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Original Article

Aberrant promoter methylation of SH3GL2 gene in vulvar squamous cell carcinoma correlates with clinicopathological characteristics and HPV infection status

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Abstract: Objective: This study attempted to examine the methylation status of SH3GL2 gene in different types of human vulvar lesions and its correlation with clinicopathological parameters. Methods: Immunohistochemical analysis was used to identify the expression status of SH3GL2 in vulvar squamous cell carcinoma (VSCC), vulvar intraepithelial neoplasia (VIN) and benign vulvar squamous epithelium tissues. Bisulfite genomic sequencing method was used to detect methylation status of the SH3GL2 gene. Clinicopathological correlation of the alterations was analysed by the chi-square tests. Results: Immunohistochemical analysis showed expression of SH3GL2 in VSCC was significantly downregulated than that in VIN and normal vulvar tissues. In accordance with higher frequency of methylation status in SH3GL2, statistical analysis showed methylation status of SH3GL2 was closely related to tumor TNM stage (P=0.003), but not related to age, tumor volume, tumor differentiation, lymph node metastasis and VIN grade. High-methylation status of SH3GL2 showed significant association with HPV infection status. Conclusions: Our results indicated that the methylation status of SH3GL2 gene was associated with the TNM staging and HPV infection status of VSCC, suggesting that it might play a synergistic role in the development of VSCC.

Keywords: SH3GL2, methylation, vulvar squamous cell carcinoma

Introduction

Vulvar squamous cell carcinoma (VSCC) is one of the malignant tumor which severely do harm to women's health, accounting for 80%-90% in female vulva malignant tumors. A large proportion of VSCC and intraepithelial neoplasias (VIN) are associated with HPV infection, mainly type 16 and 18 [1]. Previous study we have demonstrated that a loss of chromosome 9P22 as frequent genetic changes in VSCC samples [2]. SH3GL2 gene is located at 9p22, containing 9 exons, encoding 352 amino acid protein, which has a C-terminal SH3 domain (amino acid residues 295-344) [3]. SH3GL2 mainly through the SH3 domain protein and other downstream proteins plays an important biological role in cell proliferation, differentiation and other physiological functions in combination [4]. Loss of SH3GL2 has been reported as a prognostic factor in several cancers, such as urothelial carcinoma [5], ovarian cancer [6], head and neck squamous cell carcinoma [7] and is involved in inactivation of the SH3GL2 gene expression. Epigenetic silencing of SH3GL2 gene by promoter hypermethylation is frequently shown as an important mechanism in regulation of intracellular signal transduction pathway [8]. However, the expression status of SH3GL2 gene and its role in VSCC pathogenesis have not yet been elucidated. In this study, we analyzed SH3GL2 methylation status and explored its relation to VSCC progression and HPV infection status.

Materials and methods

Tissue samples

35 cases of VIN and 52 cases of VSCC (Including 11 cases of para-carcinoma tissue) were

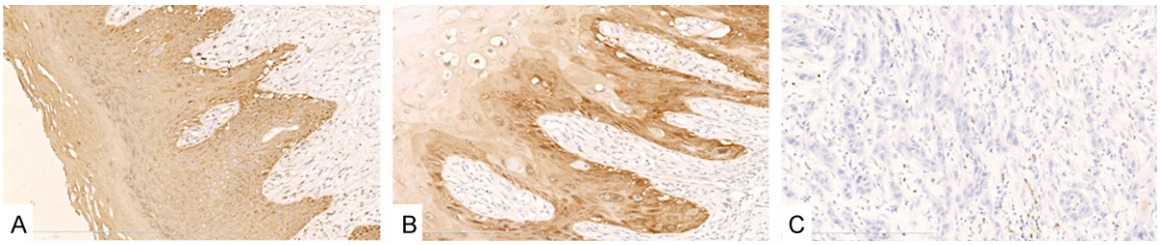

Figure 1. Immunohistochemical findings. SH3GL2 protein showed cytoplasm staining in vulva normal tissue (A) and vulvar intraepithelial neoplasia (B), negative staining in VSCC (C). (Original magnification ×200, scale bar, 300 μm).

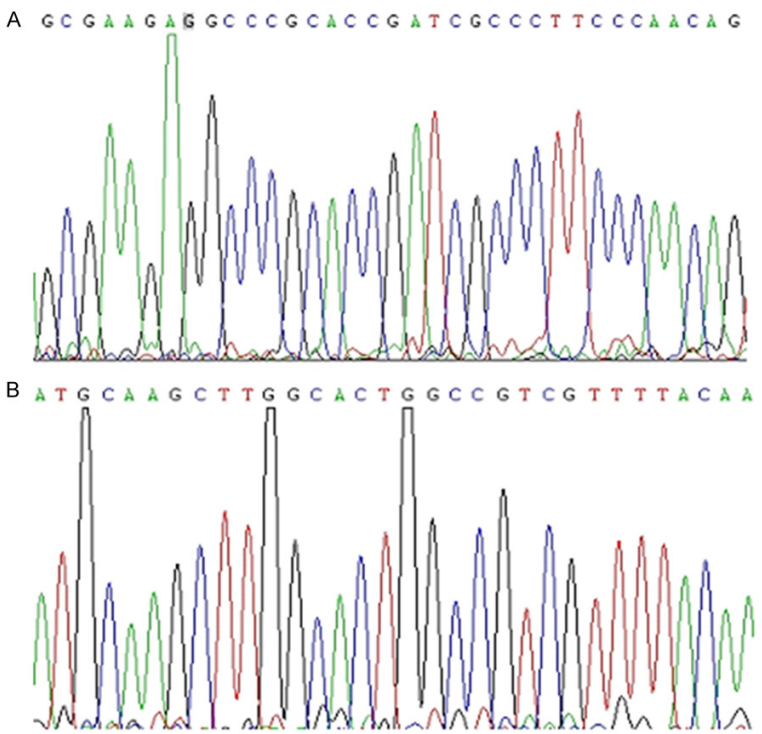

Figure 2. Examples of direct sequencing chromatogram. Bisulfite treatment. A. Methylation was found in vulvar squamous cell carcinoma. B. Methylation was found in vulva normal tissue.

obtained from the Department of Obstetrics and Gynecology, Shengjing Hospital affiliated with China Medical University from 2005 to 2013. TNM-staging and grading were assessed according to the International Federation of Gynecology and Obstetrics (FIGO, 2009) staging system and The WHO Classification of Tumours of Female Reproductive Organs. 52 cases of VSCC include Keratinizing carcinomas 36 cases, non-Keratinizing carcinomas 8 cases, Verrucous carcinomas 4 cases; Basal cell carcinoma 4 cases; tumor diameter <2 cm 16 cases, ≥2 cm 36 cases; Clinical pathological stage show 14 cases of stage I, 18 cases of stage II, 20 cases of stage III; lymph node metastasis 13 cases, without lymph node metastasis 39 cases. 35 cases of VIN include Low-grade squamous intraepithelial lesion (VIN 1) 16 cases and High-grade squamous intraepithelial lesion (VIN 2/3) 19 cases. All of the enrolled patients underwent curative surgical resection without having chemotherapy or radiation therapy. The study was approved by the Medical Research Ethics Committee of China Medical University and the informed consent was obtained from all patients.

Immunohistochemistry staining

The specimens were formalin-fixed and paraffin-embedded. Serial sections 4-μm thick were cut and mounted on aminopropyltriethoxysilane-coated glass slides. The sections were incubated with the monoclonal mouse anti-human antibody directed against SH3GL2 (diluted 1:100, Santa Cruz company) at 4°C overnight and were then treated with 3% H_2O_2 and 5% rabbit serum at 37°C for 1 h. Next, the sections were incubated with the secondary antibody and streptavidin-peroxidase complex for 30 min (SP kit, MaiXin technologies company, China). Slides were visualized using 3,3-diaminobenzidine. Sections were stained with non-specific antibodies to provide a negative control for each reaction. Staining intensity was scored semiquantitatively from 0 to 3 (0= nega-

Table 1. The correlation between SH3GL2 protein levels and gene methylation in different types of vulvar tissues

VSCC	SH3GL2 expression		χ^2	*P* value
	Positive	Negative		
SH3GL2 methylation level				0.000
Methylation	6	22	17.2	
No methylation	19	5	58	

tive, 1= weak positive, 2= strong positive). The percentage of positive staining showed in the cytoplasm staining was respectively scored as 0, 1, 2 (0= negative, 1-50%=1, 51-75%=2, ≥76%=3). The product of these two scores as the final score for each sample staining, Score ≤1 was considered negative expression, while Score >1 was considered Positive expression.

Methylation assay-bisulfite genomic sequencing PCR (BSP) combined with TA clone for sequencing

Genomic DNA was isolated from tissue samples with a DNA extraction reagent kit (Bioteke, Beijing, China) to carry out bisulfite treatment, so that unmethylated cytosine (C) was converted to thymine (T), and methylated C unchanged. The process was according to the manufacturer's protocol, and quantified.

BSP reaction PCR amplification of DNA was modified as a template. Sequences of methylation-specific primers in the exon 2 of SH3GL2 gene were: 5'-TAGTTTTYGGGGGTAGGAAT-3' (forward), downstream: 5'-CACCRACATAATA CAAAAAACC-3' (reverse). The conditions for PCR amplification were at 40 cycles of 94°C for 40 s, 56°C (annealing) for 40 s, then 72°C for 40 s.

Cutting gel block containing the target DNA fragment from electrophoresis plate, using DNA Purification Kit (Qiangen company) to recover purified PCR product, TA cloning and sequencing the PCR product accurately determined the methylation state in DNA sequences CpG sites. The process was according to the manufacturer's protocol.

CpG site unmethylated cytosine (C) converted to thymine (T), the methylated cytosine (C) constant. A single "C" and overlapping "C" and "T" at the corresponding CpG site was considered as methylation, a single "T" as no methylation.

Statistical analysis

SPSS 16.0 statistical software was used for data analyzing and processing: Data was given as mean ± SE. Statistical analysis was performed using Student's t-test for unpaired samples and comparing the differences of SH3GL2 protein expression levels in VSCC, VIN and para-cancer. Pearson correlation analysis was used to evaluate the relationships between the expression of SH3GL2 and gene methylation in VSCC and cancer-adjacent normal tissues. The χ^2 test was used to analyze the relationships between SH3GL2 expression and clinicopathological features and HPV infection status in VSCC. All *P* values less than 0.05 were considered statistically significant.

Results

Immunohistochemical results

SH3GL2 protein was mainly expressed in the cytoplasm. SH3GL2 Protein expression was positive in all 11 cases of cancer-adjacent normal tissues. The rate of positive SH3GL2 protein expression in VSCC and VIN was 48.08% (25/52) and 65.71% (23/35), respectively (**Figure 1**). SH3GL2 Protein expression was significantly lower compared with normal vulva epidermal cells, and the difference was statistically significant (χ^2=9.995, P=0.001). The positive rate in VSCC was much lower than that in VIN, but the difference was not statistically significant (χ^2=2.631, P=0.080).

MSP results

SH3GL2 abnormal gene methylation was detected in 28/52 (53.8%) cases of VSCC, while only 2 cases (18.2%) in cancer-adjacent vulva tissues (**Figure 2**). SH3GL2 promoter methylation rates in VSCC was significantly higher than the control group (P<0.05). SH3GL2 expression levels in the methylation negative group were higher than that in the methylation positive group. There was negative correlation between SH3GL2 protein levels and gene methylation (χ^2=17.258, P=0.000) (**Table 1**). It appeared that SH3GL2 protein downregulated or absented through gene methylation. HPV16, 18 positive rate was significantly higher in patients with SH3GL2 gene methylation than the negative, and correlation analysis of HPV

Table 2. Relationship between SH3GL2 gene promoter methylation levels and clinicopathological factors of vulva lesions

Clinicopathological Factors	NO.	Methylation level		χ^2	*P* value
		Yes	%		
Vulva normal tissue	11	2	18.2		
VSCC	52	28	53.8	4.630	0.032
Tumor size					
<2 cm	16	7	43.8	0.017	0.565
≥2 cm	36	21	58.3		
Age					
<60	22	12	54.5	0.008	0.578
≥60	30	16	53.3		
HPV 16/18 infection					
+	38	24	63.2	4.924	0.028
-	14	4	28.6		
Differentiation					
Well	40	21	52.5	0.126	0.492
Poor-moderate	12	7	58.3		
lymph node metastasis					
Yes	13	10	76.9	3.714	0.052
No	39	18	46.2		
TNM stage					
I + II	32	12	37.5	8.945	0.003
III	20	16	80		
VIN					
Low-grade	16	6	37.5	0.002	0.621
High-grade	19	7	36.8		

positive rate and SH3GL2 gene methylation showed closely related (r=4.924, P=0.028). Statistics showed that there was no statistically significance of SH3GL2 gene aberrant methylation in age, tumor size, histological differentiation degree (well vs. poor-moderate differentiated, P=0.492) of VSCC and difference VIN grade (P=0.621). SH3GL2 promoter methylation was 46.2% (18/39) in no lymph node metastasis group, which was much lower than VSCC with lymph node metastasis, but there were no significant statistical significance differences between the two groups. SH3GL2 gene methylation level is significantly associated with TNM stage (I + II vs. III, P=0.003) (**Table 2**).

Discussion

Current researches have found that SH3GL2 expression reduced in several malignant tumors, but there were no relevant reports in vulva squamous cell carcinoma. In this study, we demonstrated that SH3GL2 also presented in normal vulva epidermal tissues by immunohistochemistry, and expressed in the cytoplasm, but the expression was significantly decreased in VIN and VSCC, which the difference was statistically significant. This can be inferred that SH3GL2 acts as a tumor suppressor gene in VSCC development process.

DNA methylation connected the methyl groups of S-adenosine tryptophan methyl sulfide (SAM) to the five-carbon position of CpG island cytosine ring, and the whole process was catalyzed by DNA methy transferase (DNMT) [9]. CpG island hypermethylation in Promoter region can cause tumor suppressor gene silence, and lead to tumor development [10]. In order to investigate the reason of SH3GL2 downregulation or absence in VSCC due to gene aberrant methylation, we predicted SH3GL2 CpG island location through software analysis, and found the gene promoter methylation in VSCC by Bisulfite sequencing PCR and T's and A's method. Moreover there was significant difference compared with the control tissues. Therefore, we hypothesized that aberrant methylation might be the main reason of SH3GL2 downregulation or absence in VSCC.

Recently, some studies have found that SH3GL2 gene methylation participated in a variety of tumors occurrence and development. SH3GL2 loss was found to be associated with poor prognosis of urothelial carcinoma patients [5]. SH3GL2 was also frequently deleted in laryngeal squamous cell carcinoma which was closely related to tumor development and progression [11]. Furthermore, by statistical analysis of the relationships between SH3GL2 promoter methylation and clinicopathological parameters of VSCC. We found that SH3GL2 promoter methylation rates decreased with the TNM staging increase of VSCC, and the differ-

ence was statistically significant. This result suggests that SH3GL2 promoter methylation may be related to the development of VSCC.

Currently as we known, HPV16, 18, 6 and 11 infections are the main cause of HPV-related disease in anal, genital, head and neck and lung and other body parts [12]. 20% to 60% Human papillomavirus (HPV) deoxyribonucleic acid (DNA) can be detected in vulvar carcinoma, among which HPV16 is the major type [13]. We discussed the relationship between SH3GL2 promoter methylation rates and HPV16, 18 infection status, and the results showed that SH3GL2 promoter methylation was closely related to HPV16, 18 subtype infection status. Patients with SH3GL2 promoter methylation were more susceptible to HPV16, 18 infection, and the difference was statistically significant. But further studies are needed to discuss whether SH3GL2 promoter methylation participates in HPV infection of VSCC and its mechanism.

In conclusion, this study demonstrated that SH3GL2 was all positive expression in paracancer, downregulated or absented in VSCC, which was related to SH3GL2 gene aberrant methylation. In addition, SH3GL2 positive expression rate decreased with TNM staging increase of VSCC, and was associated with HPV infection status. This study suggests that SH3GL2 expression silencing caused by gene abnormal methylation may play an important role in the development of VSCC. The research of SH3GL2 gene demethylation, to restore endogenous protein expression will help to provide a new direction and idea for VSCC treatment.

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Disclosure of conflict of interest

None.

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