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*Original Article*

# High expression of flotillin-1 is associated with lymph node metastasis and poor prognosis in vulvar squamous cell carcinoma

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**Abstract:** We previously reported that flotillin-1 facilitates lymph node metastasis in cervical cancer, but the role flotillin-1 plays in carcinogenesis and metastasis in vulvar squamous cell carcinoma remains unclear. In the current study, we performed immunohistochemistry (IHC) staining in 70 paraffin-embed vulvar squamous cell carcinoma tissues. Expression of flotillin-1 protein is significantly up-regulated in vulvar squamous cell carcinoma tissues. Expression profile of flotillin-1 protein associates with FIGO stage, inguinal and/or femoral lymph node metastasis, extracapsular nodal spread and numbers of metastatic lymph node. High expression of flotillin-1 protein predicts poor PFS and OS in patients with vulvar squamous cell carcinoma and serves as an independent prognostic factor predicting poor PFS. These data implicate that flotillin-1 may play an oncogenic role in vulvar squamous cell carcinoma by facilitating inguinal and/or femoral lymph node metastasis; high expression of flotillin-1 protein may serve not only as a potential predictor of lymph node metastasis, but also as a promising prognostic risk factor for patients with vulvar squamous cell carcinoma.

**Keywords:** Flotillin-1, lymph node metastasis, prognostic factor, vulvar squamous cell carcinoma

## Introduction

Vulvar cancer is a rare cancer which accounts for less than 5% of malignancies of the female genital tract, with estimated 5,150 new cases and 1,080 deaths in the United States in 2015 [1]. Vulvar squamous cell carcinoma (VSCC) is the most common histological type which constitutes 95% of vulvar cancer [2].

Lymph node metastasis is the most important prognostic risk factor for vulvar squamous cell carcinoma [2]. Diagnosed at early stage, prognosis of patients with vulvar squamous cell carcinoma could be quite favorable after primary surgery, with overall 5-year survival rates approaching to 93% when Inguinal and/or femoral lymph node metastasis are not detected. Unfortunately, the rates decrease dramatically to 25% when lymph nodes metastasis are present [3]. Moreover, recurrent disease in inguinal and/or femoral lymph nodes predicts a remarkably poor prognosis and is almost always fatal [4], while the molecular mechanism of lymph node metastasis remains unclear.

Lipid rafts are small microdomains of cell membrane that serve as platforms for various transmembrane signal transduction pathways involving in a variety of cellular biological activities [5]. Flotillins, consisting of two homologous members, flotillin-1 and flotillin-2, are known as scaffolding proteins as well as markers of lipid rafts [6]. Evidence has been accumulating that flotillins play a pivot role not only in a diversity of physiologic processes ranging from membrane receptor signaling to cell proliferation and adhesion, but also in diverse pathologic processes, such as recently revealed, tumorigenesis and metastasis [7-9]. Specifically, dysregulation of

**Table 1.** Clinical/pathological characteristics of the Study Cohort (N=70)

| Clinicopathological Features | No. of Patients (%) |
|---|---|
| Age | Year |
| Range | 32-83 |
| Mean | 58.23±11.8 |
| Median | 60 |
| Tumor size, cm | |
| ≤3 | 39 (55.7) |
| >3 | 31 (44.3) |
| FIGO stage | |
| I | 38 (54.3) |
| II | 2 (2.9) |
| III | 20 (28.6) |
| IV | 8 (11.4) |
| Grade | |
| 1 | 50 (71.4) |
| 2 | 14 (20.0) |
| 3 | 6 (8.6) |
| Depth of Invasion, mm | |
| ≤1 | 13 (18.6) |
| >1 | 57 (81.4) |
| Lymph node metastasis | |
| No | 56 (80.0) |
| Yes | 14 (20.0) |
| Extracapsular nodal spread | |
| No | 56 (80.0) |
| Yes | 14 (20.0) |
| Numbers of metastatic lymph node | |
| 0 | 38 (54.3) |
| 1 | 13 (18.6) |
| ≥2 | 19 (27.1) |
| Recurrence | |
| No | 54 (77.1) |
| Yes | 16 (22.9) |
| Vital status | |
| Alive | 47 (67.1) |
| Dead | 23 (32.9) |
| Expression of flontilin-1 | |
| Low or none | 50 (71.4) |
| High | 20 (28.56) |

flotillin-1 has been discovered in a serial of epithelium-originated cancers, including hepatocellular carcinoma, non-small cell lung cancer and tongue squamous cell cancer [10-12]. Moreover, our group reported quite recently that flotillin-1 is significantly up-regulated in early stage cervical cancer and elevated expression of flotillin-1 is significantly associated pelvic lymph node metastasis; 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 [13]. These data inspired us to further investigate the possible role flotillin-1 plays in the tumorigenesis and metastasis of vulvar squamous cell carcinoma, whose lymph node metastasis pattern is quite similar to that of squamous cell carcinoma of cervix.

## Material and methods

### *Patients and tissue specimens*

Total 70 paraffin-embedded cancer tissue samples were taken from patients received radical vulvectomy and complete inguinofemoral lymphadenectomy in department of gynecologic oncology, the third affiliated hospital of Kunming medical university (Yunnan tumor hospital), Kunming, China, from January 1995 to December 2005. The last follow-up was carried out in June 2015, with the mean observation period of 51.1 months (4.1-179.3 months). Clinical stage was re-assessed according to the revised FIGO staging for carcinoma of the vulva [14] and tumor differentiation grades were defined according to the criteria of the World Health Organization [15]. Clinical/pathological features of the study cohort were outlined in **Table 1**. This research was approved by the Human Ethics Committee and the Research Ethics Committee of the Third Affiliated Hospital of Kunming Medical University (Yunnan Tumor Hospital). All samples were obtained with the consent of each patient, and their privacy was maintained. Follow-up data were collected retrospectively through medical records.

### *Immunohistochemistry (IHC) staining for flotillin-1 protein in paraffin-embedded tissues*

Immunohistochemistry (IHC) staining was performed using Histostain-Plus Kits (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. Generally, 5 μm thick slides were baked for 1 h at 60°C, then were deparaffined with xylene and rehydrated via graded alcohols. Endogenous peroxidase activity was blocked

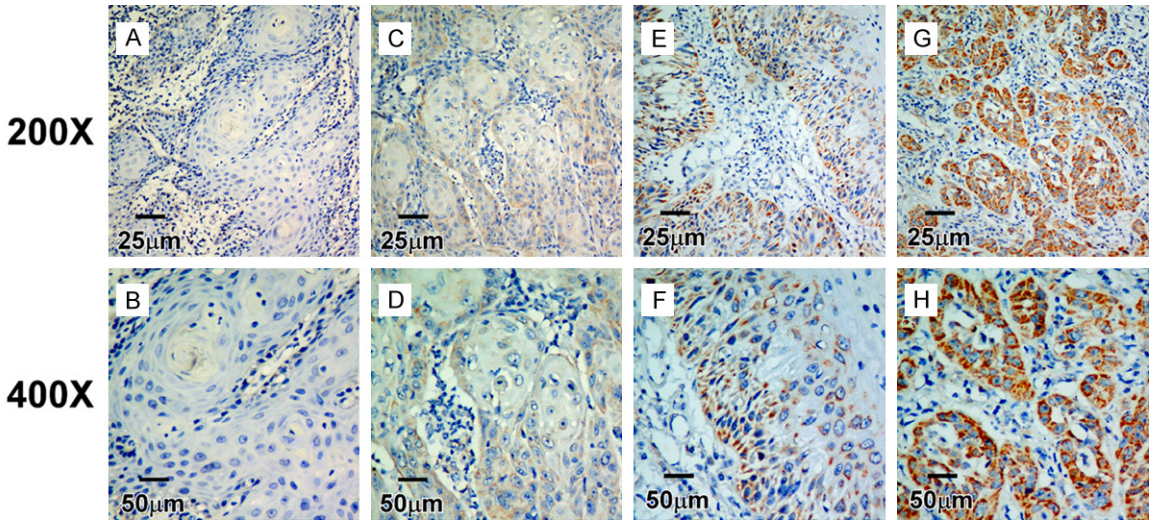


**Figure 1.** Expression of flotillin-1 protein in 70 paraffin-embedded vulvar squamous cell carcinoma tissues by immunohistochemistry (IHC) staining. A and B: Flotillin-1 expression is not detectable in vulvar squamous cell carcinoma tissues (this patient also did not present lymph node metastasis); C and D: Representative images of weak flotillin-1 staining in vulvar squamous cell carcinoma tissues; E and F: Representative images of moderate flotillin-1 staining in vulvar squamous cell carcinoma tissues; G and H: Representative images of strong flotillin-1 staining in vulvar squamous cell carcinoma tissues.

with 3% hydrogen peroxide for 10 minutes. For antigen retrieval, slides were boiled by pressure cooker in 10 mM EDTA antigenic retrieval buffer (pH 8.0). Then slides were incubated with anti-flotillin-1 antibody at a dilution of 1:400 (Sigma-Aldrich, Saint Louis, MO) overnight at 4°C in a moist chamber. The next day, the slides were treated by HRP conjugated anti-rabbit secondary antibody for 30 min at 37°C and developed for 5 min in diaminobenzidine (DAB) solution. The nucleus was counterstained using Meyer's hematoxylin. Negative control was obtained by replacing the primary antibody with normal rabbit IgG. Known immunostaining positive and negative slides were used as controls.

*Evaluation of IHC staining*

Two pathologists (Z Zhang 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 [16]; both nuclear and cytoplasmic expression were scored as positive staining. The average IRS for each case was assigned as the staining result for the patient. The specimens were rescored if the difference between the scores determined by the two pathologists was greater than 3. 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.

*Statistical analysis*

The correlation between flotillin-1 expression and clinical/pathological characteristics was assessed using Pearson's $\chi^2$ test and Spearman correlation analysis. Overall survival rates (OS) was defined as the time from diagnosis to the date of cancer-related death or last follow-up, and progression free survival rates (PFS) was defined as the time from diagnosis to the onset of recurrence. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. Multivariate survival analysis was performed for all of the parameters that were significant in the Univariate analysis using the Cox regression model. All statistical analyses were carried out with SPSS software package (IBM, standard version 22.0). A two-sided probability value of less than 0.05 was considered statistically significant.

**Table 2.** Clinical/pathological characteristics of patients with vulvar squamous cell carcinoma and flotillin-1 expression profile (N=70)

| Clinicopathological parameters | *N* *No. (%)* | Expression Level of flotillin-1 | | $X^2$ test P |
|---|---|---|---|---|
| | | Low or non No. (%) | High No. (%) | |
| Age (years) | | | | 0.449 |
| ≤60 | 37 (52.9) | 25 (35.7) | 12 (17.1) | |
| >60 | 33 (47.1) | 25 (35.7) | 8 (11.4) | |
| Tumor size (cm) | | | | 0.094 |
| ≤3 | 39 (55.7) | 31 (44.3) | 8 (11.4) | |
| >3 | 31 (44.3) | 19 (27.1) | 12 (17.1) | |
| FIGO stage | | | | <0.001 |
| I | 38 (55.9) | 36 (53.0) | 2 (2.9) | |
| II | 2 (2.9) | 0 (0.0) | 2 (2.9) | |
| III | 20 (29.4) | 13 (19.1) | 7 (10.3) | |
| IV | 8 (11.8) | 0 (0.0) | 8 (11.8) | |
| Grade | | | | 0.181 |
| 1 | 50 (71.4) | 38 (54.3) | 12 (17.1) | |
| 2 | 14 (20.0) | 8 (11.4) | 6 (8.6) | |
| 3 | 6 (8.6) | 4 (5.7) | 2 (2.9) | |
| Depth of Invasion, mm | | | | 0.056 |
| ≤1 | 13 (18.6) | 12 (17.1) | 1 (1.4) | |
| >1 | 57 (81.4) | 38 (54.3) | 19 (27.1) | |
| Lymph node metastasis | | | | <0.001 |
| No | 38 (54.3) | 36 (51.4) | 2 (2.9) | |
| Yes | 32 (45.7) | 14 (20.0) | 18 (25.7) | |
| Extracapsular nodal spread | | | | <0.001 |
| No | 56 (80.0) | 48 (68.6) | 8 (11.4) | |
| Yes | 14 (20.0) | 2 (2.9) | 12 (17.1) | |
| Numbers of metastatic lymph node | | | | <0.001 |
| 0 | 38 (54.3) | 36 (51.4) | 2 (2.9) | |
| 1 | 13 (18.6) | 9 (12.9) | 4 (5.7) | |
| ≥2 | 19 (27.1) | 5 (7.1) | 14 (20.0) | |
| Recurrence | | | | <0.001 |
| No | 54 (77.1) | 45 (64.3) | 9 (12.9) | |
| Yes | 16 (22.9) | 5 (7.1) | 11 (15.7) | |
| Vital status | | | | <0.001 |
| Alive | 47 (67.1) | 40 (57.1) | 7 (10.0) | |
| Death from VC | 23 (32.9) | 10 (14.3) | 13 (18.6) | |

## Results

*High expression of flotillin-1 protein is detected in vulvar squamous cell carcinoma tissues.*

A cohort of 70 paraffin-embedded vulvar squamous cell carcinoma tissues is detected for flotillin-1 protein expression by Immunohistochemistry (IHC) staining. Positive staining of flotillin-1 was detected in 87.1% (61/70) of vulvar squamous cell carcinoma tissues, while marginal or negative staining could be detected in normal vulva tissues or in areas surrounding the cancerous tissues among all tumor sections (**Figure 1**).

*Flotillin-1 protein expression profile significantly correlates with Inguinal and/or femoral lymph node metastasis in vulvar squamous cell carcinoma*

Correlation between flotillin-1 protein expression profile and clinical/pathological characteristics in patients with vulvar squamous cell carcinoma was further investigated. There is no statistically significant correlation between flotillin-1 protein expression and age, tumor size (cut-off as 3 cm), differentiation grade, and depth of invasion (cut-off as 1 mm). However, significant correlation with flotillin-1 protein expression and FIGO stage (P<0.001), inguinal and/or femoral lymph node metastasis (P< 0.001), extracapsular nodal spread (P<0.001), and numbers of metastatic lymph node (P<0.001, categorized as: 0, 1, and ≥2) are detected (**Tables 2** and **3**).

*High expression of flotillin-1 protein predicts poor PFS and OS in patients with vulvar squamous cell carcinoma*

In this study cohort, both progression free survival rates (PFS) and overall survival rates (OS) of patients with high flotillin-1 expression are significantly lower than that of patients with low flotillin-1 expression (P<0.001, respectively, log-rank test, **Figure 2A** and **2C**). The same pat-

**Table 3.** Spearman correlation between flotillin-1 expression level and clinical/pathological characteristics

| Variables | Flotillin-1 expression level | |
|---|---|---|
| | Spearman Correlation | *p*-Value |
| FIGO stage | 0.610 | <0.001 |
| Lymph node metastasis | 0.594 | <0.001 |
| Extracapsular nodal spread | 0.632 | <0.001 |
| Numbers of metastatic lymph node* | 0.640 | <0.001 |

*Categorized as: 0, 1, and ≥2.

tern is detected in patients with Inguinal and/or femoral lymph node metastasis (P=0.002, respectively, log-rank test, **Figure 2B** and **2D**). Interestingly, there are only two patients with high expression of flotillin-1 failed to be detected with inguinal and/or femoral lymph node metastasis (**Figure 2B** and **2D**). Cox regression model revealed extracapsular nodal spread (relative risk: 11.093, CI: 1.690-72.814, P= 0.012) and flotillin-1 expression level (relative risk: 5.002, CI: 1.160-21.560, P=0.031) serve as independent prognostic factors for poor PFS (**Table 4**), but only extracapsular nodal spread (relative risk: 8.048, CI: 1.404-46.128, P= 0.019) serves as independent prognostic factors for poor OS (Supplemental Table 1).

## Discussion

Here is the first report to demonstrate that expression of flotillin-1 protein is significantly up-regulated in vulvar squamous cell carcinoma tissues. Expression profile of flotillin-1 protein associates with FIGO stage, inguinal and/or femoral lymph node metastasis, extracapsular nodal spread and numbers of metastatic lymph node. High expression of flotillin-1 protein predicts poor PFS and OS in patients with vulvar squamous cell carcinoma and serves as an independent prognostic factor predicting poor PFS. These data implicate that flotillin-1 may play an oncogenic role in vulvar squamous cell carcinoma by facilitating inguinal and/or femoral lymph node metastasis; high expression of flotillin-1 protein may serve not only as a potential predictor of lymph node metastasis, but also as a promising prognostic risk factor for patients with vulvar squamous cell carcinoma.

Belonging to lipid raft family, flotillin-1 is a caveolae-associated, integral membrane protein which involves in a wave of cellular signal transduction pathways [17]. Quite recently, increasing studies had been presenting that flotillin-1 may also play a crucial role in tumorigenesis and metastasis of a wide range of human cancers, including hepatocellular carcinoma, non-small cell lung cancer, tongue squamous cell cancer, and gastric cancer [10-12, 18-20]. In our previous study, we found expression level of flotillin-1 is significantly up-regulated in cervical cancer cell lines and cancer tissues at both protein and mRNA levels; Elevated expression of flotillin-1 protein served as an independent prognostic factor which predicted poor overall survival time in patients with early stage cervical cancer, even in patients without lymph node metastasis and recurrence [13]. In the current study, we showed that flotillin-1 protein is significantly up-regulated in vulvar squamous cell carcinoma tissues; What's more, high expression of flotillin-1 protein predicted poor PFS and OS in patients with vulvar squamous cell carcinoma and served as an independent prognostic factor predicting poor PFS. These data suggested flotillin-1 may also play an oncogenic role in vulvar squamous cell carcinoma.

In the last two decades, the standard of primary surgery for patients with early stage vulvar squamous cell carcinoma has been shifted from radical to more limited, with especially the adoption of sentinel lymph node (SLN) biopsy for selected patients, replacing complete inguinofemoral lymphadenectomy which resulted in up to two-thirds of patients experiencing wound breakdown, lymphocytic formation, and/or lymphedema [21]. However, with the introduction of SLN biopsy, the whole pictures of inguinal and femoral lymph node status of SLN negative patients were beyond elucidation, since the false negative rates of patients with negative SLN could reach to 5.9-8.3%, based on two large multiple center trials [22, 23]. That means up to 8.3% of lymph node metastasis would be missed even in well selected, early stage patients. To address this issue, we only selected patients who received their primary surgery before 2006 when complete inguinofemoral lymphadenectomy remained the standard care in our institution, which may provide us with more accurate information

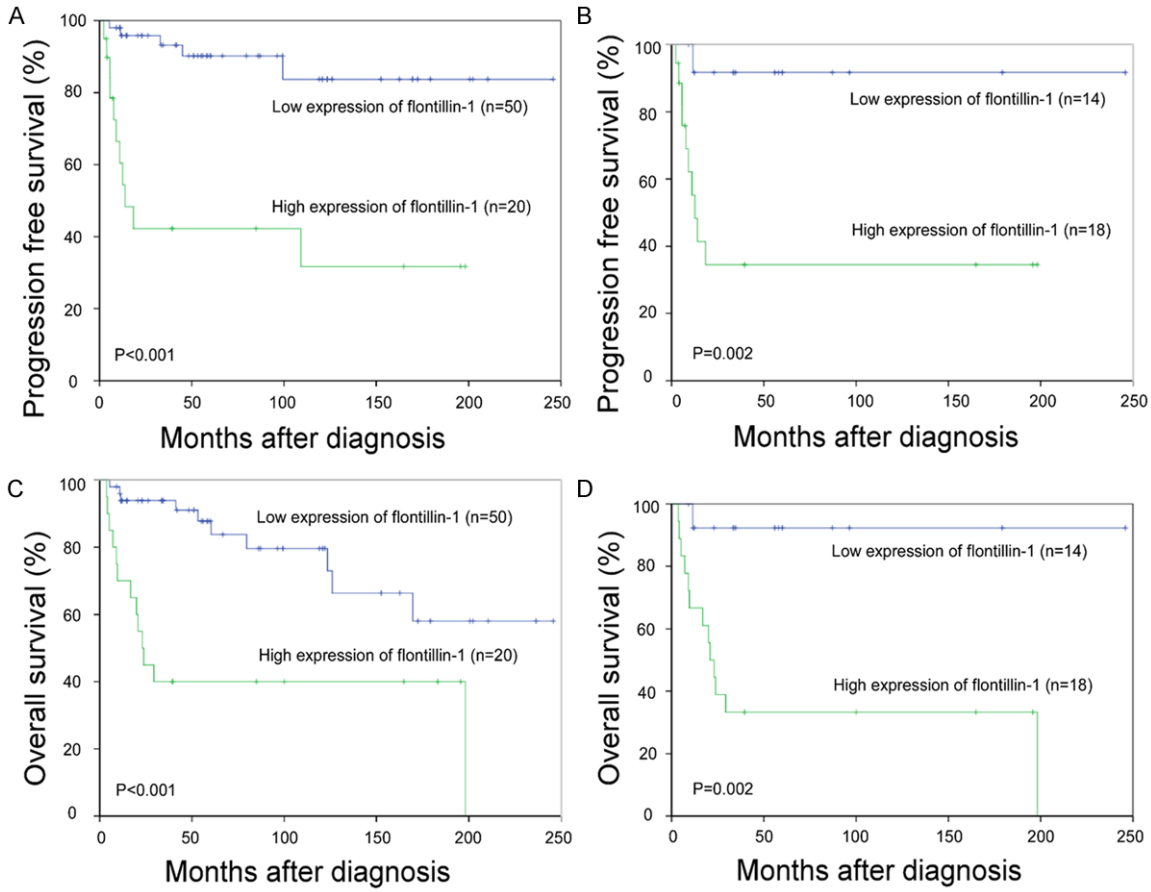


**Figure 2.** Kaplan-Meier curves with univariate analyses (log-rank) in vulvar squamous cell carcinoma patients with low flotillin-1 expression versus high flotillin-1 expression. A: High flotillin-1 expression versus low flotillin-1 expression for progression free survival rates of all patients; B: High flotillin-1 expression versus low flotillin-1 expression for progression free rates of patients with inguinal and/or femoral lymph node metastasis; C: High flotillin-1 expression versus low flotillin-1 expression for overall survival rates of all patients; D: High flotillin-1 expression versus low flotillin-1 expression for overall survival rates of patients with Inguinal and/or femoral lymph node metastasis.

concerning the metastasis of inguinal and/or femoral lymph node in the trade of relatively small sample size.

Although lymph node status remains the strongest prognostic risk factor for vulvar squamous cell carcinoma [24-27], extracapsular nodal spread [24, 27], numbers of metastatic lymph node [28] are also reported to be very important predictors of survival, that's why FIGO had incorporated extracapsular nodal spread and numbers of metastatic lymph node into its latest edition of staging system for carcinoma of the vulva since 2014 [14]. However, till now, the molecular mechanism of lymph node metastasis of vulvar squamous cell carcinoma still remains a mystery. As a matter of fact, in the present study, we demonstrated expression profile of flotillin-1 protein associates with FIGO stage, inguinal and/or femoral lymph node metastasis, extracapsular nodal spread and numbers of metastatic lymph node in vulvar squamous cell carcinoma, consisting with our previous finding that elevated flotillin-1 protein expression in early stage cervical cancer is significantly associated with pelvic lymph node metastasis, up- and down-regulation of flotillin-1 in cervical cancer cells remarkably affected cellular motility and invasion, respectively [13], we hypnotized that flotillin-1 may facilitate lymph node metastasis in vulvar squamous cell carcinoma as well. Some publications have revealed that down-regulation of flotillin-1 inhibited proliferation and tumorigenicity of breast cancer cells both in vitro and in vivo, through suppressing Akt activity and enhancing tran-

**Table 4.** Univariate and multivariate analyses of various prognostic parameters in patients with vulva carcinoma by Cox-regression model-Progression free survival

| | | Univariate analyses | | Multivariate analyses | |
|---|---|---|---|---|---|
| | No. patients | *p* | Relative risk (95% confidence interval) | *p* | Relative risk (95% confidence interval) |
| Tumor size (cm) | | 0.045 | 2.954 (1.023-8.528) | | |
| ≤3 | 39 | | | | |
| >3 | 31 | | | | |
| FIGO stage | | 0.008 | 1.854 (1.173-2.932) | | |
| I | 38 | | | | |
| II | 2 | | | | |
| III | 20 | | | | |
| IV | 8 | | | | |
| Lymph node metastasis | | 0.013 | 3.831 (1.322-11.105) | | |
| No | 38 | | | | |
| Yes | 32 | | | | |
| Extracapsular nodal spread | 56 | 0.001 | 2.715 (1.511-4.480) | 0.012 | 11.093 (1.690-72.814) |
| No | 14 | | | | |
| Yes | | | | | |
| Numbers of metastatic lymph node | 38 | <0.001 | 13.465 (4.444-40.792) | | |
| 0 | 13 | | | | |
| 1 | 19 | | | | |
| ≥2 | | | | | |
| Expression Level of flotillin-1 | | <0.001 | 8.538 (2.947-24.732) | 0.031 | 5.002 (1.160-21.560) |
| Low or none | 50 | | | | |
| High | 20 | | | | |

scriptional activity of FOXO3a [29]; depletion of flotillin-1 leads to internalization and degradation of ErbB2 and ErbB3, receptor tyrosine kinases whose overexpression occur in up to 50-70% of human breast cancers [30, 31]. Moreover, fotillin-1 could promote cell growth and metastasis through NF-κB signaling pathway both in esophageal squamous cell carcinoma cells and oral squamous cell carcinoma cells [32, 33]. Our previous data also suggested that fotillin-1 facilitates cervical cancer cell metastasis through EMT regulated by the Wnt/β-catenin and NF-κB pathways [13]. However, whether fotillin-1 could also regulate cell mobility and invasion in vulvar squamous cell carcinoma and the possible mechanism, still needs to be elucidated.

In summary, our data indicated, for the first time, that expression of flotillin-1 protein is significantly up-regulated in vulvar squamous cell carcinoma tissues; expression profile of flotillin-1 protein associates with FIGO stage, inguinal and/or femoral lymph node metastasis, extracapsular nodal spread and numbers of metastatic lymph node. High expression of flotillin-1 protein predicted poor PFS and OS in patients with vulvar squamous cell carcinoma and served as an independent prognostic factor predicting poor PFS. These discoveries intimates flotillin-1 may harbor the potential not only to be a novel prognostic risk factor, but also a promising therapeutic target in patients with vulvar squamous cell carcinoma, especially in patients with lymph node metastasis.

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## Disclosure of conflict of interest

None.

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**Supplemental Table 1.** Univariate and multivariate analyses of various prognostic parameters in patients with vulva carcinoma by Cox-regression model-Overall Survival

| | | Univariate analyses | | Multivariate analyses | |
|---|---|---|---|---|---|
| | No. patients | *p* | Relative risk (95% confidence interval) | *p* | Relative risk (95% confidence interval) |
| FIGO stage | | 0.014 | 1.577 (1.096-2.268) | | |
| I | 38 | | | | |
| II | 2 | | | | |
| III | 20 | | | | |
| IV | 8 | | | | |
| Lymph node metastasis | | 0.027 | 2.585 (1.113-6.004) | | |
| No | 38 | | | | |
| Yes | 32 | | | | |
| Extracapsular nodal spread | 56 | <0.001 | 10.332 (4.055-26.322) | 0.019 | 8.048 (1.404-46.128) |
| No | 14 | | | | |
| Yes | | | | | |
| Numbers of metastatic lymph node | 38 | 0.001 | 2.263 (1.395-3.670) | | |
| 0 | 13 | | | | |
| 1 | 19 | | | | |
| ≥2 | | | | | |
| Expression Level of flotillin-1 | | 0.001 | 4.302 (1.879-9.852) | | |
| Low or none | 50 | | | | |
| High | 20 | | | | |