Original Article
Elevated expression of flotillin-1 is associated with lymph node metastasis and poor prognosis in early-stage cervical cancer

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Abstract: Accumulating evidence has revealed that the expression of the lipid raft protein flotillin-1 is elevated in various human cancers, but the role flotillin-1 plays in the carcinogenesis of cervical cancer remains unclear. The expression profile of flotillin-1 was assayed using real-time PCR, western blotting, and immunohistochemical (IHC) staining in cervical cancer cell lines and cancer tissues with paired adjacent noncancerous cervical tissues. The expression of flotillin-1 protein was detected by IHC staining in a large cohort of 308 paraffin-embedded cervical cancer tissues. Ectopic expression and the short hairpin RNA interference approach were employed to determine the role of flotillin-1 in cervical cancer cell metastasis and the possible mechanism involved. Flotillin-1 expression protein and mRNA were significantly upregulated in cervical cancer cell lines and cancer tissues; elevated expression of flotillin-1 protein in early-stage cervical cancer was significantly associated with pelvic lymph node metastasis (P < 0.001), and was an independent predictive factor of poor overall survival. Moreover, flotillin-1 up- and downregulation remarkably affected cervical cancer cell motility and invasion, respectively, through epithelial-mesenchymal transition (EMT) regulated by the Wnt/β-catenin and nuclear factor-κB (NF-κB) pathways. Our results suggest that flotillin-1 facilitates cervical cancer cell metastasis through Wnt/β-catenin and NF-κB pathway-regulated EMT and that the flotillin-1 expression profile serves not only as novel predictor of pelvic lymph node metastasis, but also as neoteric risk factor for patients with early-stage cervical cancer.

Keywords: Flotillin-1, cervical cancer, lymph node metastasis, prognosis, EMT, Wnt/β-catenin and NF-κB pathways

Introduction

Cervical cancer is the fourth most common cancer and the fourth most frequent cause of cancer death in women globally, with 527,600 new cases and 265,700 deaths in 2012 [1]. Moreover, nearly 90% of new cases occur in developing countries [1]. In China, the largest developing country in the world and where neither nationwide screening programs nor vaccines against human papillomavirus are available, there were 87,982 new cases and 23,375 deaths from cervical cancer in 2011 [2], rendering cervical cancer one of the heaviest health burdens for Chinese women.

Although not included in the International Federation of Gynecology and Obstetrics (FIGO) staging system, it has been determined that retroperitoneal lymph node metastasis is the most important prognostic risk factor in early-stage cervical cancer (FIGO stage IB-IIA), which is an indication for adjuvant pelvic radiation plus concurrent cisplatin-based chemotherapy after primary surgery [3]. The survival rates of patients with stage I cancer are reported as 80-98%, but decrease dramatically to 50% when lymph node metastasis is present [4]. Unfortunately, sufficiently accurate models for predicting lymph node metastasis both preoperatively and intraoperatively remain beyond

Table 1. Clinical/pathological characteristics of the study cohort (n = 308)

Clinical/pathological Characteristics	No. of Patients (%)
Age	Years
Range	20-71
Mean	41.7±9.1
Median	40
SCCA, ng/ml*	
≤ 1.5	191 (66.4)
> 1.5	97 (33.7)
FIGO stage	
IB1	173 (56.2)
IB2	74 (24.0)
IIA1	28 (9.1)
IIA2	33 (10.7)
Histological type	
Squamous cell carcinoma	271 (88.0)
Adenocarcinoma	21 (6.8)
Adenosquamous	16 (5.2)
Differentiation grade	
G1	21 (6.9)
G2	118 (38.6)
G3	167 (54.6)
Tumor size, cm	
< 4	196 (65.3)
≥ 4	104 (34.7)
Deep stromal Invasion	
No	135 (45.9)
Yes	159 (54.1)
Positive parametrium	
No	301 (97.7)
Yes	7 (2.3)
Positive surgical margin	
No	286 (92.9)
Yes	22 (7.1)
LVSI**	
No	257 (83.4)
Yes	51 (16.6)
Lymph node metastasis	
No	257 (83.4)
Yes	51 (16.6)
Follow up years	
Range	0.1-11
Mean	6.0±2.1
Median	6
Recurrence	
No	266 (86.4)
Yes	42 (13.6)
Vital status at follow-up	
Alive	274 (88.6)
Death from CC	34 (11.0)
Expression of flontilin-1	
Low or none	192 (60.8)
High	116 (36.7)

*SCCA: Squamous cell carcinoma antigen. **LVSI: Lymphovascular space involvement.

clinical usage, while the molecular mechanism of lymph node metastasis remains a conundrum.

Lipid rafts are stero- and sphingolipid-enriched small cell membrane microdomains that serve as signaling and sorting platforms for numerous molecules involved in various biological processes [5]. Flotillins, or reggies, have two homologous members: flotillin-1/reggie-2 and flotillin-2/reggie-1, and are scaffolding proteins of lipid rafts and are generally considered lipid microdomain markers [6]. Evolutionarily conserved and universally expressed, flotillins are involved in various cellular processes, including membrane protein recruiting, membrane receptor signaling, cell proliferation, cell adhesion, and as recently revealed, tumorigenesis [7-9]. In particular, flotillin-1 dysregulation has been found in several cancers of epithelial origin, namely breast cancer, hepatocellular carcinoma, and non-small cell lung cancer [10-13]. However, whether flotillin-1 dysregulation also occurs in cervical cancer and the possible role flotillin-1 plays in cervical cancer metastasis remains to be elucidated.

Material and methods

Patients and tissue specimens

The study cohort enrolled 308 patients with early-stage cervical cancer (FIGO stage IB-IIA) who underwent radical hysterectomy and lymphadenectomy at the Department of Gynecologic Oncology, the Third Affiliated Hospital of Kunming Medical University (Yunnan Tumor Hospital), Kunming, China, from January 2004 to December 2009. Patients who received neoadjuvant chemotherapy and/or radiation prior to surgery were excluded. Clinical stage was assessed according to the revised FIGO staging for carcinoma of the cervix [14]; tumor pathological type and differentiation grade were defined according to World Health Organization criteria [15]. **Table 1** outlines the clinical/pathological features of the study cohort. The Human Ethics Committee and the Research Ethics Committee of the Third Affiliated Hospital of Kunming Medical University (Yunnan Tumor Hospital) approved this study. All samples were obtained with the consent of each patient, and their privacy was maintained.

Follow-up data were collected retrospectively through medical records.

Cell culture

Primary human foreskin keratinocyte (hKC) cells and four cervical cancer cell lines stored at our laboratory were originally obtained from American Type Culture Collection (Manassas, VA). HeLa, C33A, SiHa, and hKC cells were cultured in Eagle's minimum essential medium (GIBCO BRL, Rockville, MD); Ca Ski cells were cultured in RPMI 1640 medium (GIBCO BRL). All cell lines were supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT).

Vectors, retroviral infection, and transfection

Flotillin-1 overexpression and silencing plasmids were constructed as previously described [16], as was retroviral infection [16]. Stable cell lines expressing vector, flotillin-1, scramble, or flotillin-1 RNA interference (RNAi) were selected for 10 days with puromycin (0.5 μg/mL). Reporter plasmids containing wild-type (CCTTGATC; TOP-flash) or mutant (CCTTTGGCC; FOP-flash) T cell factor/lymphocyte enhancer factor (TCF/LEF) DNA binding sites (Upstate Biotechnology, Lake Placid, NY) were used to quantitatively examine β-catenin/TCF activity. Phosphorylated nuclear factor-κB-luciferase (pNF-κB-luc) and control plasmids (Clontech, Mountain View, CA) were used to determine NF-κB activity. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols.

RNA extraction and real-time quantitative PCR

Total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's manual. Complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase (Promega, Fitchburg, WI). Real-time PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green I dye (Roche, Penzberg, Germany). Expression data were normalized to the geometric mean of the housekeeping gene glyceraldehyde-3-phosphate (*GAPDH*) to control the variability in expression levels and were calculated as $2^{-[(Ct \text{ of gene})-(Ct \text{ of } GAPDH)]}$, where

Ct represents the threshold cycle for each transcript. Table S1 lists the probe sequences used for the real-time PCR.

Western blotting

Western blotting was performed as previously described [16] with anti-flotillin-1 (Sigma-Aldrich, Saint Louis, MO) and anti-E-cadherin, anti-Vimentin and anti-N-cadherin (BD Transduction Laboratories, Lexington, UK) mouse monoclonal antibodies. The membranes were stripped and re-probed with anti-GAPDH mouse monoclonal antibody (Sigma-Aldrich) as the loading control.

Immunohistochemical (IHC) staining

IHC staining was performed as previously described [16] using Histostain-Plus Kits (Invitrogen) according to the manufacturer's protocols. Known immunostaining-positive and -negative slides were used as the controls.

Evaluation of IHC staining

Two pathologists (W. Lin and C. Yang) blinded to the clinical covariates evaluated the flotillin-1 staining independently. The staining results were scored based on: (a) percentage of positive tumor cells in the tumor tissue: 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), 4 (> 75%); and (b) staining intensity: 0 (no staining), 1 (weak staining), 2 (moderate staining), 3 (strong staining). The immunoreactivity score (IRS, ranging 0-12) was then calculated by multiplying the percentage score by the intensity score [17]; both nuclear and cytoplasmic expression were scored as positive staining. The final score was stratified as - (IRS = 0), + (IRS = 1-4), ++ (IRS = 5-8), and +++ (IRS = 9-12). In this study, - to + was considered low expression; ++ to +++ was considered high expression.

Transwell migration assay

Cells (1×10^4) were plated on the top side of Matrigel-coated polycarbonate Transwell filters (BD Biosciences, San Jose, CA) and incubated at 37°C for 20 h. Cells inside the upper chamber were removed with cotton swabs. Cells that migrated to the bottom surface of the membrane were fixed in 1% paraformaldehyde, stained with hematoxylin, and 10 random fields
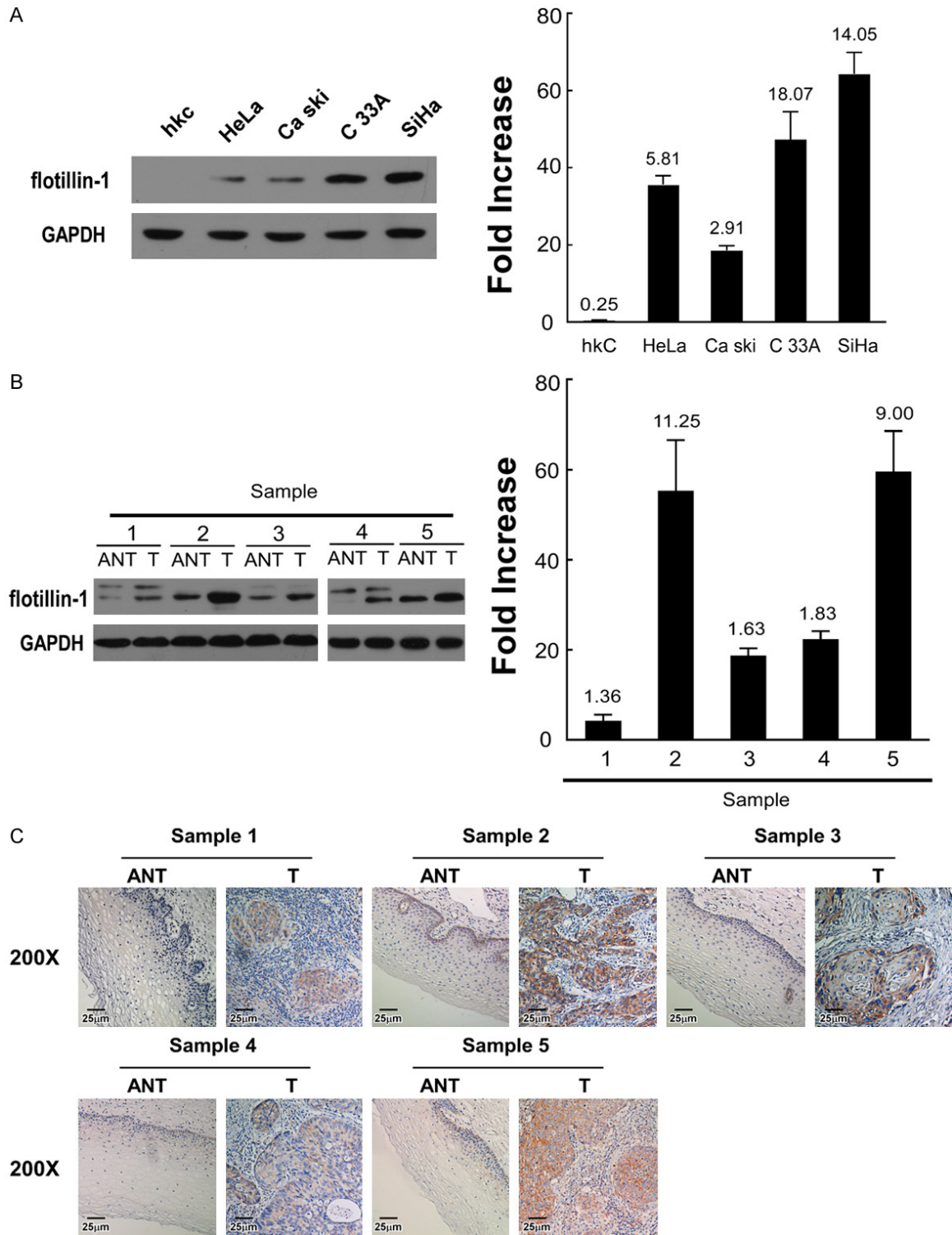
Figure 1. Expression of flotillin-1 protein and mRNA in cells and tissues. A. Expression of flotillin-1 protein (left) and mRNA (right) in hKC cells and HeLa, Ca Ski, C33A, and SiHa cervical cancer cells. B. Expression of flotillin-1 protein (left) and mRNA (right) in five paired cervical cancer (T) and adjacent noncancerous cervical tissues (ANT). Expression levels were normalized by GAPDH. Error bars represent SD calculated from three parallel experiments. C. IHC assay of flotillin-1 protein expression in five paired cervical cancer (T) and adjacent noncancerous cervical tissues (ANT).
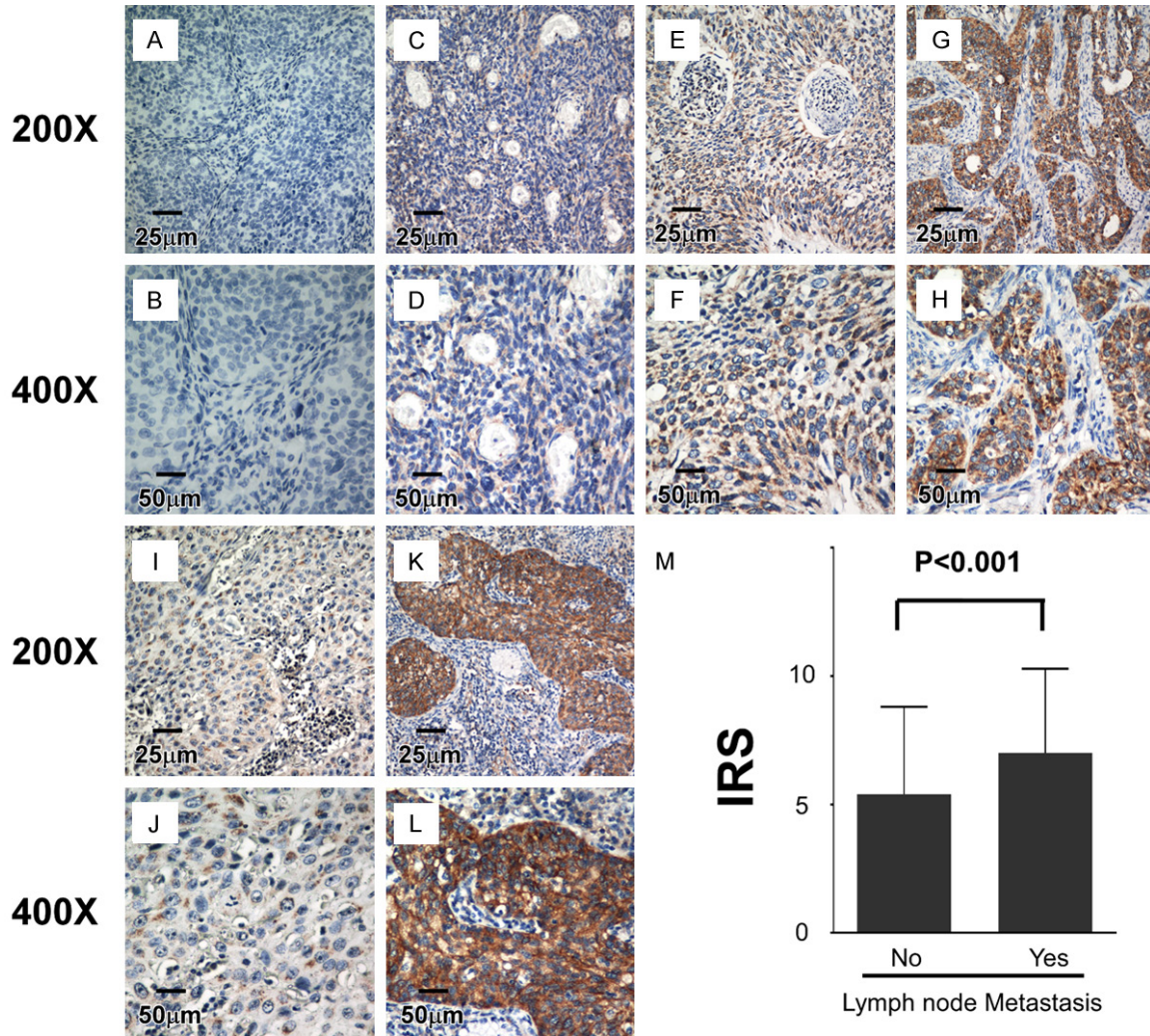
Figure 2. IHC assay of flotillin-1 protein expression in 308 paraffin-embedded cervical cancer tissues. A, B. Flotillin-1 expression was not detectable in cervical cancer tissues (this patient also did not present lymph node metastasis). C, D. Representative images of weak flotillin-1 staining in cervical cancer tissues. E, F. Representative images of moderate flotillin-1 staining in cervical cancer tissues. G, H. Representative images of strong flotillin-1 staining in cervical cancer tissues. I, J. Representative images of moderate flotillin-1 staining in cervical cancer tissues without lymph node metastasis. K, L. Representative images of strong flotillin-1 staining in cervical cancer tissues with lymph node metastasis. M. Average IRS of flotillin-1 in patients with and without lymph node metastasis. Error bars represent SD; *p*-value was calculated by Spearman correlation analysis.

per well were counted under × 200 magnification. Three independent experiments were performed and the data are presented as the mean ± standard deviation (SD).

Wound healing assay

Cells were seeded on 6-well plates with Eagle's minimum essential medium containing 10% FBS and grown to monolayer confluence. Straight wounds were created in the monolayers using a sterile pipette tip. Images were captured and documented at 0, 12, 24, and 36 h

after wounding using an inverted (Olympus, Shinjuku, Tokyo, Japan) IX50 microscope with 10 × objective lens and Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Each experiment was repeated three times.

Luciferase assay

Cells (3×10^4) were seeded in triplicate in 48-well plates and allowed to settle for 24 h. Reporter plasmid (0.1 μg) or control luciferase plasmid (0.1 μg) plus 1 ng pRL-TK *Renilla* plasmid (Promega) were transfected into the cells.

Table 2. Clinical/pathological characteristics of patients with cervical cancer and flotillin-1 expression profile (n = 308)

Characteristics	N (%)	Low or non no. (%)	High no. (%)	χ² test P
Age (years)				0.788
≤ 40	144 (46.8)	86 (27.9)	58 (18.8)	
> 40	164 (53.2)	106 (34.4)	58 (18.8)	
SCCA (ng/ml)*				0.727
≤ 1.5	191 (66.4)	124 (43.1)	67 (23.3)	
> 1.5	97 (33.7)	58 (20.1)	39 (13.5)	
FIGO stage				0.500
IB1	173 (56.2)	109 (35.4)	64 (20.8)	
IB2	74 (24.0)	49 (15.9)	25 (8.1)	
IIA1	28 (9.1)	14 (4.5)	14 (4.5)	
IIA2	33 (10.7)	20 (6.5)	13 (4.2)	
Histological type	308			0.082
Squamous cell carcinoma	271 (88.0)	175 (56.8)	96 (31.2)	
Adenocarcinoma	21 (6.8)	9 (2.9)	12 (3.9)	
Adenosquamous	16 (5.2)	8 (2.6)	8 (2.6)	
Differentiation grade				0.636
G1	21 (6.9)	12 (3.9)	9 (2.9)	
G2	118 (38.6)	77 (25.2)	41 (13.4)	
G3	167 (54.6)	101 (33.0)	66 (21.6)	
Tumor size, cm				0.427
< 4	196 (65.3)	119 (39.7)	77 (25.7)	
≥ 4	104 (34.7)	68 (22.7)	36 (12.0)	
Deep stromal Invasion				0.592
No	135 (45.9)	87 (29.6)	48 (1.3)	
Yes	159 (54.1)	96 (32.7)	63 (21.4)	
Positive parametrium				0.282
No	301 (97.7)	189 (61.4)	112 (36.4)	
Yes	7 (2.3)	3 (1.0)	4 (1.3)	
Positive surgical margin				0.896
No	286 (92.9)	178 (58.8)	108 (35.1)	
Yes	22 (7.1)	14 (4.5)	8 (2.6)	
LVSI**				0.681
No	257 (83.4)	184 (58.4)	110 (25.0)	
Yes	51 (16.6)	8 (3.9)	6 (12.7)	
Lymph node metastasis				< 0.001
No	257 (83.4)	180 (58.4)	77 (25.0)	
Yes	51 (16.6)	12 (3.9)	39 (12.7)	
Recurrence				0.685
No	266 (86.4)	167 (70.2)	99 (29.8)	
Yes	42 (13.6)	25 (43.9)	17 (56.1)	
Vital status				< 0.001
Alive	274 (88.6)	183 (59.4)	91 (29.5)	
Death from CC	34 (11.0)	9 (2.9)	25 (8.1)	

*SCCA: Squamous cell carcinoma antigen. **LVSI: Lymph vascular space involvement.

At 48 h after transfection, luciferase and *Renilla* activity was measured using a Dual Luciferase Reporter Assay Kit (Promega).

Statistical analysis

The correlation between flotillin-1 expression and clinical/pathological characteristics was assessed using Pearson's χ² test and Spearman correlation analysis. Overall survival (OS) was defined as the time from diagnosis to the date of cancer-related death; progression-free survival (PFS) was defined as the time from diagnosis to the onset of recurrence. Survival curves were plotted using the Kaplan-Meier method and compared by the log-rank test. Multivariate analysis was performed for all parameters that were significant in the univariate analysis using the Cox regression model. All statistical analyses were carried out with SPSS software package (IBM, Armonk, NY, standard version 22.0). A 2-sided *p*-value < 0.05 was considered statistically significant.

Results

Flotillin-1 expression is significantly upregulated in cervical cancer cell lines and cervical cancer tissues

Both flotillin-1 protein and mRNA are significantly upregulated in the four cervical cancer cell lines (HeLa, Ca Ski, C33A, SiHa) and five fresh cervical cancer tissues as compared to the hKC cells and the paired adjacent noncancerous cervical tissues, respectively (**Figure
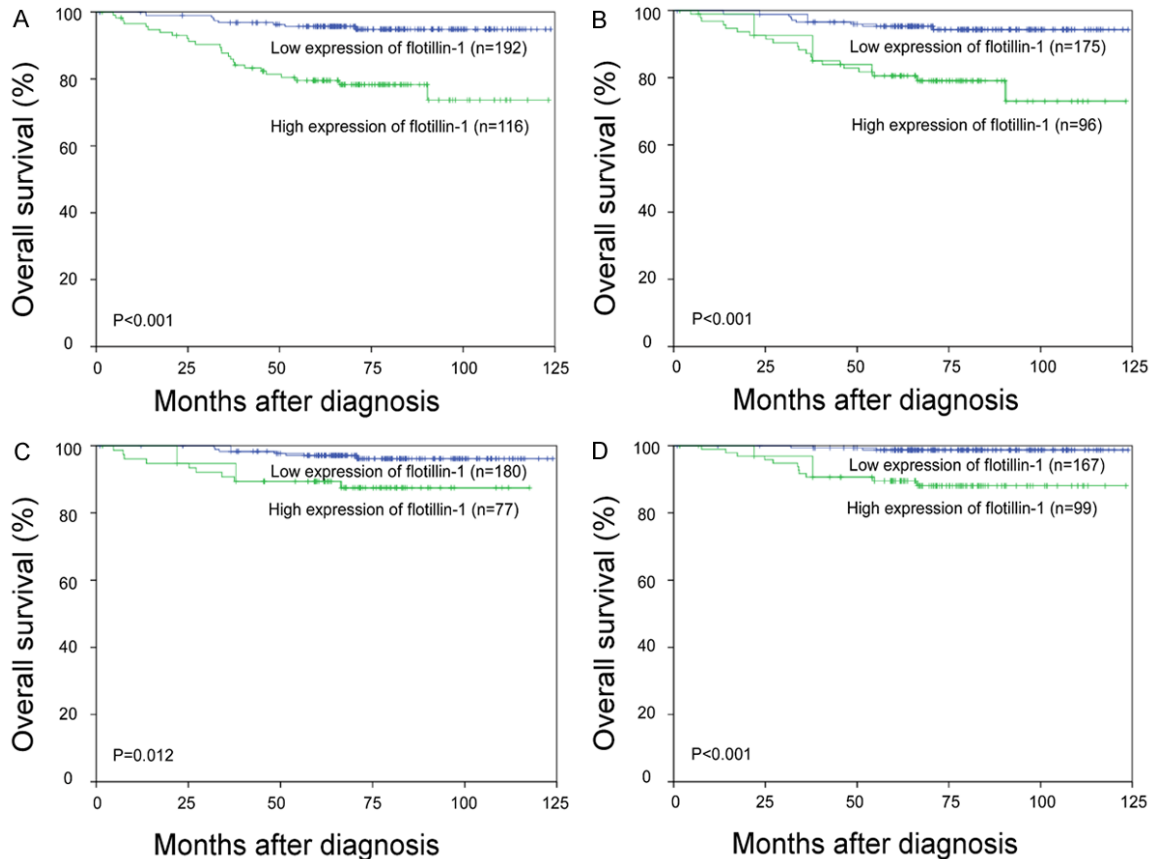
Figure 3. Kaplan-Meier curves with univariate analyses (log-rank) in cervical cancer patients with low versus high flotillin-1 expression. A. OS curves of all patients. B. OS curves of patients with squamous cell carcinoma; C. OS curves of patients without lymph node metastasis. D. OS curves of patients without recurrence.

1). IHC staining detected flotillin-1 protein in 93.8% (289/306) of paraffin-embedded cervical cancer tissues, while marginal or negative staining was detected in control normal cervical tissues and normal areas surrounding the cancerous tissues among all tumor sections (**Figure 2A-H**).

Flotillin-1 protein expression profile correlates with pelvic lymph node metastasis in early-stage cervical cancer

We studied the correlation between the flotillin-1 protein expression profile and clinical/pathological characteristics in patients with early-stage cervical cancer further. There was no statistically significant correlation between flotillin-1 protein expression and age, squamous cell carcinoma antigen (SCCA) level, FIGO stage, differentiation grade, tumor size (cut-off, 4 cm), deep stromal invasion, positive parame-

trium, positive surgical margin, and lymphovascular space involvement (LVSI). However, flotillin-1 protein expression and histological type were significantly correlated (P = 0.031), and most importantly, lymph node metastasis (P < 0.001) was correlated (**Table 2**, Table S2; **Figure 2I-M**).

Elevated expression of flotillin-1 protein predicts poor OS in patients with early-stage cervical cancer

In this cohort, the OS rate of patients with high flotillin-1 expression was significantly lower than that of patients with low flotillin-1 expression (P < 0.001, log-rank test, **Figure 3A**). The same pattern was detected in patients with squamous cell carcinoma of the cervix (P < 0.001, log-rank test, **Figure 3B**). Even in patients without lymph node metastasis (P = 0.012, log-rank test, **Figure 3C**) or without recurrence (P < 0.01, log-rank test, **Figure 3D**),

Table 3. Cox regression model univariate and multivariate analyses of prognostic parameters in patients with cervical cancer

		Univariate analysis		Multivariate analysis		
	No. patients	p	Regression coefficient (SE)	p	Relative risk	95% confidence interval
Lymph node metastasis		0.021	1.180 (0.512)	0.001	3.304	1.581-6.901
No	254					
Yes	51					
Site of recurrence		0.045	0.852 (0.425)	< 0.001	3.179	2.345-4.308
No	266					
Local	13					
Distant	19					
Both	10					
Expression Level of flotillin-1		0.025	1.117 (0.499)	0.004	3.423	1.495-7.837
Low or none	192					
High	116					

low flotillin-1 expression also demonstrated a survival advantage over high flotillin-1 expression. However, no impact of flotillin-1 expression on PFS was detected in this study cohort (Figure S1). The multivariate Cox regression model revealed that pelvic lymph node metastasis (relative risk: 3.304, confidence interval [CI]: 1.581-6.901, P = 0.021), site of recurrence (relative risk: 3.179, CI: 2.345-4.308, P = 0.045), and flotillin-1 expression level (relative risk: 3.423, CI: 1.495-7.837, P = 0.025) were independent prognostic factors for poor OS (**Table 3**).

Flotillin-1 promotes cervical cancer cell motility and invasion through Wnt/β-catenin and NF-κB pathway-regulated epithelial-mesenchymal transition (EMT)

To further identify the role of flotillin-1 in cervical cancer metastasis and the possible mechanism, flotillin-1 cDNA and two flotillin-1 short hairpin RNAs (shRNAs) were used to respectively express flotillin-1 ectopically and to suppress endogenous flotillin-1 stably in HeLa and SiHa cells. As expected, the Transwell migration assay and wound healing assay both indicated that, compared to cells transfected with control vectors, ectopic expression of flotillin-1 strikingly enhanced HeLa and SiHa cell motility and invasion, while flotillin-1 ablation dramatically inhibited it (**Figure 4A** and **4B**). Western blotting further revealed that flotillin-1 overexpression enhanced the expression of mesenchymal markers including Vimentin and N-cadherin,
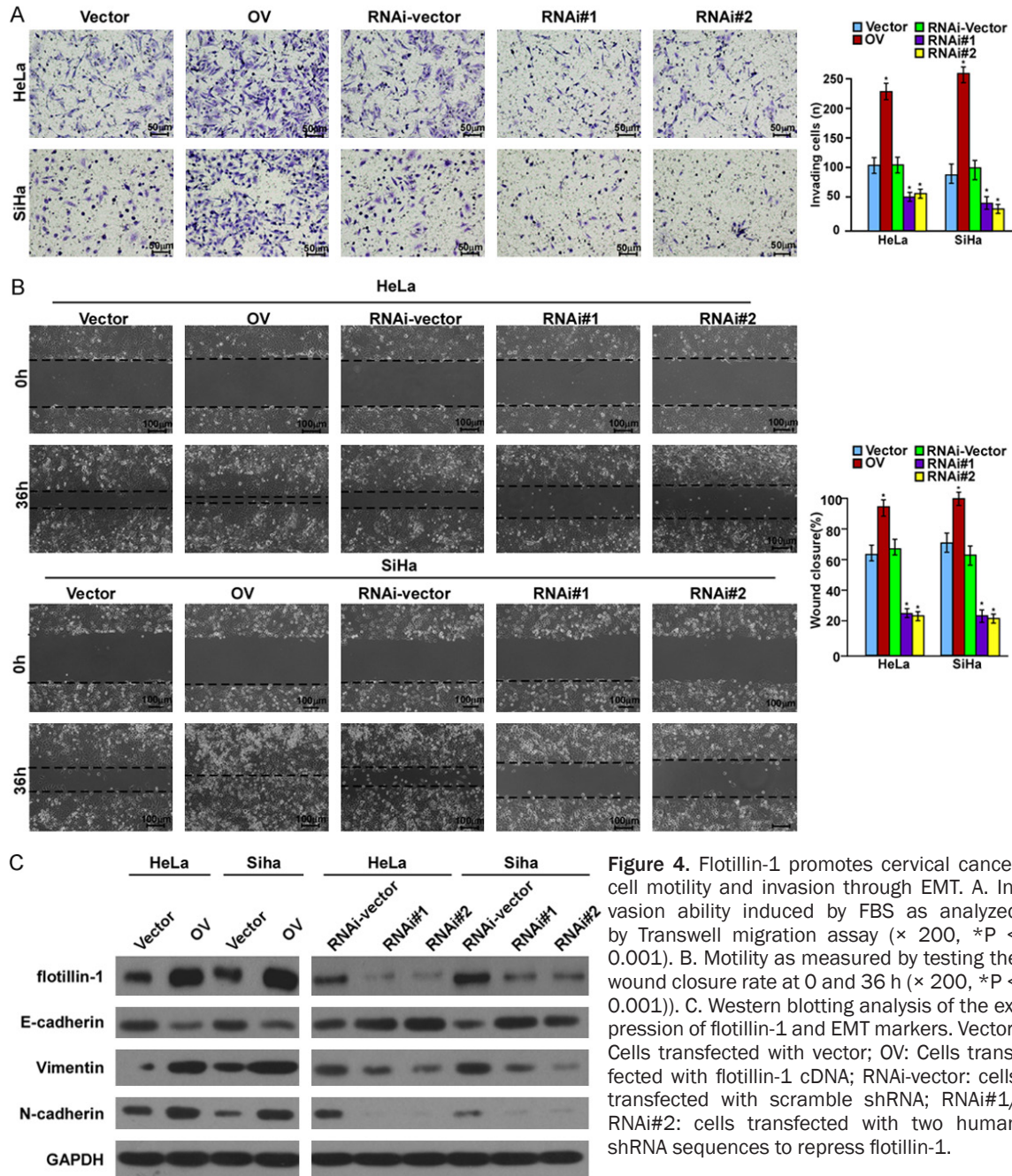
but inhibited the expression of epithelial markers, such as E-catenin (**Figure 4C**), while knockdown of flotillin-1 reversed it (**Figure 4C**). The TOP-flash/FOP-flash Wnt reporter assay confirmed that the canonical (β-catenin-dependent) Wnt pathway was highly activated in cells expressing ectopic flotillin-1 and was inactivated in cells with suppressed endogenous flotillin-1 expression (**Figure 5A**). In addition, the expression levels of a series of Wnt/β-catenin target genes were upregulated following flotillin-1 overexpression and were downregulated when flotillin-1 was silenced (**Figure 5B**). Moreover, ectopic expression of flotillin-1 significantly enhanced the NF-κB luciferase activity as well as the expression levels of a number of well-known downstream genes of the NF-κB pathway, while the ablation of flotillin-1 reduced it (**Figure 5C** and **5D**).

Discussion

This is the first report to demonstrate that flotillin-1 expression is significantly upregulated in cervical cancer cell lines and cancer tissues at both protein and mRNA level in comparison with that of hKC cells and paired adjacent non-cancerous cervical tissues. Elevated expression of flotillin-1 protein is associated with pelvic lymph node metastasis, and the expression profile of flotillin-1 serves as an independent prognostic factor that predicts poor OS time in patients with early-stage cervical cancer even when there is no lymph node metastasis or recurrence. Moreover, the up- and downregula-

Figure 4. Flotillin-1 promotes cervical cancer cell motility and invasion through EMT. A. Invasion ability induced by FBS as analyzed by Transwell migration assay (× 200, *P < 0.001). B. Motility as measured by testing the wound closure rate at 0 and 36 h (× 200, *P < 0.001)). C. Western blotting analysis of the expression of flotillin-1 and EMT markers. Vector: Cells transfected with vector; OV: Cells transfected with flotillin-1 cDNA; RNAi-vector: cells transfected with scramble shRNA; RNAi#1/ RNAi#2: cells transfected with two human shRNA sequences to repress flotillin-1.

tion of flotillin-1 remarkably affected cervical cancer cell motility and invasion, respectively, possibly through Wnt/β-catenin and NF-κB pathway-regulated EMT. These data imply that flotillin-1 may facilitate cervical cancer metastasis and that the flotillin-1 expression profile acts not only as a novel predictor of pelvic lymph node metastasis, but also as neoteric prognostic risk factor for patients with early-stage cervical cancer.

Radical hysterectomy plus bilateral pelvic lymph node dissection with (or without) para-aortic lymph node sampling remains the standard primary treatment for patients with early-stage cervical cancer (FIGO stage IB-IIA), as retroperitoneal lymph node metastasis is the major prognostic risk factor that determines whether a patient needs adjuvant pelvic radiation plus concurrent cisplatin-based chemotherapy after surgery [3, 18]. In fact, the occur-
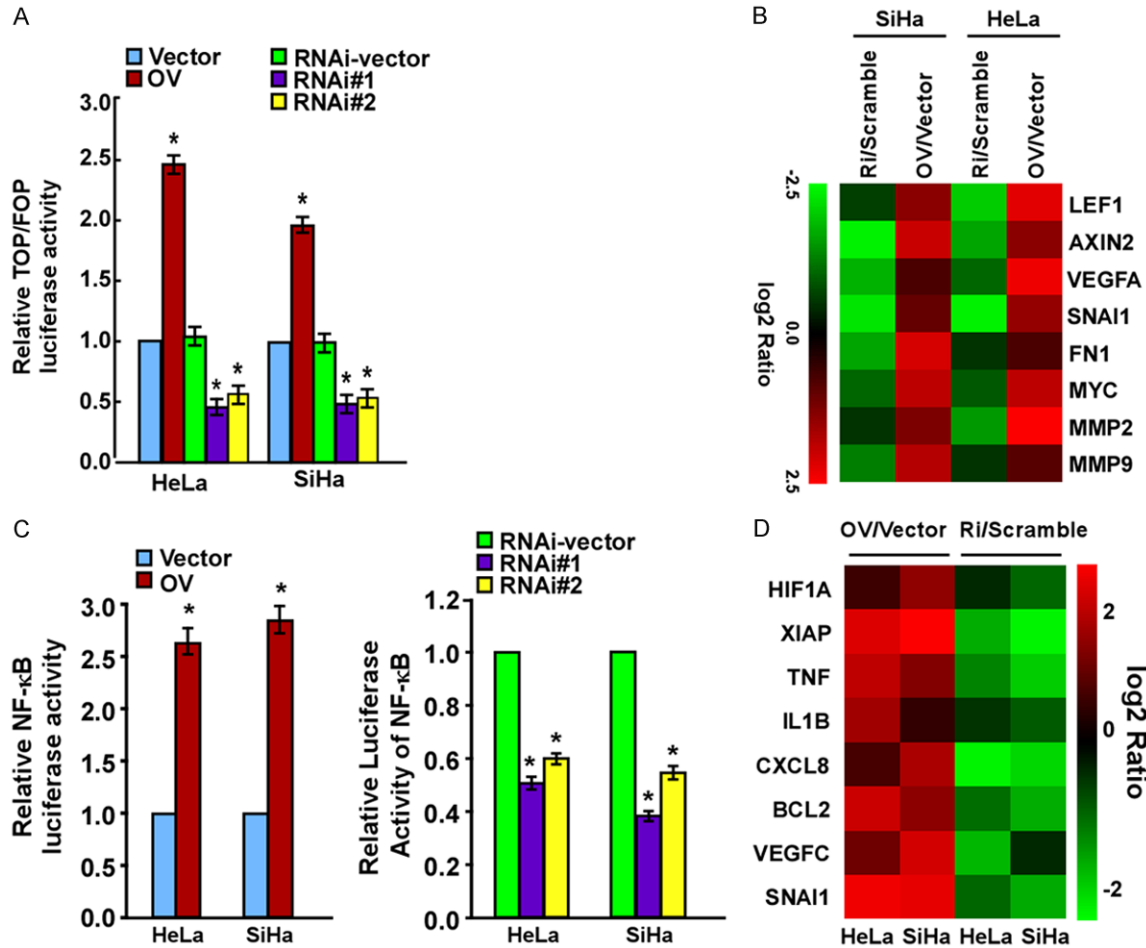
Figure 5. Flotillin-1 activates the NF-κB and Wnt/β-catenin pathways in cervical cancer cells. A. Luciferase reporter activities analyzed in cells according to the TOP-flash versus FOP-flash intensity scale. B. Real-time PCR indicates an apparent overlap between Wnt/β-catenin-dependent gene expression and flotillin-1-regulated gene expression. Pseudo-color represents the intensity scale of flotillin-1 shRNA versus RNAi vector or flotillin-1 cDNA vs. Vector, generated by $\log_2$ transformation. C. NF-κB luciferase reporter activity. D. Real-time PCR indicates an apparent overlap between NF-κB-dependent gene expression and flotillin-1-regulated gene expression. Pseudo-color represents the intensity scale of flotillin-1 cDNA vs. Vector or flotillin-1 shRNA versus RNAi-vector, generated by $\log_2$ transformation. Vector: cells transfected with vector; OV: cells transfected with flotillin-1 cDNA; RNAi-vector/Scramble: cells transfected with scramble shRNA; RNAi#1/RNAi#2/Ri: cells transfected with two human shRNA sequences to repress flotillin-1.

rence of postoperative complications caused by lymphadenectomy, especially pelvic lymphocyte, can reach 25%, while oncologists increasingly value the potential anti-cancer immunity of intact lymph nodes [4]. Unfortunately, there are no accurate and efficient models for gynecologic oncologists to evaluate pelvic lymph node metastasis both preoperatively and intraoperatively. Although recent data suggest that sentinel lymph node mapping and biopsy may be the most promising method [19, 20], whether the technique has been sufficiently validated for routine use is still debated [21, 22]. In the present study, our data demonstrate that the

expression level of flotillin-1 protein is significantly associated with lymph node metastasis in early-stage cervical cancer, shedding light on the possibility of predicting retroperitoneal lymph node metastasis preoperatively by investigating flotillin-1 protein expression levels in biopsy samples, subsequently allowing the adoption of a more personalized treatment algorithm. For patients with significantly elevated flotillin-1 protein expression, implying high lymph node metastasis risk, definitive radiation with concurrent cisplatin-containing chemotherapy instead of surgery should be advised to prevent overlying complications; for those with

low flotillin-1 protein expression, suggesting low lymph node metastasis risk, radical hysterectomy alone could be considered to preserve intact lymph nodes, and further, anti-cancer immunity.

Furthermore, the univariate and multivariate analyses suggested that the level of flotillin-1 protein expression is an independent prognostic factor that predicts poor OS in early-stage cervical cancer patients, even in patients without lymph node metastasis or recurrence, suggesting that patients with high flotillin-1 protein expression may require more intensive treatment to obtain a desirable prognosis and closer surveillance to detect early recurrence even when there are no other risk factors, such as lymph node metastasis, at the time.

Flotillin-1 encodes a caveolae-associated integral membrane protein that belongs to the lipid raft family, and is involved in vesicular trafficking and signal transduction [23]. Quite recently, an increasing number of publications have reported the essential roles of flotillin-1 in tumorigenesis. Flotillin-1 overexpression predicts poor prognosis in hepatocellular carcinoma [12, 24], non-small cell lung cancer [25], soft tissue sarcomas [25], tongue squamous cell cancer [26], clear cell renal cell carcinoma [27], and gastric cancer [28]; silencing flotillin-1 inhibits breast cancer cell proliferation and tumorigenicity both in vitro and in vivo [11], while flotillin-1 depletion leads to ErbB2 and ErbB3 internalization and degradation [29, 30]. Moreover, flotillin-1 promotes cell growth and metastasis by activating the NF-κB signaling pathway in both esophageal squamous cell carcinoma cells and oral squamous cell carcinoma cells [16, 31]. In the present study, there was elevated flotillin-1 expression in cervical cancer cell lines and cancer tissues compared to hKC cells and normal cervical tissues, whereas cervical cancer cell motility and invasion were strikingly enhanced following the ectopic expression of flotillin-1, and were dramatically inhibited by the ablation of flotillin-1 as compared to cells transfected with control vectors, suggesting that flotillin-1 may play an oncogenic role in cervical cancer as well.

EMT contributes to metastasis of a variety of human cancer cells [32]. Multiple signaling pathways regulate EMT, with the NF-κB and Wnt/β-catenin pathways playing a pivotal role

[32]. In agreement with previous reports [16, 31], we found that flotillin-1 facilitated cancer cell metastasis through NF-κB pathway-regulated EMT; activation of the canonical (β-catenin-dependent) Wnt pathway, which is active in cervical cancer [33], may also play a role in flotillin-1-induced EMT. However, the detailed molecular mechanism underlying flotillin-1 interaction with the Wnt/β-catenin and NF-κB pathways to induce EMT in cervical cancer cells, which facilitates their metastasis, requires further investigation in our subsequent studies.

Taken together, our data first demonstrate that flotillin-1 expression is significantly upregulated in early-stage cervical cancer and that elevated flotillin-1 is significantly associated with pelvic lymph node metastasis and poor OS. Flotillin-1 promoted cervical cancer cell motility and invasion via Wnt/β-catenin and NF-κB pathway-regulated EMT. Our data first suggest that flotillin-1 may serve not only as a novel predictor of pelvic lymph node metastasis, but also as a neoteric prognostic risk factor for patients with early-stage cervical cancer.

Disclosure of conflict of interest

None.

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Table S1. Probe sequences used for real-time PCR

Name	Sequence (5' to 3')
Flotillin-1-Up	CTGCTCCTCAATCTGCTGCT
Flotillin-1-Down	GAAGAAGGCCGCCTATGAC
GAPDH-Up	AGAGGCAGGGATGATGTTCTG
GAPDH-Down	AGAGGCAGGGATGATGTTCTG
Flotillin-1-shRNA#1	CCCTCAATGTCAAGAGTGAAA
Flotillin-1-shRNA#2	ACAGAGAGATTACGAACTGAA
LEF1-Up	CACTGTAAGTGATGAGGGGG
LEF1-Down	TGGATCTCTTTCTCCACCCA
AXIN2-Up	CTGGTGCAAAGACATAGCCA
AXIN2-Down	AGTGTGAGGTCCACGGAAAC
VEGFA-Up	AGCTGCGCTGATAGACATCC
VEGFA-Down	CTACCTCCACCATGCCAAGT
SNAI1-Up	AGGTTGGAGCGGTCAGC
SNAI1-Down	CCTTCTCTAGGCCCTGGCT
FN1-Up	ACCTCGGTGTTGTAAGGTGG
FN1-Down	CCATAAAGGGCAACCAAGAG
MYC-Up	CACCGAGTCGTAGTCGAGGT
MYC -Down	TTTCGGGTAGTGGAAAACCA
MMP2-Up	GGAAAGCCAGGATCCATTTT
MMP2-Down	ATGCCGCCTTTAACTGGAG
MMP9-Up	TTGGTCCACCTGGTTCAACT
MMP9-Down	ACGACGTCTTCCAGTACCGA
HIF1A-Up	TGGCTGCATCTCGAGACTTT
HIF1A-Down	GAAGACATCGCGGGGAC
XIAP-Up	CCGGCCCAAGTGAAAAGGTG
XIAP-Down	TTTGTAGACTGCGTGGCACT
TNF-Up	AGATGATCTGACTGCCTGGG
TNF-Down	CTGCTGCACTTTGGAGTGAT
IL1B-Up	AAGCCCTTGCTGTAGTGGTG
IL1B-Down	GAAGCTGATGGCCCTAAACA
CXCL8-Up	AAATTTGGGGTGGAAAGGTT
CXCL8-Down	TCCTGATTTCTGCAGCTCTGT
BCL2-Up	GAGAAATCAAACAGAGGCCG
BCL2-Down	CTGAGTACCTGAACCGGCA
VEGFC-Up	CGGACTCGACCTCTCGG
VEGFC-Down	TGGACACAGACCGTAACTGC

Table S2. Spearman correlation analysis between flotillin-1 expression level and clinical/pathological characteristics

Variables	Flotillin-1 expression level	
	Spearman Correlation	*P*-value
Histological type	0.123	0.031
Lymph node metastasis	0.357	< 0.001

Figure S1. Kaplan-Meier curves with univariate analyses (log-rank) in patients with low versus high flotillin-1 expression. A. PFS curves of all patients. B. PFS curves of patients with squamous cell carcinoma. C. PFS curves of patients without lymph node metastasis.