=0.371$, respectively).

Discussion

The Snail family proteins, firstly identified in *Drosophila*, are zinc finger transcriptional regulators. Snail and Slug are considered major transcription factors, regulating EMT in various cancers [21-25]. Both nuclear and cytoplasmic staining of Snail family proteins was analyzed in this study because the subcellular localization of transcription factors is closely correlated with its function in tumor progression. Snail and Slug bind directly to the E-cadherin promoter of E-cadherin; these proteins also up-regulate mesenchymal makers, including vimentin and MMP, ultimately promoting the occurrence of EMT [26]. In this study, Snail nuclear staining was found to be closely related to the decreased expression of E-cadherin and the increased expression of vimentin, which also confirms the findings that the down-regulated expression of E-cadherin increases the expression of Snail in SiHa and CaSki cells [27]. In our study, Snail nuclear expression was significantly associated with lymph node metastasis of SCC. Thus, nuclear Snail protein appears to be a potential indicator of EMT and may prove useful in evaluating the progression of SCC.

The aberrant expression of Slug is associated with invasive and migratory potential in various carcinomas [28-34]. Little is known about the expression of Slug in cervical cancers and its association with various clinicopathological factors. Although the expression of Slug in SiHa cells has been reported [30], this study showed low nucleus Slug expression in most of the cervical carcinoma tissues sampled. In addition, unlike Snail, Slug expression was not linked with the expression of E-cadherin, vimentin, histological grade or nodal metastasis in the investigated cervical tumors. Similar findings have been reported in renal cell carcinoma by Mikami [23]; suggesting that Slug might not participate in EMT in cervical cancer.

Smuc, a novel member of the Snail family of proteins, may regulate bHLH transcription factors by controlling differentiation processes through the competition of zinc finger domains for E-box binding [35-38]. Smuc mRNA is also expressed in skin melanotic melanoma, lung epidermoid carcinoma and germ cell tumors [18,39,40]. Nevertheless, little is known about the Smuc protein in the progress of human tumors, especially at the onset of EMT. Herein, Smuc nuclear staining was found to be associated with the decrease of E-cadherin and the increase of vimentin. Smuc nuclear expression is also positive associated with nodal metastasis. Thus, Smuc may also (in addition to Slug) be involved in the EMT in cervical carcinoma.

FIGURE 3. Immunohistochemistry of Slug, E-cadherin and vimentin in cervical tissues. Snail (upper panel), aberrant E-cadherin (middle panel) and vimentin (lower panel) was shown in serial sections (400× magnification). SCC1 is indicated as cancer tissues with significant EMT (high expression of vimentin and low expression of E-cadherin); SCC2 is indicated as cancer tissues without or with only weak EMT (low expression of vimentin and high expression of E-cadherin).

FIGURE 4. Scatter diagram of IHC staining score for the nuclear and cytoplasm expression of Slug in cervical carcinoma tissue with different expression levels of E-cadherin and vimentin. “-” : negative expression; “+” : low expression; “++” : high expression.

FIGURE 5. Immunohistochemistry of Smuc, E-cadherin, and vimentin in cervical tissues. Smuc (upper panel), aberrant E-cadherin (middle panel) and vimentin (lower panel) was shown in serial sections (400× magnification). SCC1 is indicated as cancer tissues with significant EMT (high expression of vimentin and low expression of E-cadherin); SCC2 is indicated as cancer tissues without or weak EMT (low expression of vimentin and high expression of E-cadherin).

FIGURE 6. Scatter diagram of IHC staining score for the nuclear and cytoplasm expression of Smuc in cervical carcinoma tissue with different expression levels of E-cadherin and vimentin. “-” : negative expression; “+” : low expression; “++” : high expression.

In summary, the nuclear accumulation of Snail and Smuc might take part in tumor progression by promoting EMT via regulation the expression of E-cadherin and vimentin.

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