Original Article
Slit2/Robo1 is downregulated in cervical carcinoma and inhibits tumor progression by regulating β-catenin

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Abstract: Objectives: To determine the expression and clinical value of Slit2 and its receptor Robo1 in cervical squamous carcinoma (SCC), cervical intraepithelial neoplasia (CIN) and normal cervical epithelium (NCE) samples. In addition, the roles and the possible mechanisms of Slit2 in tumor progression of cervical carcinoma were also investigated. Methods: Immunohistochemical (IHC) staining and real time PCR were carried out to detect the expression of Slit2/Robo1 in 55 cases of SCC, 30 cases of CIN I-II, 30 cases of CIN III and 20 cases of NCE. Cell proliferation ability was monitored by using CCK-8 assay after Slit2 was inhibited in Hela cells. Immunobloting and IHC methods were used to detect the expression of β-catenin in Hela cells and cervical carcinomas, respectively. Results: The expression of Slit2 and Robo1 gradually declined in NCE, CIN and SCC tissues and the differences were of statistical significance (P<0.05). We also found that Slit2 and Robo1 protein expression positively correlated (P<0.05) with each other in cervical carcinomas. Besides, we surprisingly found that Slit2 expression positively correlated with clinical stage, the depth of invasion and lymph node metastasis of cervical carcinoma. Inhibition of Slit2 in Hela cells promoted cell proliferation and the expression of β-catenin was elevated. Finally we found that Slit2 and β-catenin expression was negatively correlated in cervical carcinoma (P<0.05). Conclusion: Slit2/Robo1 expression maintained at low levels in cervical carcinoma specimens and exerted an anti-proliferating function by negatively regulating β-catenin. The tumor suppressing Slit2/Robo1 might serve as potential therapeutic targets in cervical carcinoma treatment.

Keywords: Slit2, Robo1, cervical carcinoma, cell proliferation, β-catenin

Introduction

Slit guidance ligand 2 (slit 2) is a member of the slit family of secreted glycoproteins, which are ligands for the Robo (roundabout guidance receptor) family of immunoglobulin receptors. Slit/Robo signaling was first identified in nervous systems and established as an extracellular signature to guide axon path finding, promote axon branching and control neuronal migration [1]. Studies also indicated that it may also have functions during other cell migration processes including leukocyte migration [2], and several cell signaling pathways including cell proliferation, cell motility and angiogenesis [3-5]. Many reports had also suggested that aberrant Slit/Robo expression in cells can lead to cancer development, progression and metastasis.

Cervical carcinoma constitutes a significant public health problem, especially in developing countries, like Mexico [6]. It remains to be one of the leading causes of cancer-related death in women despite much efforts have been made in screening, diagnosis, prevention, and treatment. Several key factors that contribute to cervical carcinoma development have already been identified, such as Human Papillomavirus (HPV) and intrinsic genetic factors, the latter show great potential for use as susceptibility or prognosis factors. In cervical carcinoma, the expression of Slit proteins (Slit 1-3) and Robo1, 3 were downregulated or completely suppressed [7]. Since there are still few studies concerning the expression, function, especially the clinical value of Slit2/Robo1 in cervical carcinoma, we set to explore the above questions and also to discuss the

putative mechanism underlying its anti-tumor activities.

Methods and materials

Patients and tissue samples

The study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Fujian Medical University. Written informed consent from all patients was obtained prior to participation in the study. A total of 20 normal cervical epithelium (NCE) tissue samples, 60 cervical intraepithelial neoplasia (CIN, CIN I-II 30 and CIN III 30) tissue samples and 55 cervical squamous carcinoma (SCC) tissue samples were obtained from patients from 2009.9 to 2011.6 for immunohistochemistry assay. A total of 20 NCE tissue samples, 20 CIN III tissue samples and 20 SCC tissue samples were obtained from patients from 2011.6 to 2011.12 for RNA isolation and PCR assay. All samples were immediately dissected, formalin-fixed and paraffin-embedded for IHC or snap-frozen, and kept at -80°C for qRT-PCR until use.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissue sections from tissue samples were selected. Robo1 and Slit2 were stained by SP method. In brief, 4 μm tissue sections were subsequently dewaxed and rehydrated using xylene and graded alcohol washes. Antigen retrieval was performed at 121°C for 3 min, by using pH6.0 citrate buffer. After blocking with hydrogen peroxide and normal horse serum, the sections were incubated with primary antibody against Robo1 or Slit2 for 15 h at 4°C. The sections were then incubated with biotinylated secondary immunoglobulin and peroxidase-conjugated streptavidin. The enzyme substrate was DAB.

Cell lines and cell culture

Human cervical carcinoma cell lines Hela were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). Hela cells were cultured in DMEM medium (Gibco) supplemented with 10% FBS (Gibco), 100 units/ml of penicillin, and 100 mg/ml of streptomycin at 37°C, a humidified atmosphere containing 5% CO_2 incubator.

Transfections and short interfering RNAs

Hela cells were plated in 6-well plate and cultured in DMEM full medium until cell density reached 60% prior to small interfering RNAs (siRNAs) transfection. When transfecting cells with siRNA, Lipofectamine 2000 (Invitrogen) were employed according to the manufacturer's instructions. The sequences of the siRNAs were synthesized and purchased from GenePharma (Shanghai, China). The cells were harvested 48 h after transfection.

Cell proliferation assay

Logarithmic growth phase cells were seeded at a density of 5×10^3 cells per well in a 96-well plate containing 100 μl DMEM full medium. Cell Counting Kit-8 (Dojindo) reagents was added at 12 h, 24 h, 48 h, and 72 h after seeding (0 h) and incubated at 37°C for 2 h. The data of optical density of OD450 were measured by a microplate reader (Molecular Device). Each experiment was performed at least three times with six replicates.

Reverse transcript PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR)

The total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The specific expression of *Slit2* gene in tissue samples was assessed using RT-PCR and qRT-PCR. RT-PCR was performed in a regular way. The primers for RT-PCR are as follows:

Slit2: F: 5'-GCAGTAAAGTCCTCTCTACTTTAG-3'; R: 5'-CAGTAACTTGACATGTTTCTTTAGG-3'. *β-actin*: F: 5'-TGACGGGGTCACCCACACTGTGC-3'; R: 5'-CTAGAAGCATTTGCGGTGGACGA-3'.

Real-time PCR was performed on ABI7500 Two Step Real-Time PCR System using the comparative Ct quantization method. Real-time Master Mix (Toyobo) was used to detect and quantify the expression level of genes. β-actin was as internal control for RT-PCR or qRT-PCR.

Western blotting

Total proteins were extracted by RIPA buffer (Beyotime). 40 μg protein were separated by SDS-PAGE and transferred onto NC membrane. Membranes were blocked by 5% non-fat milk for 1 h at RT and incubated at 4°C overnight
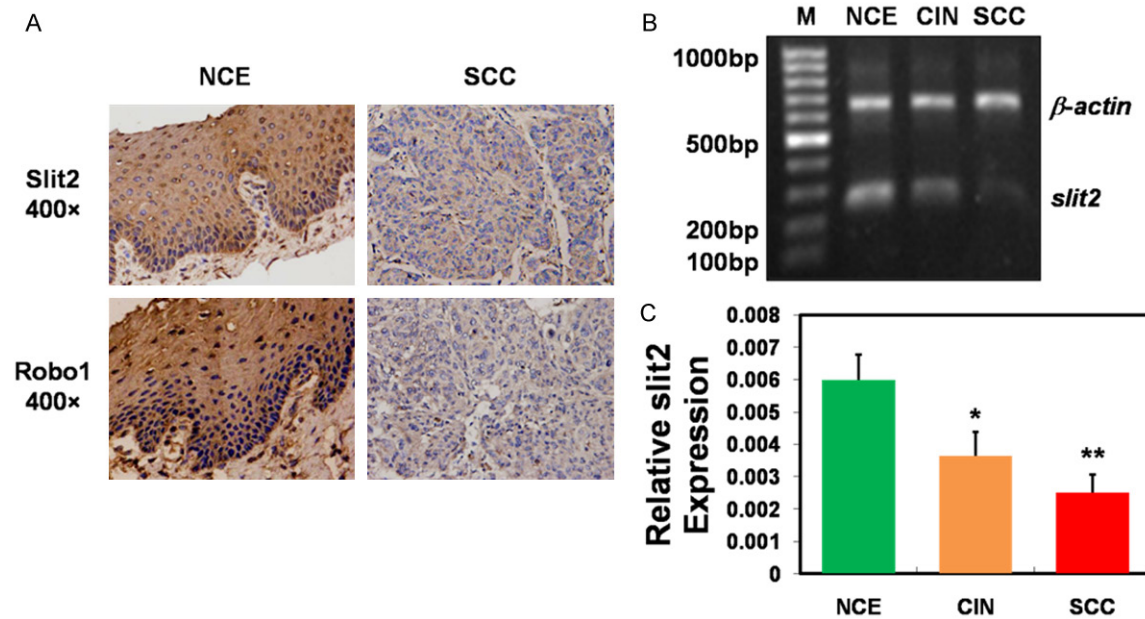
Figure 1. Robo1 and Slit2 was downregulated in human cervical squamous carcinoma samples. A. IHC analysis of the expression of Slit2 and Robo1 protein in normal cervical epithelium (NCE) and cervical squamous carcinoma (SCC) tissue samples. B. RT-PCR analysis of expression of *Slit2* mRNA expression in NCE, cervical intraepithelial neoplasia (CIN III) and SCC tissue samples. C. QRT-PCR analysis of expression of *Slit2* mRNA expression in NCE, CIN III and SCC tissue samples (N=20). *: *P*<0.05, **: *P*<0.01.

Table 1. Difference among positive expressions of Slit2 and Robo1 in NCE, CIN and SCC

Category	N	Slit2		Robo1	
		+%	κ^2	+%	κ^2
NCE	20	95.0	23.210*	90.0	17.642*
CIN	60	55.0		66.7	
SCC	55	32.7		40.0	
CIN I-II	30	70.0	5.455*	80.0	4.800 *
CIN III	30	40.0		53.3	

*: P<0.05.

with primary antibodies. The next day, membranes were washed with PBST and incubated with secondary antibodies conjugated to HRP. Immunoreactive bands were visualized and analyzed using a chemiluminescent substrate (Thermo) and automatic chemical luminescence analysis system (Bio-Rad). The antibodies used in this study were β-actin (1:6000, Sigma), ROBO1 (1:1000, Santa Cruz), Slit2 (1:1000, Santa Cruz) and β-catenin (1:1000, Cell Signaling Technology).

Statistical analysis

Statistical analysis was performed using SPSS software v18.0. Two-tailed Student's t-test and Chi-square analysis were performed to analyze the data. The results are expressed as the mean ± S.D. A *P* value less than 0.05 was considered to be statistically significant.

Results

Slit2/Robo was downregulated in cervical carcinoma

By utilizing the tissue array containing 155 cases of cervical carcinoma and normal cervical tissues (55 cases of SCC, 30 cases of CIN I-II, 30 cases of CIN III and 20 cases of NCE), we performed IHC staining to determine the protein expression of both Slit2 and its receptor Robo1. Brown granules in cytoplasm indicated for positive protein attribution. As shown in **Figure 1A**, we observed strong positive signals of both Slit2 and Robo1 in NCE tissues, whereas weak expression of both proteins in SCCs. We then analyzed the expression concentrations of these two proteins in SCC, CIN and NCE tissues, and found a fine gradually downregulation of Slit2 and Robo1 in NCE, CIN and SCCs, and the differences were of significance between each other groups (P<0.05, **Table 1**). Besides, we also found that Slit2 and Robo1 were more decreased in CIN III than CIN

Table 2. Correlation of Slit2 expression with clinicopathologic features of cervical carcinoma

Features	N	Slit2		κ^2	P
		+ (%)	- (%)		
Age/yr					
≥45	29	9 (31.0%)	20 (69.0%)	0.080	0.778
<45	26	9 (34.6%)	17 (65.4%)		
Clinical stage					
Ia-Ib	20	10 (50.0%)	10 (50.0%)	4.259	0.039*
IIa	35	8 (22.9%)	27 (77.1%)		
Depth of myometrium invasion					
≤1/2	12	8 (66.7%)	4 (33.3%)	6.180	0.013*
>1/2	43	10 (23.3%)	33 (76.7%)		
Differentiation					
Well-moderate	31	10 (32.3%)	21 (67.7%)	0.007	0.933
Poor	24	8 (33.3%)	16 (66.7%)		
Volume/cm					
≥4	19	6 (31.6%)	13 (68.4%)	0.017	0.895
<4	36	12 (33.3%)	24 (66.7%)		
Lymphnode metastasis					
Positive	17	2 (11.8%)	15 (88.2%)	4.911	0.027*
Negative	38	16 (42.1%)	22 (57.9%)		

*: $P<0.05$.

I-II ($P<0.05$, **Table 1**). On the other hand, we isolated RNA from 60 cases of fresh clinical specimens containing 20 cases of NCE, 20 cases of CIN III and 20 cases of SCC. Reverse Transcript PCR and quantitative Real time PCR was done for Slit2 mRNA expression in these specimens. As shown in **Figure 1B** and **1C**, we found Slit2 mRNA presented similar downregulation in cervical carcinomas like that of IHC results. All the above data demonstrated that Slit2 and Robo1 were obviously downregulated in higher grades of cervical carcinomas than lower grades and normal tissues.

Association of Slit2 expression with clinical-pathological features

To evaluate the clinical value of Slit2 in cervical carcinoma, we used κ^2 test to statistically analyzed the association of its protein expression with the detailed clinicalpathological features among SCC patients including age, clinical stage, the depth of invasion, lymphnode metastasis, tumor differention and tumor volume. Surprisingly, we found that Slit2 expression positively and significantly correlated with clinical stage, the depth of invasion and lymphnode metastasis, but not correlated with other features of these patients (**Table 2**). This finding suggested that Slit2 might be served as a potent prognosis indicator in cervical carcinoma.

Slit2/Robo1 inhibited hela cell proliferation and negatively regulated β-catenin expression

Since we had already found a downregulation of Slit2/Robo1 in cervical carcinoma, we next wanted to better understand their roles in tumor progression of this disease. We ordered two independent siRNA oligos targeting to Slit2 and performed transfection experiments in Hela cells. Robo1 was inhibited similarly by another two siRNAs. As a result, we found that both Slit2 and Robo1 suppression could finely accelerated cell proliferation of Hela cells as determined by CCK-8 assy (**Figure 2A**, **2B**). Confirmedly, Slit2/Ro-bo1 could be silenced over 50-70% than negative control (NC)-treated cells (**Figure 2C**, **2D**). Since numerous reports had considered that Slit2 exerted its anti-cancer function by deregulating β-catenin [8-10], one of the major oncogenic pathways responsible for malignant behaviors of cancer cells, we detected the expression of β-catenin in Slit2/Robo1-inhibited cells. As shown in **Figure 2E-H**, we found that both mRNA and protein levels of β-catenin were increased, which might partially explained for the pro-growth activities of Sli2 and Robo1 in Hela cells.

Slit2 protein negatively correlated with β-catenin in cervical carcinoma

Finally, we performed IHC staining for β-catenin expression in cervical carcinoma and analyzed the correlation of its expression with Slit2. The results came out that β-catenin expression was negatively correlated with the expression of Slit2 in cervical carcinoma ($P<0.05$, **Table 3**), which was in line with our cellular results in
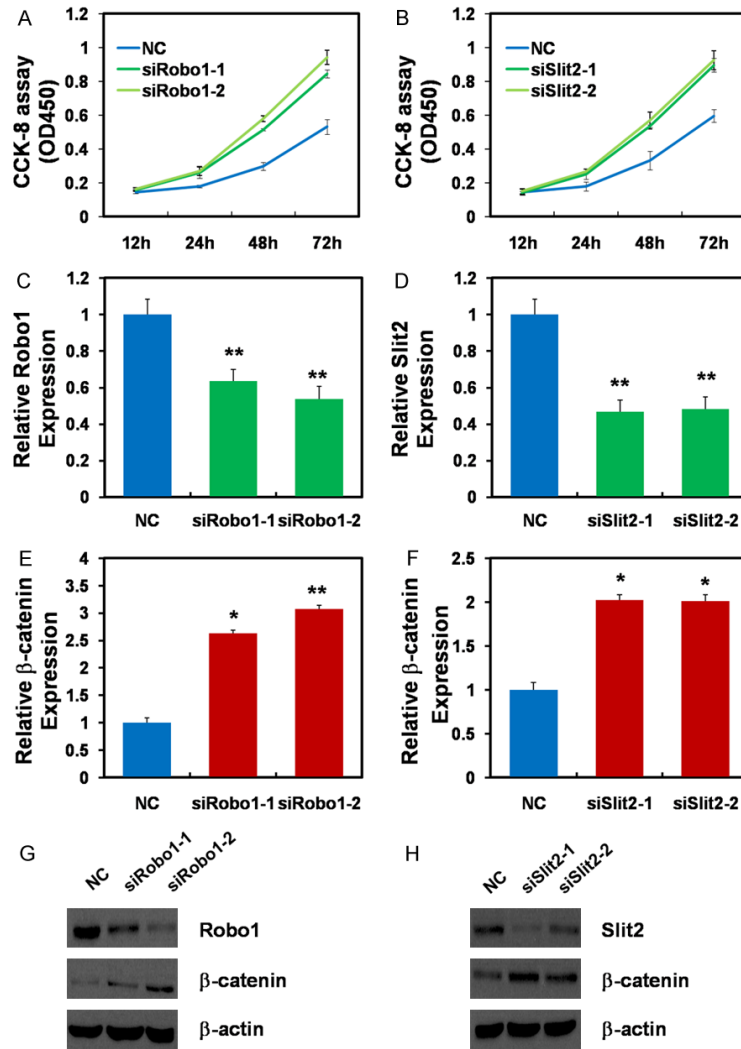
Figure 2. Slit2/Robo1 inhibited Hela cell proliferation through negatively regulated β-catenin expression. A, B. CCK-8 assay were performed in Hela cells transfected with Slit2/Robo1 siRNAs. C, D. Expression of Slit2/Robo1 mRNA level was detected by qRT-PCR in Hela cells treated with Slit2/Robo1 siRNAs and negative controls (NC). E, F. Expression of β-catenin mRNA level was detected by qRT-PCR in Hela cells treated with Slit2/ROBO1 siRNAs and negative controls (NC). G, H. Inactivation of Slit2/Robo1 with siRNAs significantly elevated the expression of β-catenin in Hela cells. *: $P<0.05$, **: $P<0.01$.

as promising early diagnostic and directed therapeutic targets. Facing and developing more efficient therapy targets for this disease are urgently desired. Slit2 is known to function through Robo receptors as a repulsive axon guidance cue. Robo1 and Robo4 are two receptors of the secretory protein Slit2, in which Robo1 is mainly expressed in tumoral cells. It is also known that the activation or suppression of the Slit/Robo pathway modulates several oncogenic signaling pathways that are associated with the development and progression of cancer [12].

In our study, we observed a downregulation of Slit2/Robo1 in cervical carcinoma tissues, with the lowest expression level in advanced forms of cervical carcinoma. We also found that the decreased expression of Slit2 was correlated with higher clinical stages, the depth of invasion and lymph node metastasis of cervical carcinoma, which suggested the critical clinical value of Slit2 in prognosis of this disease. Functionally, we performed siRNA-mediated gene silencing of Slit2/Robo1 in Hela cells, and found that Slit2/Robo1 inhibition could lead to faster cell proliferation. The pro-growth function of Slit2/Robo1 might be interpreted by the negative regula-

Figure 2E-H. However, the internal mechanisms remained largely unclear and needs to be studied in future.

Discussion

Cervical carcinoma remains as the 4th leading cause of death and the 2nd leading cause of mortality in females worldwide aged 19-39 years [11]. Molecular pathways involved in cervical malignant transformation have presented

tion of β-catenin, and our IHC method finally verified that Slit2 expression inversely correlated with β-catenin protein.

It might be of little importance that we found a downregulation of Slit2/Robo1 in cervical carcinoma, since a previous report [7] had already concluded from their studies. They also demonstrated that the suppression of Slit/Robo proteins in cervical cancer is due to the promoter hypermethylation, which is an early event in

Table 3. Correlations of Slit2 expression with Robo1 and β-catenin expressions in SCC

Group	Expression	N	Slit2 + (%)	-	P
Robo1	+	22	17 (77.27)	5	0.000*
	-	33	1 (0.03)	32	
β-catenin	+	42	6 (14.29)	36	0.000*
	-	13	12 (92.31)	1	

*: P<0.001.

tumor progression [7]. We did not look back to the mechanisms governing the downregulation of Slit2/Robo1, but concentrated on the function of Slit2/Robo1 inhibition in cervical carcinoma cells. We mainly performed gene silencing experiments because the large molecular weight of Slit2/Robo1. We found that either Slit2 or Robo1 suppression could lead to enhanced cell proliferation capacity of Hela cells. Nevertheless, we did not evaluate other biological roles of Slit2/Robo1 pathway such as metastasis processes. According to the correlation analysis results between Slit2 expression and clinicalpathological features of cervical carcinoma patients, we thought that Slit2/Robo1 might also impact on tumor cell migration and invasion. Indeed, in other kinds of human solid tumors. Indeed, current reports suggest that Slit2 and its receptor Robo1 inhibit metastasis in breast cancer [13], fibrosarcoma and squamous cell carcinoma [14]. Another question needed to be resolved is how Slit2/Robo1 negatively regulated β-catenin expression. Substantial work is under way for better illustrating Slit2/Robo1 in cervical carcinoma.

In summary, our data established that Slit2 and its receptor Robo1 were both downgulated in cervical carcinoma, and inhibited cell proliferation at least through targeting β-catenin.

Disclosure of conflict of interest

None.

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