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Original Article

Ubc9 expression predicts chemoresistance in breast cancer

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Abstract

Ubiquitin-conjugating enzyme 9 (Ubc9), the sole conjugating enzyme for sumoylation, regulates protein function and plays an important role in tumorigenesis. Whether Ubc9 is involved in the chemoresistance of breast cancer remains unknown. In this study, we aimed to evaluate the contribution of Ubc9 in the chemoresistance of breast cancer. Immunohistochemistry (IHC) was used to examine the expression level of Ubc9. Chi-square test, Wilcoxon test, and one-way ANOVA were applied to analyze the relationship between Ubc9 expression, clinicopathologic features, and clinical response to neoadjuvant chemotherapy. The significance of variables for survival was analyzed by the Cox proportional hazards model in a multivariate analysis. Kaplan-Meier survival curves were plotted and log-rank test was performed. The proportion of Ubc9-positive cells was higher in invasive ductal carcinoma than in normal breast tissues [(48.48 ± 17.94)% vs. (5.82 ± 2.80)%, $P < 0.001$]. High Ubc9 expression was associated with poor differentiation ($\chi^2 = 6.538$, $P = 0.038$), larger tumor size ($\chi^2 = 4.701$, $P = 0.030$), advanced clinical stage ($\chi^2 = 4.651$, $P = 0.031$), lymph node metastasis ($\chi^2 = 9.913$, $P = 0.010$), basal-like phenotype ($\chi^2 = 8.660$, $P = 0.034$), and poor clinical response to neoadjuvant chemotherapy ($\chi^2 = 11.09$, $P = 0.001$). The expected 6-year cumulative disease-free survival rate was 87.32% in patients with low Ubc9 expression compared to 68.78% in those with high Ubc9 expression ($\chi^2 = 4.289$, $P = 0.038$). These data indicate that high Ubc9 expression correlates with poor response to chemotherapy and poor clinical prognosis.

Key words Ubc9, breast cancer, chemoresistance

Chemotherapy plays a critical role in treating breast cancer. However, some patients with breast cancer still develop distal metastasis following chemotherapy. The mechanisms determining chemoresistance of breast cancers are complicated, with a variety of molecules involved. Among these mechanisms, the overexpression of P-glycoprotein (P-gp)[1] and the presence of breast tumor-initiating cells (BT-ICs)[2] were believed to contribute to the development of primary and acquired multidrug resistance (MDR) and, thus, to treatment failure in 90% of patients with metastatic breast cancers[3]. Unfortunately, application of P-gp inhibitors, including cyclosporin A, valspodar, and verapamil, has not been successful in several phase III clinical trials[4,5]. In addition, modification in the intracellular targets for chemotherapeutic drugs, including altered expression of tubulin isotypes[6], has been implicated in drug resistance to taxanes and vinca alkaloids[7]. Moreover, the efficacy of microtubule inhibitors is limited by cumulative toxicities such as neurotoxicity[8]. Therefore, it is necessary to identify other targets or anti-cancer strategies to reverse chemoresistance of breast cancer.

Recently, increasing evidence demonstrated that sumoylation is a multistep process analogous to ubiquitin pathway and involves maturation, activation, conjugation, ligation, and de-conjugation steps. Sumoylation, which includes small ubiquitin-like modifier (SUMO) protein addition or removal from other proteins, is a post-translational modification that plays an important role in diverse cellular processes, including transcriptional regulation, nuclear transport, cell cycle control, and maintenance of genome integrity through modulating

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protein-protein interactions of target proteins [9-11]. Ubiquitin-conjugating enzyme 9 (Ubc9), the sole E2-conjugating enzyme for sumoylation, plays an important role in tumorigenesis and progression [12]. Ubc9 has been reported to be expressed at high levels in advanced melanomas and was found to shield melanoma cells from apoptosis. Blocking Ubc9 expression sensitized melanoma cells to the cytotoxic effects of chemotherapeutic drugs [13]. Ubc9 was also reported to be expressed at higher levels in head and neck tumor and lung tumor specimens than in the matched normal tissues [14]. However, whether Ubc9 is involved in the chemoresistance of breast cancer remains unknown. In this study, we aimed to investigate the contribution of Ubc9 in the chemoresistance of breast cancer. Our clinical data showed a strong association between high Ubc9 expression level and poor response to chemotherapy as well as poor prognosis in breast cancer.

Materials and Methods

Ethics statement

The study was approved by the Internal Review Board and the Ethics Board of the Sun Yat-sen Memorial Hospital. Patients provided written consent so that their samples and clinical data could be used for investigational purposes.

Patients and tumor samples

Tumor tissues of primary invasive ductal breast carcinoma were obtained from female patients with stage IIB and III disease prior to preoperative neoadjuvant chemotherapy in the Breast Tumor Center, Sun Yat-sen Memorial Hospital, Sun Yat-sen University between January 2005 and December 2009. All patients underwent preoperative neoadjuvant chemotherapy with 2 to 6 cycles of FEC regimen [5-fluorouracil (500 mg/m^2), epirubucin (90 mg/m^2), and cyclophosphamide (500 mg/m^2)]. Subsequently, the patients underwent total mastectomy or breast conservation. The patients with breast conservation underwent postoperative radiotherapy delivered to the entire breast using photon beams (median dose, 50 Gy) and a boost to the primary tumor bed using electron beams (median dose, 16 Gy). According to the clinical response to neoadjuvant chemotherapy determined by the Response Evaluation Criteria in Solid Tumors (RECIST), adjuvant FEC was administered to patients with clinical complete response (cCR) and partial response (PR), whereas taxotere (75 mg/m^2) was administered to patients with stable disease (SD) and disease progression (PD). Endocrine therapy was administered to patients with positive hormonal receptor.

Immunohistochemistry

The level of Ubc9 in paraffin-embedded tissue sections was detected with immunohistochemical staining. Goat monoclonal Ubc9 antibody (ab21193, Abcam) (1:200 dilution) was used as primary antibody. Goat IgG was added as negative control in each case. Paraffin-embedded tissues were pretreated at 65℃ for 2 h, followed by deparaffinization using standard procedures. Antigen retrieval was carried out in antigen retrieval solution (10 mmol/L Tris, 1 mmol/L EDTA, pH 9.0) before 2% sheep serum was added. Then, slides were incubated with Ubc9 antibody for more than 16 h at 4℃ overnight. After 3 washes with PBST and further incubation with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 37℃, positive signals were detected with the chromogen 3,3'-diaminobenzidine (DAB). Positive expression of Ubc9 (in brown) was mainly detected in the cytoplasm and/or nucleus. Staining scores were determined based on both the intensity and the proportion of Ubc9-positive cells in five random fields under a ×40 objective. The proportion of malignant cells that were positively stained was assigned a score from 0 to 4 (0 score, 0–4%; 1 score, 5%–24%; 2 scores, 25%–49%; 3 scores, 50%–74%; 4 scores, ⩾ 75%) and termed category A. The average intensity, corresponding to the presence of negative, weak, intermediate, and strong staining, was given a score from 0 to 3, respectively, and termed category B. Scores from category A were multiplied by scores from category B (A × B) to form a final score. Final scores less than 6 (median value of the 121 samples) were defined as low expression, whereas scores more than or equal to 6 were considered high expression[15,16]. According to the report of two pathologists with expertise in breast cancer pathology, estrogen receptor (ER) and progesterone receptor (PR) were immunostained by using monoclonal antibodies, and nuclear staining of more than 1% of cells was considered positive. Human epidermal growth factor receptor 2 (Her-2) status was evaluated by immunohistochemistry and strong expression (+++) of Her-2 was defined as Her-2 positivity. ER, PR, Her-2, and Ubc9 staining were independently reviewed by the two pathologists, and the average was used for the analyses.

Statistical analysis

SPSS13.0 was adopted for data analysis. Chi-square test, Wilcoxon test, and one-way ANOVA were applied to analyze the relationship between Ubc9

expression, clinicopathologic features, and clinical response to neoadjuvant chemotherapy. The significance of various variables for survival was analyzed by the Cox proportional hazards model in a multivariate analysis. Survival curves were plotted using the Kaplan-Meier method and compared with the log-rank test. $P < 0.05$ was considered statistically significant.

Results

Primary tumor tissues and corresponding peritumoral normal breast tissues were obtained from 121 women with invasive ductal breast carcinoma, aged 28 to 56 years with a median of 48 years.

Ubc9 is up-regulated in breast cancer tissues compared to matched normal breast tissues

To determine whether Ubc9 plays any role in breast cancer tumorigenesis, we assessed Ubc9 expression in breast cancer tissues with immunohistochemical staining. Ubc9-positive ($Ubc9^+$) cells were almost absent in normal breast tissues, but Ubc9 was highly expressed in breast cancer cells, primarily in the cytoplasm and/or nucleus (Figure 1). The percentage of $Ubc9^+$ cells was about 8-fold higher in breast cancer tumors than in normal breast tissues [(48.48 ± 17.94)% vs. (5.82 ± 2.80)%, $P < 0.001$]. These data suggested that Ubc9 was up-regulated in breast cancer tissues compared to matched normal breast tissues.

High Ubc9 expression associates with a more aggressive phenotype of breast cancer

We also analyzed the association between Ubc9 expression level and clinicopathologic features of breast cancer. Then, taking account of multivariate variables, Ubc9 expression was associated with several clinicopathologic features (Table 1). High Ubc9 expression was associated with poor differentiation ($\chi^2 = 6.538$, $P = 0.038$), larger tumor size ($\chi^2 = 4.701$, $P =$

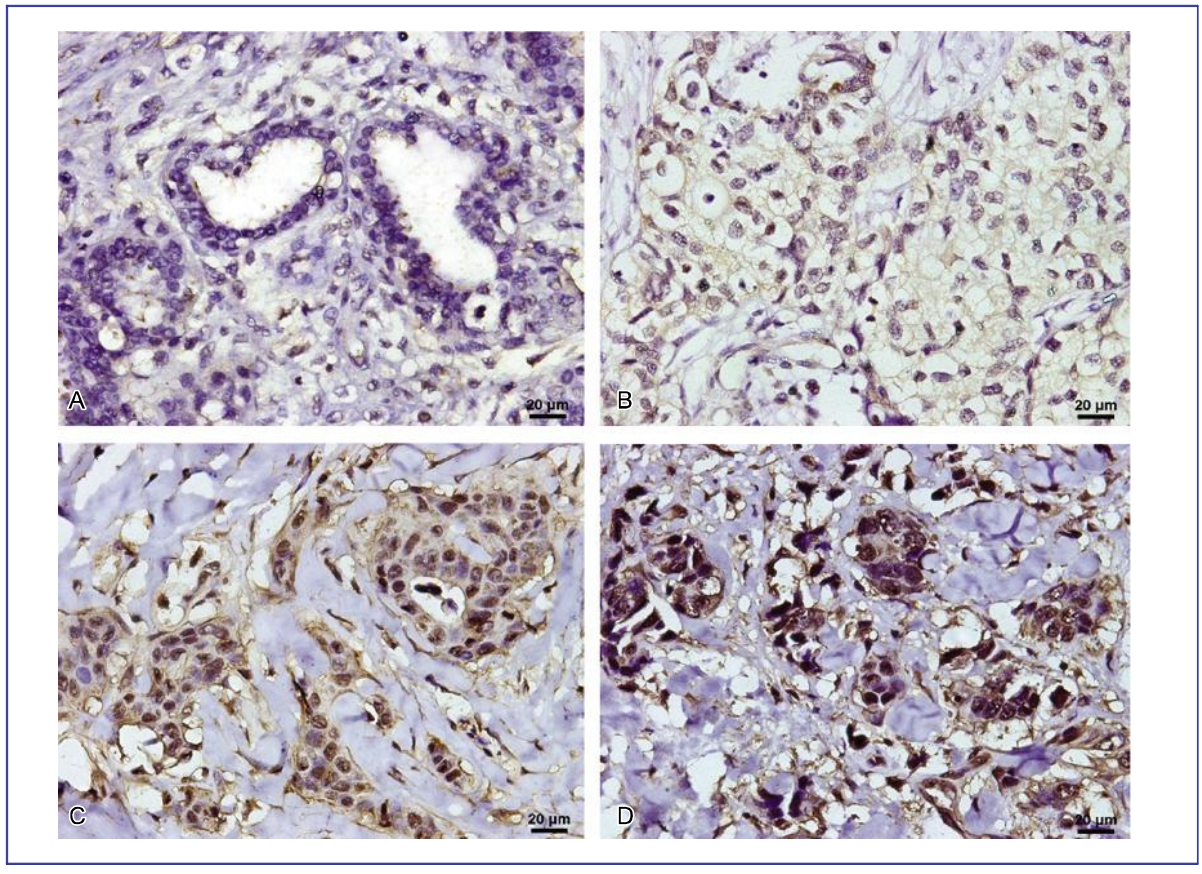

Figure 1. **Expression of ubiquitin-conjugating enzyme 9 (Ubc9) protein in normal breast and invasive ductal breast carcinoma tissues (SP).** A, no expression of Ubc9 protein is seen in normal breast tissues. B, weak cytoplasmic and nuclear expression of Ubc9 protein in invasive ductal carcinoma tissues. Ubc9-positive cells are stained in brown. C, moderate cytoplasmic and nuclear expression of Ubc9 protein in invasive ductal carcinoma tissues. D, strong cytoplasmic and nuclear expression of Ubc9 protein in invasive ductal carcinoma tissues.

Table 1. Association between Ubc9 expression and clinicopathologic features of breast cancer

Item	Total No.	Ubc9 expression [No. (%)]		χ^2	*P*
		Low	High		
Age (years)				1.086	0.297
⩽48	59	23 (19.01)	36 (29.75)		
>48	62	30 (24.79)	32 (26.45)		
Nottingham grade				6.538	0.038
Grade I	14	11 (9.09)	3 (2.48)		
Grade II	47	20 (16.53)	27 (22.32)		
Grade III	60	22 (18.18)	38 (31.40)		
Tumor size				4.701	0.030
2–5 cm	105	50 (41.32)	55 (45.45)		
>5 cm	16	3 (2.48)	13 (10.75)		
Clinical stage				4.651	0.031
IIB	42	24 (19.83)	18 (14.88)		
III	79	29 (23.97)	50 (41.32)		
Lymph node status				9.913	0.010
pN1	22	13 (10.74)	9 (7.44)		
pN2	51	27 (22.32)	24 (19.83)		
pN3	48	13 (10.74)	35 (28.93)		
ER				3.620	0.057
+	98	47 (38.84)	51 (42.15)		
–	23	6 (4.96)	17 (14.05)		
PR				1.945	0.163
+	66	30 (24.79)	36 (29.75)		
–	55	32 (26.45)	23 (19.01)		
Sorlie classification*				8.660	0.034
Luminal A	80	42 (34.71)	38 (31.40)		
Luminal B	18	5 (4.13)	13 (10.74)		
HER-2(+)	10	4 (3.31)	6 (4.96)		
Basal-like	13	2 (1.65)	11 (9.09)		

*Sorlie classification as ER(+)/Her-2(–) (Luminal A), ER(+)/Her-2(+) (Luminal B), ER(–)/Her-2(+) (HER–2+), and ER(–)/Her-2(–) (Basal-like).

0.030), advanced clinical stage (χ^2 = 4.651, *P* = 0.031), and lymph node metastasis (χ^2 = 9.913, *P* = 0.010). In contrast, Ubc9 expression was not associated with age (χ^2 = 1.086, *P* = 0.297), ER status (χ^2 = 3.620, *P* = 0.057), or PR status (χ^2 = 1.945, *P* = 0.163). However, Ubc9 expression level was up-regulated in breast cancers with basal-like phenotype (χ^2 = 8.660, *P* = 0.034) that were clinically characterized as more aggressive and less responsive to standard treatment[17]. Collectively, our data suggested that high Ubc9 expression associated with more aggressive phenotype of breast cancers.

High Ubc9 expression associates with poor clinical response to neoadjuvant chemotherapy

Because Ubc9 expression was associated with the clinicopathologic status of breast cancer, we hypothesized that Ubc9 expression may affect tumor drug responsiveness[18]. Therefore, we investigated Ubc9 expression in breast tumors before neoadjuvant chemotherapy to assess whether Ubc9 plays a role in intrinsic chemoresistance of breast cancer. According to the RECIST, 33 out of 53 patients with low Ubc9 expression achieved cCR or PR after chemotherapy, whereas only 22 out of 68 patients with high Ubc9 expression had PD or SD. The clinical response rate, including cCR and PR, was higher in patients with low Ubc9 expression than in those with high Ubc9 expression (27.27% vs.18.18%, χ^2 = 11.09, *P* = 0.001) (Table 2). The proportion of Ubc9-positive cells was significantly lower in chemosensitive tumors (cCR and PR) than in chemoresistant tumors (PD and SD) (41.53% vs. 54.27%, *P* = 0.001). These data indicated that high Ubc9 expression associated with poor clinical response to neoadjuvant chemotherapy.

High Ubc9 expression associates with poor clinical prognosis

To evaluate the value of Ubc9 in predicting prognosis in breast cancer, we compared the treatment

paradigms between patients with high and low Ubc9 expression. We found that treatments including chemotherapy regimens, excision options, and endocrine therapy were comparable between two groups (Table 3). The Cox proportional hazards regression model showed that clinical stage (χ^2 = 7.324, P < 0.001), lymph node metastasis (χ^2 = 3.958, P = 0.047), and Ubc9 expression (χ^2 = 3.900, P = 0.048) were independent prognostic factors for survival of breast cancer patients (Table 4).

Further, disease-free survival (DFS) curves were evaluated between two groups with the Kaplan-Meier method. The patients were followed up for 10 to 72 months after operation, with a median of 45 months. Distant metastasis was not found in these patients upon diagnosis, but was identified in 20 (16.52%) patients during follow-up: 8 had liver and/or bone metastasis, 7 had bone metastasis, 4 had lung metastasis, and 1 had brain metastasis. The 2- and 4-year DFS rates of

Table 2. Clinical response to neoadjuvant chemotherapy in patients with high or low Ubc9 expression

Response to chemotherapy	Total No.	Ubc9 expression [No. (%)]	
		Low	High
cCR	14	8 (6.61)	6 (4.96)
PR	41	25 (20.66)	16 (13.22)
SD	27	10 (8.26)	17 (14.06)
PD	39	10 (8.26)	29 (23.97)
Total	121	53	68

cCR, complete clinical response; PR, partial response; SD, stable disease; PD, disease progression. χ^2 test was applied for data analysis, χ^2 = 11.09, P = 0.001.

Table 3. Treatment paradigms between patients with high and low Ubc9 expression

Treatment	Total No.	Ubc9 expression [No. (%)]		P
		Low	High	
Chemotherapy regimena				0.748
FEC	72	32 (26.46)	43 (35.54)	
FEC-T	46	21 (17.37)	25 (20.66)	
Excision				0.583
Mastectomy	93	42 (34.71)	51 (42.15)	
Conservation	28	11 (9.09)	17 (14.05)	
Endocrine therapy				0.148
AI	54	27 (22.31)	27 (22.32)	
TAM	44	20 (16.53)	24 (19.83)	
None	23	6 (4.96)	17 (14.05)	
Radiotherapy				0.234
Yes	99	40 (33.06)	59 (48.76)	
No	22	13 (10.74)	9 (7.44)	

[a]FEC regimen: 5-fluorouracil (500 mg/m^2), epirubucin (90 mg/m^2), and cyclophosphamide (500 mg/m^2); FEC-T regimen: FEC followed Taxotere (75 mg/m^2). AI, aromatase inhibitors; TAM, tamoxifen.

Table 4. Multivariate Cox proportional hazard analysis of prognostic variables in 121 patients with breast cancer

Variable	Wald (χ^2)	P	Hazard ratio	95% CI
Age	0.97	0.943	0.94	0.87–1.01
Size	1.11	0.573	1.08	0.77–1.44
Grade	1.89	0.297	1.15	0.94–1.33
Stage	7.32	<0.001	6.02	2.54–9.35
Lymph node status	3.96	0.047	2.02	1.19–2.74
Ubc9 expression	3.90	0.048	4.57	1.01–7.64

patients with high Ubc9 expression were 90.46% and 79.17%, respectively, compared to 96.15% and 91.12%, respectively, in those with low Ubc9 expression. The expected cumulative 6-year DFS rate was significantly higher in patients with low Ubc9 expression than in those with high Ubc9 expression (87.32% vs. 68.78%, χ^2 = 4.289, P = 0.038) (Figure 2). These results suggest that Ubc9 can be used as a prognostic factor for breast cancers.

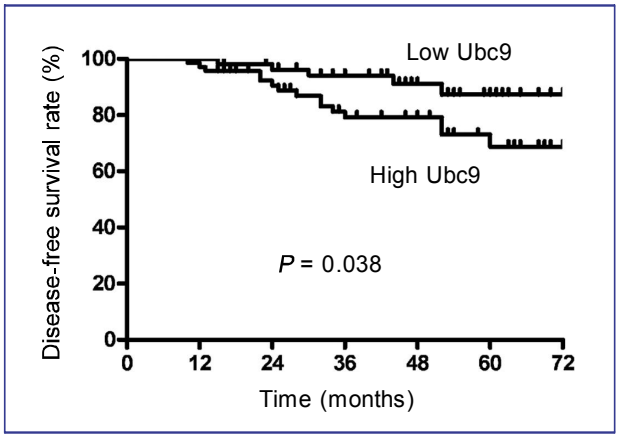

Figure 2. **The cumulative disease-free survival curves of patients with high or low Ubc9 expression.** The cumulative DFS rates were significantly higher in patients with low Ubc9 expression than in those with high Ubc9 expression (P < 0.05).

Discussion

Chemoresistance plays an important role in breast cancer recurrence and metastasis. In our study, we found that invasive ductal breast carcinomas highly expressed Ubc9, which was related with large tumor size, poor differentiation, advanced clinical stages, and lymph node metastasis. Specifically, Ubc9 was highly expressed in breast cancers with basal-like phenotype, which are less sensitive to chemotherapy. Moreover, the patients with high Ubc9 expression showed poor response to neoadjuvant chemotherapy and poor clinical prognosis, indicating that Ubc9 could serve as a predictor for chemosensitivity and prognosis of breast cancer.

The mechanisms of drug resistance in breast cancer are rather complicated and involve overexpression of ATP-binding cassette transporters, anti-apoptotic factors[19], and kinases for DNA repair [20]. However, targeting any single molecule is insufficient to reverse chemoresistance[21], suggesting that other molecules may also contribute to the sensitivity of breast cancer cells to chemotherapy. Here, we demonstrated that Ubc9, the sole E2-conjugating enzyme required for sumoylation, strongly associates with chemosensitivity and clinical prognosis in breast cancer.

SUMO modification (sumoylation) is a newly discovered form of post-translational modification, which modulates protein function through covalent attachment to lysine residues within targeted proteins. Many proteins involved in cell cycle regulation, proliferation, apoptosis, and DNA repair are targets for sumoylation. Thus, alterations in sumoylation could ultimately affect cell growth, cancer development, and drug response [22,23]. Ectopic expression of Ubc9 enhances tumor growth in animal models, suggesting that Ubc9 plays a critical role in tumorigenesis[24]. In our study, we compared the Ubc9 expression level in normal and malignant breast tissues, and found that Ubc9 was up-regulated in breast cancer like in other human malignancies[16]. Moreover, high Ubc9 expression associated with poor differentiation and lymph node metastasis, suggesting that high Ubc9 expression was associated with a more aggressive phenotype. More importantly, our clinical data showed that tumors with high Ubc9 expression poorly responded to neoadjuvant chemotherapy and experienced recurrence and metastasis more rapidly. These data indicated that poor clinical response and prognosis associated with high Ubc9 expression due to chemoresistance.

How Ubc9 is involved in chemoresistance remains unclear. A previous study showed that MCF-7 cells with mutated Ubc9 also had down-regulated Bcl-2, which was associated with a high rate of cell death and poor survival [25], thus suggesting that Ubc9 may mediate chemoresistance by regulating an anti-apoptotic protein. In our study, we found that Ubc9 expression level was up-regulated in breast cancers with basal-like phenotype, which are clinically characterized as more aggressive and less responsive to standard treatment and as being associated with poor overall patient prognosis [26]. Breast cancer cells with basal-like phenotype are known to show properties similar to BT-ICs. In our previous study, we found that Ubc9 plays a critical role in maintaining self-renewal and inhibiting apoptosis in BT-ICs [24], suggesting that there is a link between Ubc9-positive cells and BT-ICs. Therefore, the effect of Ubc9-positive cells on sensitivity to chemotherapy is probably related to their potential similarity to BT-ICs, which is chemoresistant due to overexpression of ATP-binding cassette transporter and dysregulation of signaling pathway[27].

In summary, this study provides the preliminary insight that Ubc9 expression may has some relationship with aggressive phenotype, and may serve as a predictor for clinical response to chemotherapy and poor prognosis. To make this finding more convictive, further studies are needed to enrich these results.

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