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Original Article

UCH-L1 involved in regulating the degradation of EGFR and promoting malignant properties in drug-resistant breast cancer

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Abstract: Ubiquitin carboxy terminal hydrolase-L1 (UCHL1) belongs to the UCH proteases family that deubiquitinates ubiquitin-protein conjugates in the ubiquitin-proteasome system. Our previous research showed that UCH-L1 and EGFR could regulate the expression of P-gp, CD147 and MMPs in multi-drug resistance (MDR) breast cancer cells, respectively. But it is still unclear whether direct regulation exists between the UCH-L1 and EGFR in MDR breast cancer. In order to clarify this, MDR human breast carcinoma cell line MCF7/Adr, that expresses relatively high UCH-L1, and its parental cell line MCF7, that expresses relatively low UCH-L1, were chosen for this study. We added ubiquitin proteasome inhibitor MG-132 into the culture of MCF7/Adr cells and transfected pIRES2-UCH-L1-EGFP plasmid into MCF7 cells, respectively. Using quantitative real-time polymerase chain reaction and western blot analyses, we found accompanying over-expression of UCH-L1, EGFR was up-regulated in both MCF7/ADR and MCF7 cells. Preliminary results indicated the degradation of EGFR might be regulated by ubiquitin level. So we speculated that up-regulated UCH-L1 could promote expression level of EGFR, thereby enhance the invasion and metastasis abilities of tumor cells. Moreover, to further explore the role of UCH-L1 and EGFR, we investigated the expression of UCH-L1, EGFR and P-gp in 65 local advanced breast cancer cases by immunohistochemistry assay. The result showed that the patients not responding to chemotherapy had higher UCH-L1, EGFR and P-gp expression levels and more lymph nodes metastasis. The Kaplan-Meier survival analysis showed that the patients with elevated UCH-L1 expression after chemotherapy presented shorter overall survival and disease free survival times than those with down-regulated or unchanged expression of UCH-L1. Our findings suggest that UCH-L1 may be an indicator of chemotherapy-response and poor-survival in breast cancer. UCH-L1 might be an appropriate target for improving chemo-resistant breast cancer therapy.

Keywords: UCH-L1, EGFR, breast cancer, multidrug resistance

Introduction

UCH-L1 is one member of the ubiquitin carboxy terminal hydrolase (UCH) family, which is selectively expressed in the testis/ovary and brain [1-3]. Recently some studies showed that UCH-L1 was abnormally expressed in some tumors, and correlated with cancer cell differentiation, metastasis and multi-drug resistance (MDR) [4-7]. In our past assays, we found UCH-L1 not only regulated the expression of P-gp, CD147 and MMPs, but also the ubiquitination and degradation of P-gp and CD147 in MDR breast cancer cells. P-gp and CD147 were ubiquitinated, and modification of ubiquitin was important for their degradation. We also found that EGFR could promote malignant properties of MDR breast cancer cells via up-regulating CD147, MMPs [8]. So we pondered the linkage between UCH-L1 and EGFR.

The ubiquitin-proteasome system (UPS) is a major pathway that mediates protein degradation. Many researchers have demonstrated that ubiquitination plays a crucial role in the regulation of protein turnover [9, 10]. EGFR is also reported degrading by the regulation of ubiquitin-proteasome pathway [11, 12]. In this study, the functional interaction between UCH-L1 and EGFR was examined, and their degradation

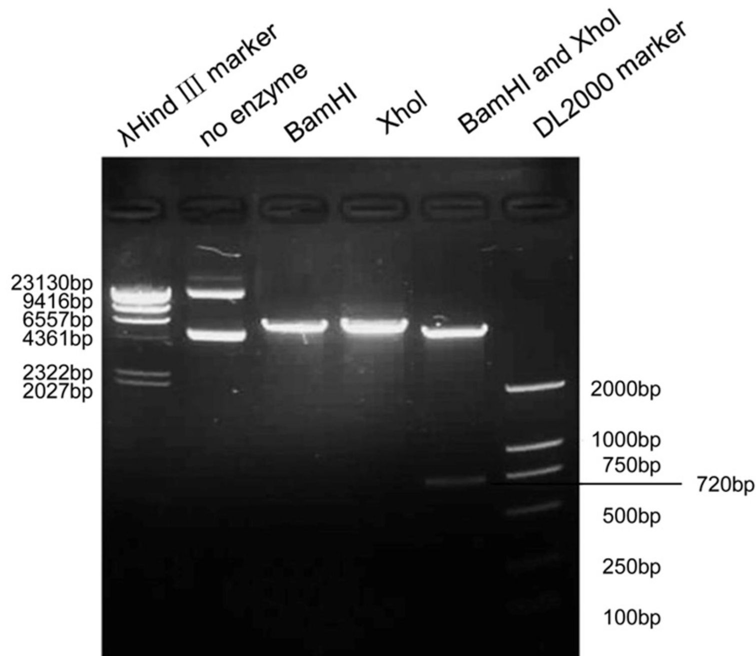

Figure 1. pIRES2-UCH-L1-EGFP plasmid was identified by digestion with restriction enzymes.

pathway was proposed. It is supposed that upregulated UCH-L1 could suppress the degradation of EGFR via ubiquitin-proteasome pathway, and then enhance the expression of P-gp, CD147 and MMPs, which directly resulted in promoting malignant properties of MDR breast cancer cells. This might provide further insight into the mechanism underlying the linkage between drug resistance and tumor metastasis.

Materials and methods

Cell culture

The MDR breast cell line MCF7/Adr was cultured in RPMI-1640 medium (Gibco-BRL, Karlsruhe, Germany), and the parental cell line, MCF7, was cultured in DMEM (high glucose; Gibco-BRL) supplemented with 0.01 mg/mL bovine insulin (Sigma, St. Louis, MO, USA). All cell culture media contained 10% foetal bovine serum (FBS; PAA Laboratories, Linz, Austria), 100 U/ml penicillin and 100 μg/mL streptomycin. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. For stable MDR1 gene expression, the MCF7/Adr cells were maintained in the presence of Adriamycin (Sigma). Ubiquitin proteasome inhibitor MG-132 was purchased from Sigma.

Plasmid transfection

MCF7 cells were transfected with eukaryotic expression vectors pIRES2-UCH-L1-EGFP (kindly provided by Professor Zhigang Zhang) or empty loading control vector using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. pIRES2-UCH-L1-EGFP plasmid was identified by digestion with restriction enzymes (**Figure 1**). In brief, 80% confluent cells grown in 100-mm dishes were washed twice with Opti-MEM (Gibco-BRL) medium before the addition of 25 μg of plasmid DNA and 60 μl of Lipofectamine 2000 in 3 ml of Opti-MEM media. After 6 h incubation at 37°C, culture medium was changed to usual complete medium and cells were cultured at 37°C for another 42 h till harvested. To detect the transfection efficiency, the fluorescent microscopy (Zeiss, Oberkochen, Germany) was used to detect the green fluorescent expression of the EGFP-labelled cells.

Reverse transcription and quantitative real-time polymerase chain reaction

Total cellular RNA was extracted using the Tripure isolation reagent (Sangon, Shanghai, China). The RNA samples were subjected to reverse transcription (RT) with 2 μg of RNA, Oligo $(dT)_{18}$, dNTP, and reaction buffer supplied with M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time polymerase chain reaction (PCR) reactions were then performed in 20 μL of solution with 2 μg of cDNA, 1 mM of each forward and reverse primer and 2× SYBR green mix (Takara, Shuzo, Kyoto, Japan). Changes in the mRNA expression level were calculated following normalisation to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. Relative gene expression was determined by the fluorescence intensity ratio of the target gene to GAPDH. The primers used in the real-time PCR reactions were designed based on information from the human genome database. The following are the primers used for the specific amplification of GAPDH and

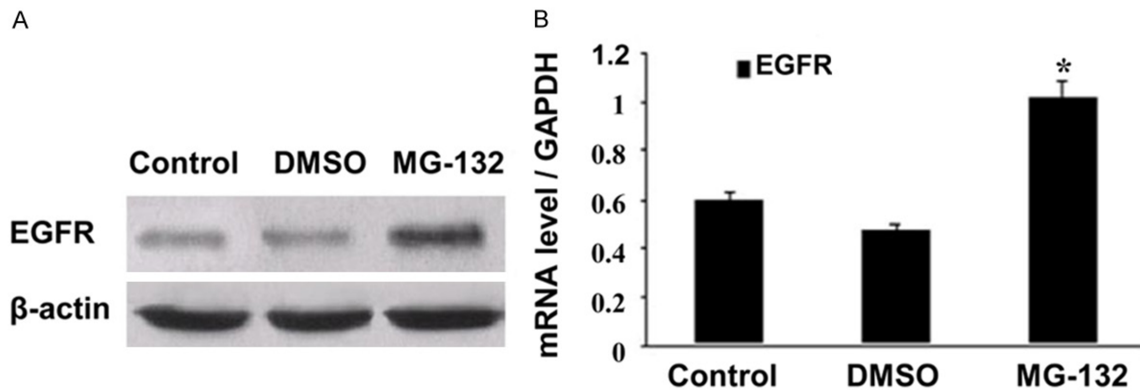

Figure 2. Ubiquitin proteasome inhibitor MG-132 blocked the degradation of EGFR in MCF7/Adr cells. after co-culture with ubiquitin proteasome inhibitor MG-132, in MCF7/Adr cells, significantly elevated expressions of EGFR were found in both protein and mRNA levels. A. EGFR protein level was assessed by western blot. B. EGFR mRNA level was assessed by real-time PCR. Bar graphs represent mean ± SEM of three independent experiments. *$P < 0.05$ vs. control cells. Results are representative of three similar experiments.

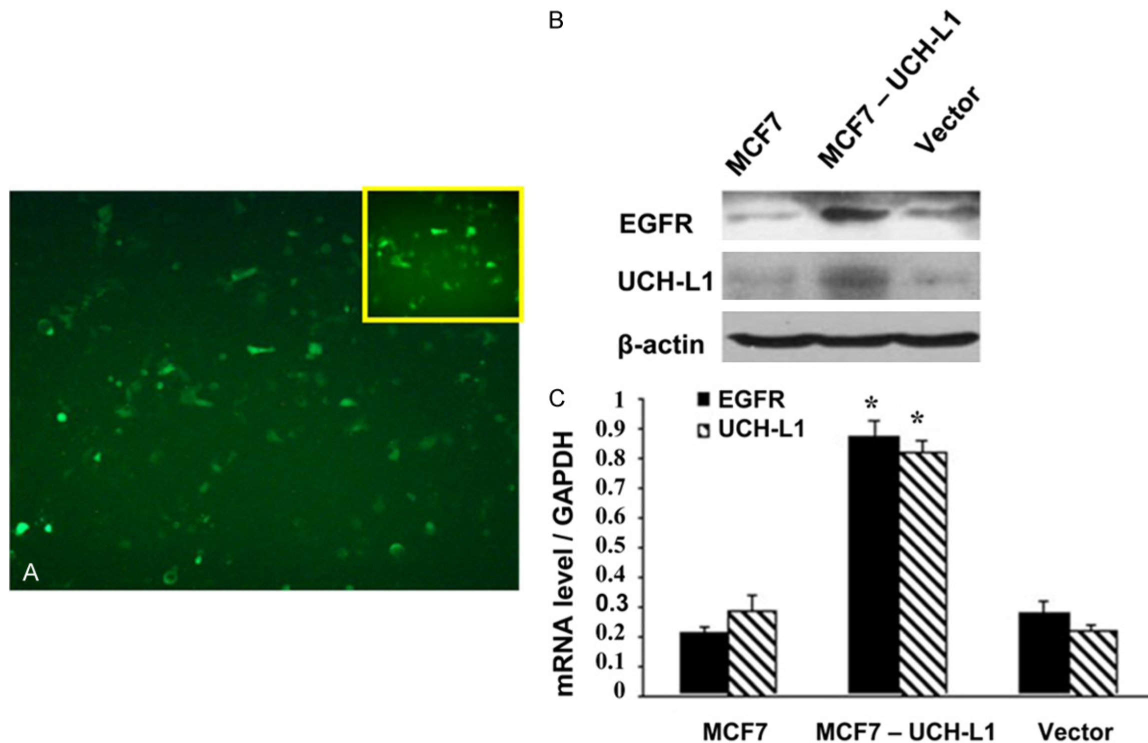

Figure 3. pIRES2-UCH-L1-EGFP plasmid caused over-expression of UCH-L1 and EGFR in MCF7 cells. A. PIRES2-UCH-L1-EGFP plasmid transfection efficiency was observed by immunofluorescence. (Original magnification ×200). B. EGFR and UCH-L1 protein level were assessed by western blot. C. EGFR and UCH-L1 mRNA level were assessed by real-time PCR. Bar graphs represent mean ± SEM of three independent experiments. *$P < 0.05$ vs. control cells. Results are representative of three similar experiments.

UCH-L1: GAPDH forward primer: 5'-AAC GGA TTT GGT CGT ATT G-3', and reverse primer: 5'-GGA AGA TGG TGA TGG GAT T-3'; UCH-L1 forward primer: 5'-GCC AAT GTC GGG TAG ATG-3', and reverse primer: 5'-CAA AGT CCC TCC CAC AGA-3'; EGFR forward primer: 5'-CCA AGG CAC GAG TAA CAA GC-3', and reverse primer: 5'-CCA AAT TCC CAA GGA CCA CC-3'.

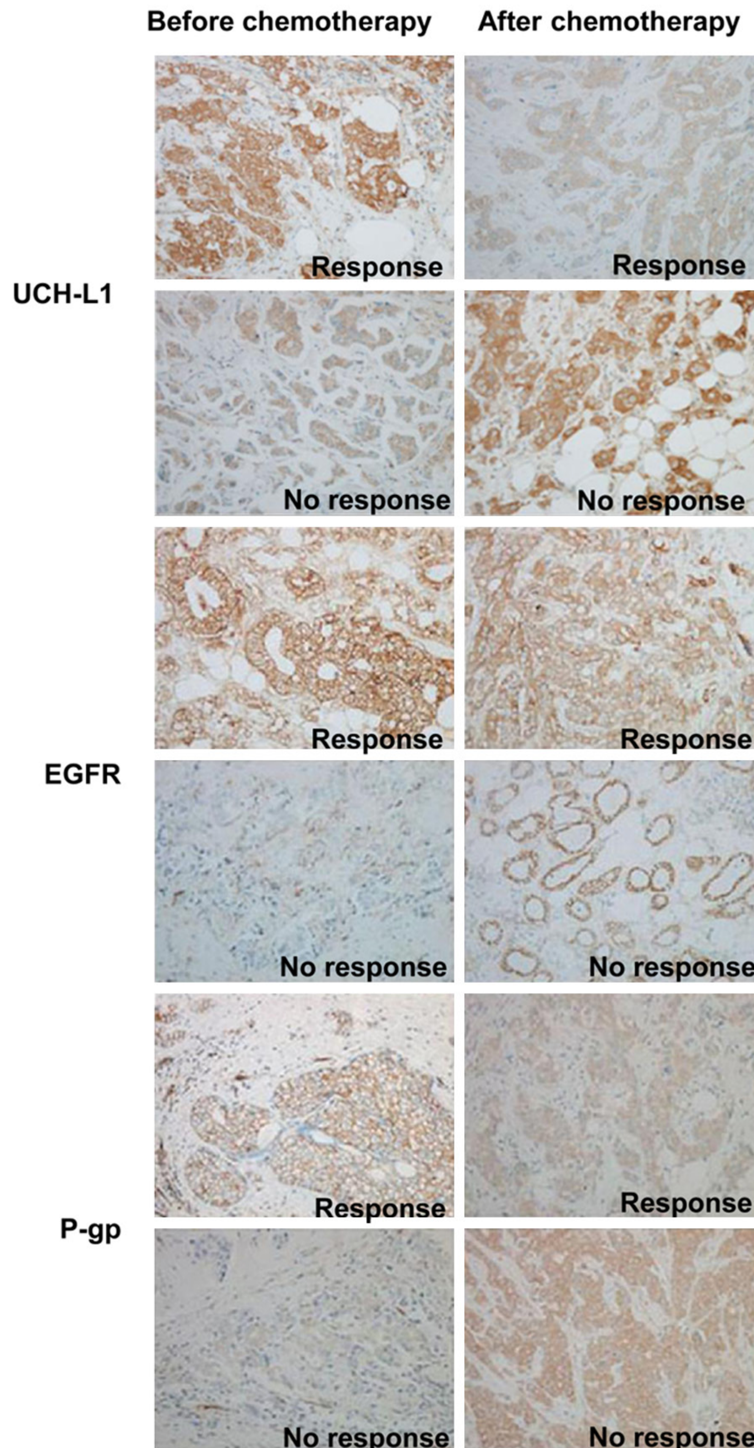

Figure 4. Expression of UCH-L1, EGFR and P-gp before and after chemotherapy was measured by immunohistochemistry in patients' tissues. Representative photographs of HE staining of UCH-L1, EGFR and P-gp of different groups are shown. (Original magnification 200×).

Western blot

The cells were collected and lysed in a modified RIPA buffer [50 mM Tris (pH 7.8), 150 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$, 1% NP-40, 0.5% sodium deoxycholate, 1 mM DTT, and 20 mM N-ethylmaleimide] containing 1 Complete Protease Inhibitor Cocktail tablet (Roche Molecular Biochemical, Indianapolis, IN, USA) per 50 mL of buffer. Total cell lysate (50 μg of protein) was resolved using standard sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% non-fat milk for 1 h at room temperature and then incubated overnight with primary antibodies against UCH-L1 (Chemicon, Temecula, CA, USA), EGFR (BD Biosciences, USA), and β-actin (Sigma) at 4°C. The membranes were then incubated for 1 h at 4°C with the appropriate HRP-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). Detection of the protein expression levels using enzyme-linked chemiluminescence (ECL; Pierce, Rockford, USA) was performed according to the manufacturer's protocol.

Patient and tissue specimens

After approval from our institutional review board, we selected 65 cases of local advanced breast cancer from a cohort of women who had undergone a core-needle biopsy (pathology-confirmed breast invasive ductal carcinoma) and neoadjuvant chemotherapy (paclitaxel 175 mg/m^2 (Bristol-Myers Squibb, USA) + epirubicin 75 mg/m^2 (Pfizer, USA), once every 3 weeks for 4 cycles) at our institution between 2009 and 2011 to be included in this study. All patients had received a radical mastectomy after 4 cycles of chemotherapy.

Table 1. Statistical quantification and comparison of IHC scores of the variation of UCH-L1, EGFR and P-gp before and after chemotherapy

	UCH-L1			EGFR			P-gp		
	Before	After	*P* value	Before	After	*P* value	Before	After	*P* value
PCR (13 cases)	2.15	N/A	N/A	0.63	N/A	N/A	0.5	N/A	N/A
Response (27 cases)	1.852	1.704	0.404	0.593	0.556	0.866	0.926	0.778	0.46
No response (25 cases)	1.84	2.44	0.001*	0.72	1.16	0.046*	1.12	1.6	0.037*

*$P < 0.05$.

Table 2. Statistical quantification and comparison of the expression of ER/PR and Ki67 and the status of LN metastasis after chemotherapy

	ER/PR (cases)		Ki67 (mean ± SD) %	LN metastasis (cases)		
	+	-		(-)	1-3 (+)	> 3 (+)
PCR (13 cases)	6	7	37.1429 ± 18.898	8	4	1
Response (27 cases)	19	8	26.214 ± 22.202	13	8	6
No response (25 cases)	15	10	35.4 ± 26.824	3	13	9
P value	0.341		0.489	0.005*		

*$P < 0.05$.

According to the Japanese Breast Cancer Society (JBCS) criteria for the histological evaluation of the therapeutic response after neoadjuvant chemotherapy [13], the cohort was divided into three groups, including pathological complete response (PCR, n = 13), partial response (n = 27) and no response (n = 25). Tissue specimens before and after chemotherapy were conserved for testing.

We ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The privacy rights of human subjects must always be observed.

Immunohistochemistry

Paraffin-embedded tissue samples from 18 murine tumours and 65 mammary carcinomas were prepared. Briefly, the slides were dehydrated in xylene and graded alcohols. Antigen retrieval of EGFR was performed with proteinase K at room temperature for 20 min. UCH-L1 and P-gp antigen retrieval were performed with 0.05 M TBS buffer and 0.01 M citrate buffer at pH 6.0 at 95°C for 20 min, respectively. Then, the slides were incubated with diluted primary antibody (anti-EGFR, 1:100 dilution; anti-UCH-L1, 1:50 dilution; anti-P-gp, 1:50 dilution) for 12 h, followed by incubation with a biotinylated secondary antibody for 1 h, peroxidase-labelled streptavidin for 15 min (LSAB-2 System; DAKO, Denmark), and diaminobenzidine and hydrogen peroxide chromogen substrate plus diaminobenzidine enhancer (DAKO) for 10 min. The slides were counter-stained with Mayer's haematoxylin. The analysis of EGFR, UCH-L1 and P-gp protein expression was performed using an EGFR rabbit polyclonal Ab (BD), a UCH-L1 rabbit polyclonal Ab (Chemicon) and a P-gp mouse monoclonal Ab (Calbiochem, USA). The positive controls for UCH-L1 and P-gp included sections of formalin-fixed, paraffin-embedded human brain tissues as indicated in the instruction manual, and the positive controls for EGFR included sections of FFPE human breast carcinoma tissues. The negative controls were incubated with an immunoglobulin fraction in place of the polyclonal primary Ab in the positive tissues mentioned above. The saturation and intensity of the immunostained cells were evaluated over three visual fields, at a power of 400× under a light microscope (Carl Zeiss, Gottingen, Germany). For the statistical analysis, according to the study by Han et al. [14] and our previous work (unpublished data), the total staining of EGFR, UCH-L1 and P-gp was scored based on the intensity and percentage of cells with EGFR, UCH-L1 and P-gp cytoplasmic staining using the following scale: score 0, negative staining for all of the tumour cells; score 1, negative/weak staining for all of the tumour cells or moderate staining in less than 10% of the cells; score 2, moderate staining in more than 30% but less than 70% of the tumour cells or strong staining within 10% of the tumour cells; score 3, moderate staining in more than 70%, or strong staining in more than 30% but less than 70% of all the tumour cells; and score 4, strong

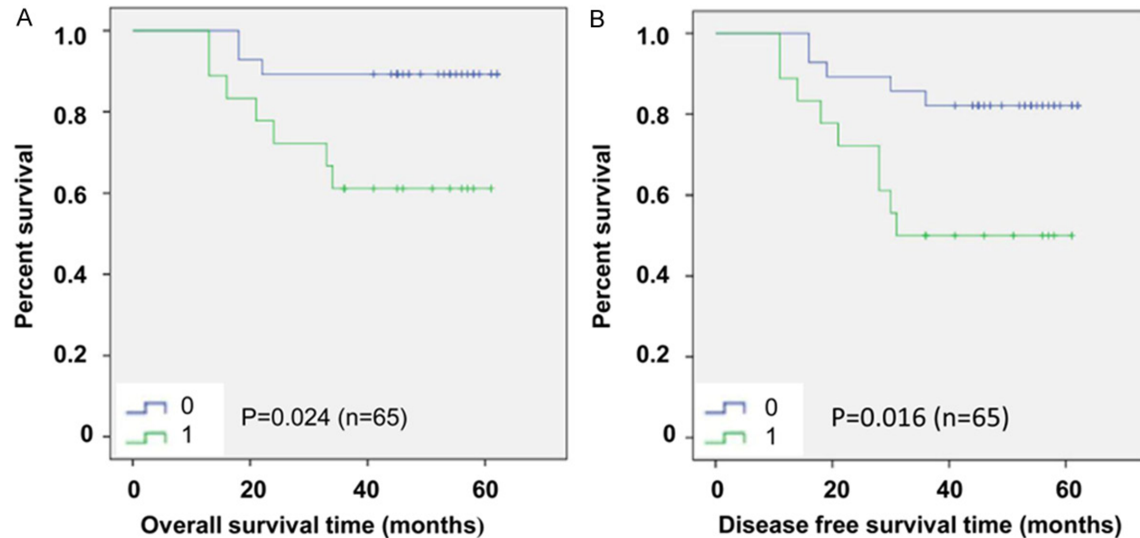

Figure 5. Kaplan-Meier plot of 65-cases survival analysis suggested that up-regulation of UCH-L1 after chemotherapy was concerned with shorter OS and DFS times in MDR breast cancer. Kaplan-Meier plot of OS and DFS of 65 patients with breast carcinomas stratified by UCH-L1 expression level was demonstrated in (A) and (B). 0: presented down-regulated or unchanged expression of UCH-L1 after chemotherapy; 1: presented up-regulated expression of UCH-L1. Data showed that there was a correlation between over-expressed UCH-L1 after chemotherapy and shorter overall survival time (A) and shorter disease-free survival time (B). A log-rank test demonstrated a significant difference between the groups ($P < 0.05$).

staining in more than 70% of all the tumour cells. The reproducibility of EGFR, UCH-L1 and P-gp staining was examined between 2 laboratories (by 2 independent pathologists) using 2 different primary antibodies.

Statistical analysis

Statistics were calculated using SPSS 19.0 software. All experiments were repeated at least three times, and the results are presented as the mean ± standard errors (SEM). The differences were analysed by ANOVA and Student's t-test. Pearson's correlation coefficients were used to determine whether two prognosis-related factors correlated with each other. Kaplan-Meier survival analysis was used to estimate the prognostic relevance, and the survival difference between groups was assessed by the log-rank test. The statistical significance was determined at $P < 0.05$ (two-tailed).

Results

Ubiquitin proteasome inhibitor MG-132 can block the degradation of EGFR in MCF7/Adr cells

In our past findings, we resolved that EGFR could regulate the expression of P-gp and CD147, and ubiquitin proteasome pathway also played an important role in the degradation of P-gp and CD147. So it is wondered whether EGFR is also regulated by ubiquitin proteasome pathway in MDR breast cancer cells. After co-culture with ubiquitin proteasome inhibitor MG-132, in MCF7/Adr cells, significantly elevated expressions of EGFR were found in both proteins and mRNA levels (**Figure 2A**, **2B**). It suggested that the block of ubiquitin proteasome pathway could affect the degradation of EGFR in MDR breast cancer cells.

pIRES2-UCH-L1-EGFP plasmid caused specific and effective up-regulation of UCH-L1 and EGFR expression in MCF7 cells

Our previous study has found both UCH-L1 and EGFR were involved in regulating the invasion/metastasis abilities of MDR breast cancer. So we wondered about the relationship between UCH-L1 and EGFR. For this purpose, we transfected pIRES2-UCH-L1-EGFP plasmid into MCF7 cells which expressing UCH-L1 in a relatively low level. The transfection efficiencies were initially evaluated using immunofluorescence analysis as shown in **Figure 3A**. In addition, real-time PCR and western blot analysis showed elevated expression of UCH-L1 in MCF7 cells, which resulted in up-regulation of EGFR (**Figure 3B**, **3C**).

UCH-L1 and EGFR are involved in MDR and metastasis process in chemo-resistant breast cancer tissue samples

65 local advanced breast cancer (LABC) cases undergoing neoadjuvant chemotherapy with the P-gp substrates, paclitaxel and epirubicin, were chosen for this study. The expression of UCH-L1, EGFR and P-gp were assayed by immunohistochemical staining (**Figure 4**). According to the chemo-response, the patients were divided into 3 groups: pathological complete response (PCR; n = 13), partial response (n = 27) and no response (n = 25). The elevated expression of UCH-L1, EGFR and P-gp after chemotherapy were observed only in the no-response group ($P < 0.05$; **Table 1**), which also exhibited an increase in lymph node metastasis ($P < 0.01$; **Table 2**). However, in the partial-response group, the expression of UCH-L1, EGFR and P-gp were decreased, and fewer lymph node metastases were found. There was no statistic correlation between UCH-L1 and ER, PR and Ki67, so was EGFR and P-gp (all $P > 0.05$; **Table 2**). In the PCR group, due to tumour missing after chemotherapy, the differential comparison before and after chemotherapy couldn't be conducted. The above results reveal that UCH-L1 and EGFR may be involved in the progression of promoting malignant properties in MDR breast cancer.

A 65-case survival analysis reveals a correlation between up-regulation of UCH-L1 and shorter OS/DFS in MDR patients

The 65 patients were divided to 2 groups according to the status of UCH-L1 after chemotherapy. One group presented up-regulated expression of UCH-L1, while the other group presented down-regulated or unchanged expression of UCH-L1. The Kaplan-Meier survival analysis showed that there was a correlation between overexpressed UCH-L1 after chemotherapy and shorter overall survival (OS) time ($P < 0.05$; **Figure 5A**) and shorter disease-free survival (DFS) time ($P < 0.05$; **Figure 5B**). It further suggested that UCH-L1 might be involved in chemotherapy resistance and poor survival.

Discussion

Ubiquitin proteasome system (UPS) participates in the turnover of many key regulatory proteins and in the development of cancer. UPS has three parts: ubiquitin, ubiquitin ligase and 26S proteolytic enzyme complex. The target protein is covalently connected to ubiquitin via ubiquitin ligase, and then is identified and hydrolyzed by 26S proteolytic enzyme complex [15]. UCH-L1 belongs to the UCH protease family that deubiquitinates ubiquitin-protein conjugates in the UPS [16]. It has a dual function: a hydrolase activity that removes small COOH-terminal ubiquitin to generate an ubiquitin monomer and a dimerization-dependent ubiquitin ligase activity [17]. So UCH-L1 can obstruct the degradation process of the target protein.

UCH-L1 over-expression has been observed in breast cancer [4]. In this study, two closely associated mammary carcinoma cell lines, MCF7 and MCF7/Adr, were chosen for comparison. Our previous experiments indicated that MCF7/Adr expressed much higher UCH-L1 than MCF7 cells [7, 18, 19]. Furthermore, the expression of UCH-L1 promoted coincided with up-regulation of MDR1 gene and increase of invasion abilities in MCF7 cells [21]. Through the role of deubiquitination, over-expressed UCH-L1 may block the degradation of P-gp and CD147, and then enhance expression of MMPs in MCF7/Adr cells. EGFR is also reported to play a very crucial part in enhancing CD147 and MMPs expression to establish favorable conditions for cells migration/invasion in MDR breast cancer cells [8]. EGFR is mostly reported degrading by autophagy-lysosome pathway, but the process can be affected by the regulation of ubiquitin.

According to above, UPS might be involved in the degradation of EGFR. We speculated through inhibiting the degradation of EGFR, UCH-L1 might infect the transcription levels of P-gp, CD147 and MMPs [20]. In this study, we found the expression of EGFR was consistent with UCH-L1 in both breast cancer cells and human tissues. When UCH-L1 plasmid was transfected into MCF7 cells, the expression of EGFR was elevated in coincidence with up-regulation of UCH-L1. Moreover, when MCF7/Adr cells were treated with ubiquitin proteasome inhibitor MG-132, the expression of EGFR was obviously up-regulated. These suggested that EGFR could degrade though ubiquitin proteasome pathway in MDR breast cancer. It is suggested that UCH-L1 plays as the deubiquitinat-

ing enzyme and inhibits the conjugating of the ubiquitin monomer and EGFR, which could further block the internalization and degradation of EGFR.

Our previous studies have shown both UCH-L1 and EGFR were involved in enhancing malignant properties in MDR breast cancer cells [8, 21]. However, there was no relevant clinical study reported. In our study, 65 local advanced breast cancer patients were conducted to verify the above findings. We found the patients not responding to chemotherapy had higher UCH-L1, EGFR and P-gp expression levels and more lymph nodes metastasis, which indicating poorer prognosis. Just the opposite, the patients who have responsed to chemotherapy presented decreasing UCH-L1, EGFR and P-gp expression and less lymph nodes metastasis. The Kaplan-Meier survival analyses showed that the patients with elevated UCH-L1 expression after chemotherapy presented shorter OS and DFS time than those with down-regulated or unchanged expression of UCH-L1. From the clinical perspective, this work revealed that high UCH-L1 and EGFR were involved in enhancing chemo-resistance and metastasis ability in breast cancer patients and lead to poorer survival.

Glogowska et al have reported that the combination of existing ErbB inhibitors and the UCH-L1 gene silencing effect of specific exon 23-encoded peptides may be a novel treatment for patients with aggressive ErbB1/2-dependent cancers [22]. This result is similar to suggest that UCH-L1 and EGFR have cooperation in promoting cancer malignant properties.

In summary, our findings indicate that UCH-L1 plays a crucial role in modulating the degradation of EGFR and promoting malignant properties in MDR breast cancer. UCH-L1 might be a novel target for improving EGFR-related chemoresistant breast cancer therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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