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Original Article

Clinical significance of methylation of *E-cadherin* and *p14ARF* gene promoters in skin squamous cell carcinoma tissues

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Abstract: Epigenetic regulation of genes by DNA methylation contributes to cancer. The present study sought to identify methylation changes in the promoters of *E-cadherin* and *p14ARF*, two genes with potential cancer roles promoting in skin squamous cell carcinoma. Skin squamous cell carcinoma specimens were collected from 40 patients and normal skin tissues were collected from 30 individuals as controls. Promoter methylation was detected for *E-cadherin* and *p14ARF* by methylation-specific PCR. Correlations between *E-cadherin* or *p14ARF* methylation and clinicopathological parameters were analyzed by the Spearman rank test. Methylation of *E-cadherin* (37.5%) and *p14ARF* (60.0%) was significantly more common in skin squamous cell carcinoma than in normal skin tissue (10.0 and 6.7%, respectively; $P < 0.05$). Additionally, *E-cadherin* and *p14ARF* methylation were positively correlated within skin squamous cell carcinoma ($r = 0.422$, $P = 0.007$). Furthermore, methylation of these gene promoters in skin squamous cell carcinoma was correlated with differentiation, lymph node metastasis, and clinical stage ($P < 0.05$). Aberrant methylation in promoters of *E-cadherin* and *p14ARF* may promote occurrence and progression of skin squamous cell carcinoma.

Keywords: Skin squamous cell carcinoma, E-cadherin, p14ARF, DNA methylation, clinicopathological parameter

Introduction

Advances in epigenetic research, revealing DNA sequence-independent mechanisms of post-translational modification, have uncovered the importance of epigenetic changes in the process of tumorigenesis. For example, many tumors exhibit tumor suppressor genes with one or more methylated CpG islands; DNA methylation serves as a mark for post-translation inactivation or dysfunction. The inactivation of tumor suppressor genes promotes tumor formation and growth [1, 2]. Indeed, abnormal methylation of gene promoters is an important contributor to cancer development because this feature has been noted in many tumor suppressor genes that control cell cycle, DNA repair, cell adhesion, and metastasis. Such changes in DNA methylation status appear to occur before tumor formation, rather than in parallel to or as a result of cell transformation. Identifying methylation changes that increase tumor susceptibility can help diagnose or prevent tumors. Importantly, recent studies have also determined that methylation changes can be identified in circulating DNA and other tissue/fluid samples, while still correlating with specific tumor development [3]. These findings indicate that methylation status can be investigated in individuals using minimally invasive methods and straightforward analyses [4, 5], making it even more attractive as a diagnostic tool.

Several genes have been demonstrated to be inactivated in tumors because of methylation changes, particularly in their promoters. Hypermethylation of *T-cadherin*, *NT5E*, and *SLIT2* have been associated with skin, breast, and ovarian cancer, respectively [6-8]. Because skin squamous cell carcinoma accounts for a large proportion of malignant neoplasms of the

Table 1. Primer sequences for methylation-specific PCR analysis

Primers	Sense sequences	Antisense sequences	Product size
E-cadherin - U	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	151 bp
E-cadherin - M	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	150 bp
p14ARF - U	TTTTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	132 bp
p14ARF - M	GTGTTAAAGGGCGGCGTAG	AAAACCCTCACTCGCGACGA	122 bp

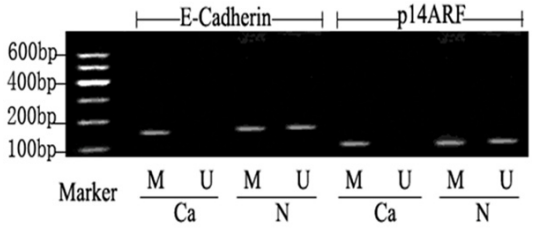

Figure 1. Electrophoretogram of *E-cadherin* and *p14ARF* methylation in skin squamous cell carcinoma and normal skin tissue. PCR amplification products were separated on 2% agarose gel. If only unmethylated bands were observed during electrophoresis of MSP products, the sample was recorded as unmethylated; if methylated bands were observed, the sample was recorded as methylated. Note: Ca = skin squamous cell carcinoma (Stage II); N = normal skin; M = methylated; U = unmethylated.

skin (about 80-90%) [9], and its incidence is increasing annually (particularly among older people), identifying genes in which methylation changes are associated with this cancer is important. E-cadherin, a cell adhesion protein, promotes stability of epithelial cell structures, promoting interaction with adjacent epithelial cells to prevent cell exfoliation. Inactivation of the *E-cadherin* gene can promote migration of tumor cells [10]. Indeed, *E-cadherin* expression is reduced in patients with endometrial carcinoma of the uterus, which significantly affects 5-year survival rates of affected patients [11]. Similarly, P14 (produced by *p14ARF*), a cyclin-dependent kinase inhibitor, is a complex regulatory factor of the cell cycle that plays an important role in inhibiting tumor occurrence and development. Inactivation of *p14ARF* is associated with a variety of tumors, such as bladder cancer, oral squamous cell carcinoma, and colon cancer [12, 13].

The potential for inactivation of *E-cadherin* and/or *p14ARF* to result in cancer makes these genes good candidates for use as biomarkers. Here, methylation-specific PCR (MSP) was used to determine the methylation status of *E-cadherin* and *p14ARF* gene promoters in 40 samples of squamous cell carcinoma of the skin to identify their potential role in this disease and discover new approaches for early diagnosis.

Patients and methods

Patients and controls

From January 1 to December 31, 2011, samples of skin squamous cell carcinoma were collected from 40 patients who received surgical resection in the First Affiliated Hospital, Harbin Medical University, Harbin, China, and had been diagnosed by pathological confirmation. Each case had detailed clinical and pathological data and none received preoperative chemotherapy or radiotherapy. Cancer patients included 25 males and 15 females, ages 39 to 73 years (mean age 53.9 ± 11.6 years). Clinical evaluation indicated that 25 cases were highly or moderately differentiated, while 15 cases were poorly differentiated, and 27 cases had no lymph node metastasis, while 13 cases had lymph node metastasis. According to Broders' pathological grading criteria for skin squamous cell carcinoma, 26 cases were grade I+II, and 14 cases were grade III+IV.

Normal tissue specimens were collected by surgical resection from 30 individuals to serve as a control group. These included 18 males and 12 females, ages 35 to 69 years (mean age 49.5 ± 10.4 years). No statistically significant difference was detected in age or gender between the two groups. All specimens were obtained under informed consent with approval by the Ethics Committee of our hospital (Identification No. HMU (Ethics) 20121103).

Methylation-specific polymerase chain reaction

Methylation-specific PCR (MSP) was used to detect the methylation status of *E-cadherin* and *p14ARF* promoter regions. Genomic DNA was isolated from tissue using proteinase K

Table 2. Methylation of *E-cadherin* and *p14ARF* in skin squamous cell carcinoma and normal skin tissues [N (%)]

Sample	N	*E-cadherin*		p14ARF	
		Unmethylated	Methylated	Unmethylated	Methylated
Skin squamous cell carcinoma	40	25 (62.5)	15 (37.5)	16 (40.0)	24 (60.0)
Normal skin tissues	30	27 (90.0)	3 (10.0)	28 (93.3)	2 (6.7)
χ^2		6.787		20.886	
P		0.009		0.001	

Data are reported as number of samples with percent in parentheses.

Table 3. Correlation between *E-cadherin* and *p14ARF* methylation in skin squamous cell carcinoma [N (%)]

E-cadherin	N	*p14ARF*	
		Unmethylated	Methylated
Unmethylated	25	14 (56.0)	11 (44.0)
Methylated	15	2 (13.3)	13 (86.7)
Total	40	16 (40.0)	24 (60.0)

Data are reported as number with percent in parentheses. r = 0.422; P = 0.007 (Spearman correlation test).

digestion and phenol/chloroform extraction. The Wizard clean-up system kit (Promega Corporation, USA) was used to purify DNA according to manufacturer instruction. Unmethylated DNA was converted using hydrosulfite modification, as follows: 2 μL DNA were added to 50 μL sterilized twice-distilled water and 3.3 μL of 3 mol/L NaOH. The reaction mix was denatured at 37°C for 15 min, 30 mL of 10 mol/L hydroquinone and 520 μL of 3 mol/L sodium bisulfite were added, and the mix was covered with 200 μL liquid paraffin. The reaction was performed at 55°C for 16 h. Subsequently, 2 μL purified and modified DNA were combined with 1 μL upstream and 1 μL downstream primers for the target genes (see **Table 1**), 12.5 μL 2 X Taq PCR MasterMix (TIANGEN, Biotech Co. Led, China), and 8.5 μL ribozyme-free water for PCR amplification. Thermal cycling conditions were as follows: predenaturation at 95°C for 5 min; 35 cycles of 94°C for 45 s, 65°C for 45 s (*E-cadherin*) or 60°C for 45 s (*p14ARF*), and 72°C for 1 min, and re-extension at 72°C for 7 min. PCR amplification products were separated on 2% agarose gel. If only unmethylated bands were observed during electrophoresis of MSP products, the sample was recorded as unmethylated (U); if methylated bands were observed, the sample was recorded as methylated (M).

Statistical methods

SPSS13.0 statistical software was used for statistical analysis. The χ^2 test was used to compare methylation status of *E-cadherin* or *p14ARF* between normal and cancer tissues, and Spearman correlation was used to analyze the relationship of methylation status between *E-cadherin* and *p14ARF*. P < 0.05 was considered to be statistically significant.

Results

Increased methylation of E-cadherin and p14ARF in skin carcinoma

Methylation of *E-cadherin* and *p14ARF* was significantly more common in skin squamous cell carcinoma tissues (37.5 and 60.0%, respectively) than in normal skin tissues (10.0 and 6.7%, respectively, P < 0.05; **Figure 1** and **Table 2**). Further, methylation of *E-cadherin* was positively correlated with methylation of *p14ARF* in skin squamous cell carcinoma tissues (r = 0.422 P = 0.007; **Table 3**).

Correlation between methylation of E-cadherin and p14ARF and clinicopathological parameters

To determine whether the increased methylation in cancer tissue correlated with disease progression, the methylation status of *E-cadherin* and *p14ARF* was compared to the clinicopathological characteristics. **Table 4** shows that methylation of both genes in skin squamous cell carcinoma tissues was correlated with differentiation degree, lymph node metastasis, and clinical stage (P < 0.05), but not with gender or age.

Discussion

Cancer results from the loss of gene/protein functions because of a variety of different fac-

Table 4. Correlation between methylation of *E-cadherin* and *p14ARF* and clinicopathological parameters in skin squamous cell carcinoma [N (%)]

Characteristic	N	*E-cadherin*				*p14ARF*			
		U	M	χ^2	P	U	M	χ^2	P
Gender									
Male	25	15 (60.0)	10 (40.0)	0.178	0.673	9 (36.0)	16 (64.0)	0.444	0.505
Female	15	10 (66.7)	5 (33.3)			7 (46.7)	8 (53.3)		
Age (years)									
< 60	25	16 (64.0)	9 (36.0)	0.064	0.800	11 (44.0)	14 (56.0)	0.444	0.505
≥ 60	15	9 (60.0)	6 (40.0)			10 (66.7)	5 (33.3)		
Pathological differentiation									
High+Moderate	25	20 (80.0)	5 (20.0)	8.711	0.003	14 (56.0)	11 (44.0)	7.111	0.008
Low	15	5(33.3)	10 (66.7)			2 (13.3)	13 (86.7)		
Lymph node metastasis									
No	27	21 (77.8)	6 (22.2)	8.274	0.004	15 (55.6)	12 (44.4)	8.376	0.004
Yes	13	4 (30.8)	9 (369.2)			1 (7.7)	12 (92.3)		
Clinical stage									
I+II	26	21 (80.8)	5 (19.2)	10.579	0.001	14 (53.8)	12 (46.2)	5.934	0.015
III+IV	14	4 (28.6)	10 (71.4)			2 (14.3)	7 (85.7)		

The χ^2 test was used to compare methylation status between normal and cancer tissues. U = unmethylated; M = methylated.

tors, including genetic, epigenetic, and environmental phenomena. In the case of E-cadherin, loss of expression has been linked to the development and progression of a wide variety of tumors, including squamous cell carcinoma [10, 14]. Recent evidence indicates that the primary mechanism of *E-cadherin* inactivation is the abnormal methylation of promoter CpG islands. A high frequency of *E-cadherin* methylation has been identified in prostate cancer and has been correlated with poor differentiation and rapid progression [15, 16]. Furthermore, a study of eyelid sebaceous gland carcinoma demonstrated increased *E-cadherin* promoter methylation, correlating to loss of protein expression and disease progression [17]. Similarly, we found that *E-cadherin* was more likely to be methylated in skin squamous cell carcinoma tissues than in normal tissues, and that this methylation correlated with disease severity. Therefore, epigenetic regulation of E-cadherin through promoter methylation appears to promote progression of skin squamous cell carcinoma, consistent with findings for other tumor types.

Loss of the tumor suppressor p14 through *pARF14* methylation has also been reported [18]. Hypermethylation of pARF14 is involved in dysregulation of the p53 and RB pathways, inhibiting their tumor suppressive functions, as previously shown in skin squamous cell carcinoma [19]. In addition, inactivation of *pARF14* appears to have a synergistic effect with Epstein-Barr virus infection to promote the occurrence of gastrointestinal tumors [20]. Here, methylation of *p14ARF* was more common in skin squamous cell carcinoma tissues than in normal skin tissues. Additionally, methylation of this gene correlated with differentiation degree, lymph node metastasis, and clinical stage, as well as with *E-cadherin* methylation. These findings suggest that aberrant *p14ARF* promoter methylation, as with *E-cadherin*, promotes squamous cell carcinoma of the skin.

In summary, *E-cadherin* and *p14ARF* methylation is abnormal in skin squamous cell carcinoma. Detection of methylation status of these genes may be useful as a biological indicator for and early diagnosis or prognosis estimation in patients with this disease. Further work in identifying a demethylation reagent to restore proper methylation status in these genes may result in new reagents to prevent *E-cadherin* and *p14ARF* methylation-related skin squamous cell carcinoma.

Disclosure of conflict of interest

None.

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