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Overexpression of Epidermal Growth Factor Receptor (EGFR) and HER-2 in Bladder Carcinoma and Its Association with Patients' Clinical Features

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Background: The aim of this study was to determine the expression of EGFR/HER-2 and investigate their association with patients' clinical features in bladder transitional cell carcinoma (BTCC).


Material/Methods: Immunohistochemistry was utilized in our study to explore the expression of EGFR/HER-2 of 56 human bladder cancer samples and 10 normal bladder samples.

Results: EGFR and HER-2 expressions were both significantly higher in bladder transitional cell carcinoma (BTCC) than that in non-cancer bladder samples; the EGFR positivity rate was 55.4% among BTCC samples and 37.5% for HER-2a. A statistically significant correlation was also present between the increasing EGFR or HER-2 expression levels and the clinical stages, pathologic grades, and tumor recurrence. The expression level of EGFR increased along with higher clinical stages and pathologic grades of BTCC, and the obviously increased expression of HER-2 was statistically associated with clinical stages and tumor recurrence. In addition, the expression level of HER-2 increased along with the higher clinical stage of BTCC. EGFR expression and HER-2 levels were positively associated in BTCC samples.

Conclusions: Our findings demonstrate that high EGFR and HER-2 expressions are dramatically increased in the BTCC tissues and are closely related to the clinical stages, pathologic grades, and tumor recurrence. Therefore, the evaluation of EGFR and HER-2 expression in BTCC may contribute to identifying patients who are at increased risk of disease progression and recurrence.

MeSH Keywords: **Genes, erbB-1 • Receptor, erbB-2 • Urinary Bladder Neoplasms**

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Background

Bladder carcinoma is the most common tumor in the urogenital system. During the past 20 years, the incidence of bladder carcinoma has been rising, and the incidence of bladder cancer is ranked 7th in men and 17th in women worldwide [1]. Bladder transitional cell carcinoma (BTCC) is the most common malignant tumor in the bladder and accounts for 90% of all malignant bladder tumors [2,3]. At present, although there has been in-depth research on the occurrence and mechanism of progression of bladder cancer, it remains unknown how treatment prevents the tumorigenesis of BTCC. It has a high recurrence rate of up to 50–70% after resection [4]. Despite surgical removal of cancer tissue, with cisplatin-based adjuvant chemotherapy after surgery, approximately half of the patients die within 4–5 years after surgery [5]. Treatment of bladder cancer has become one of the most challenging problems for urologists worldwide.

The epidermal growth factor receptor (EGFR) family has been implicated in tumor cell growth and differentiation, including EGFR, HER-2, HER-3, and HER-4 [6]. Many solid tumors have been found to overexpress EGFR, such as head and neck [7], lung [8], colon [9], breast [10], kidney [11], prostate, and bladder cancer [12]. EGFR is a tyrosine kinase transmembrane receptor that plays an important role in carcinogenesis, including cell survival, cell apoptosis, tumor metastasis, and angiogenesis via activating the EGFR signaling network [13]. HER-2 overexpression had studied extensively in breast carcinoma [14], and HER-2 is over-expressed in approximately 20% of primary invasive breast carcinomas [15] and is associated with poor prognosis [16]. Previous experiments have reported that EGFR and HER-2 are expressed in bladder cancer [17]. In the present study, we used immunohistochemistry to determine the expression of EGFR/HER-2, and to investigate their association with patients' clinical features, pathological grade, and tumor recurrence, in hope of finding a new therapeutic strategy for bladder carcinoma.

Material and Methods

Patients and tissue samples

Fifty-six bladder carcinoma samples were obtained during radical cystectomy from patients presenting with primary bladder tumors between January 2004 and December 2007 in the Second Hospital, University of South China, Central South University. This study was approved by the Institutional Review Board of the Second Hospital, University of South China, and every patient gave consent for EGFR/HER-2 examination of bladder carcinoma samples after being sufficiently informed. All samples were histopathologically diagnosed as bladder

transitional cell carcinoma, and all included patients had complete follow-up data. Meanwhile, 10 non-cancer bladder mucous membrane tissues samples were used as a control group which were obtained from morphologically normal areas as far away as possible from tumors exceeding 5 centimeters during cystectomy. These normal samples were further confirmed by histopathological after surgery. All tissues were fixed in 10% formalin and were paraffin-embedded. All the patients included were not treated by either radiotherapy, chemotherapy, or other non-operative treatment before surgery. The patient's general information including sex, age, tumor size, initially diagnosed or recurrence, tumor stage, tumor grade, and occurrence of lymph node metastasis were collected as clinical indexes. Histological cell type of the tumors was assigned according to the WHO classification: 12 were classified as grade I, 11 as grade II, and 33 as grade III. Staging was reviewed based on the UICC-TNM staging system: 10, 12, 15, and 19 tumors were stage T1, T2, T3, and T4, respectively.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections (3 μ m) were dewaxed with xylene and rehydrated through a series of graded alcohol, washed in distilled water and 0.01 phosphate-buffered saline (pH 7.2), and immersed in 10 mmol/L citrate buffer (pH 6.0), followed by antigen retrieval by heating sections for 15 min [18]. Following blocking of endogenous peroxidase activity with 0.3% hydrogen peroxide for 20 min, the sections were washed with phosphate-buffered saline (PBS) and incubated overnight in a humidified chamber at 4°C with the primary antibody (rabbit polyclonal anti-human EGFR or HER-2 antibody) at a working concentration [19]. After washing with PBS, sections were incubated with biotinylated goat anti-rabbit IgG/HRP (Wuhan Boster) for 20 min at 37°C. Then, Diaminobenzidine (DAB) was used as chromogen and the sections were subsequently counterstained with hematoxylin, then dehydrated, cleared, and mounted. Immunohistochemical staining was examined under bright-field microscopy (Ordinary optical microscope OlymPusBX40, Japanese Olympus Company).

All slides were evaluated by a double-blind method by 2 professors of the Department of Pathology at our institution (The Second Hospital, University of South China, Central South University). We found no false-positives or false-negatives when performing the immunohistochemical staining. Samples were viewed at high magnification and the total score was based on a system previously described by Fromowitz et al. [20]. Two scoring systems – staining intensity and percentage of stained cells – were included. The staining intensity was scored on a semi-quantitative 4-point scale as: 0, equivalent to the negative control; 1, weak cytoplasmic stain slightly darker than the negative control; 2, moderate stain, defined as an intensity of score 1–3; 3, intense stain, equivalent to or darker than the

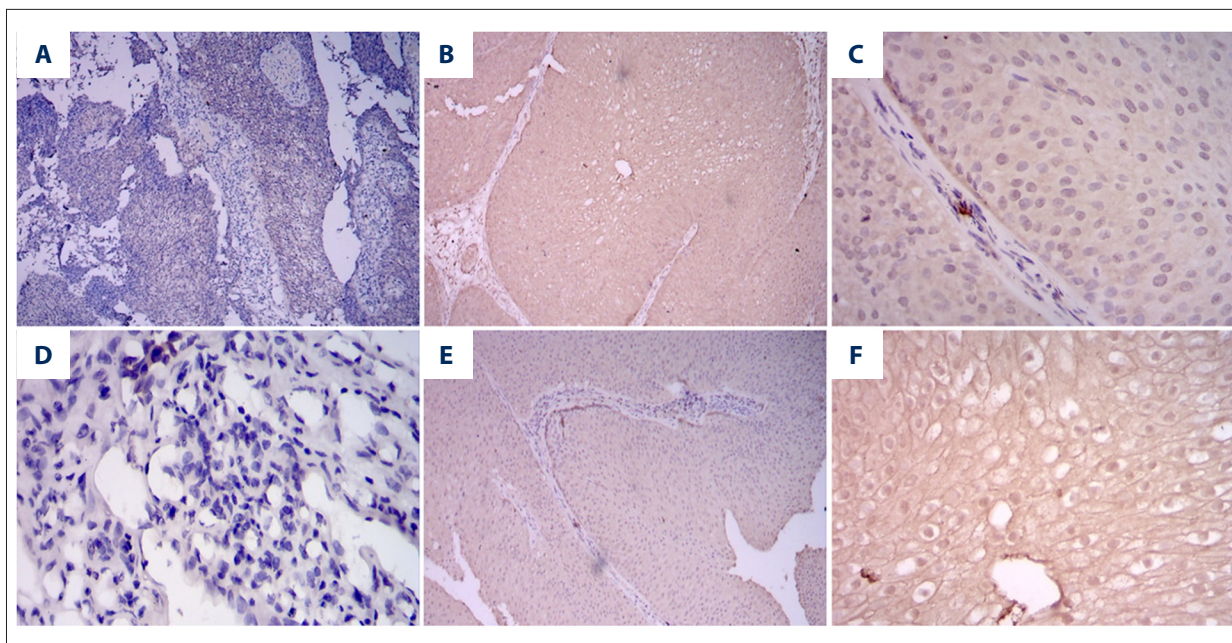


Figure 1. Expression of EGFR and HER-2 in BTCC and normal bladder tissues (A) Normal bladder tissue, negative immunostaining for EGFR (×100); (B) BTCC tissue, positive immunostaining for EGFR (×100); (C) BTCC tissue, positive immunostaining for EGFR (×400); (D) Normal bladder tissue, negative immunostaining for HER-2 (×100); (E) BTCC tissue, positive immunostaining for HER-2(×100); (F) BTCC tissue, positive immunostaining for HER-2 (×400).

Table 1. Expression of EGFR in BTCC and normal bladder tissues.

| Variables | No. of patients | EGFR | | | | P | No. of patients | HER-2 | | | | P |
|---------------|-----------------|------|----|----|-----|--------|-----------------|-------|---|----|-----|--------|
| | | - | + | ++ | +++ | | | - | + | ++ | +++ | |
| Normal tissue | 10 | 9 | 1 | 0 | 0 | P<0.05 | 10 | 10 | 0 | 0 | 0 | P<0.05 |
| BTCC | 56 | 25 | 11 | 14 | 6 | | 56 | 35 | 7 | 11 | 3 | |

BTCC – bladder transitional cell carcinoma.

positive control. The percentage of stained cells was also scored on a semi-quantitative 4-point scale as: 0 for no positive cells; 1, 0–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. Then, a composite score was obtained by multiplying the score of staining intensity and percentage of stained cells: a score of 0 was –, 1–4 was +, 5–8 was ++, and 9–12 was +++.

All statistical analyses were calculated using SPSS for Windows, v 17.0. The association between the variables was tested using the chi-square test and Fisher’s exact probability test. The relationship between EGFR and HER-2 was analyzed using non-parametric correlation (Spearman) test, and a P-value of less than 0.05 was considered significant.

Results

The expression level of EGFR/HER-2 in BTCC and normal tissues

The EGFR/HER-2 expression were located mainly in the membrane of tumor cells and was sparsely distributed in the cytoplasm of tumor cells (Figure 1). Positive immunohistochemical expression of EGFR was observed in tissue sections from 31 of 56 (55.4%) BTCC tissues: 11 cases were scored as +, 14 cases were scored as ++, and 6 cases were scored as +++. In contrast, immunohistochemical expression of EGFR was absent or very limited in 1 of 10 (10%) cancer-free controls: 9 with no staining and only 1 case was scored as +. The expression of EGFR in BTCC was markedly higher than that in non-cancer bladder tissues (P<0.05; Table 1). Likewise, positive immunohistochemical expression of HER-2 was observed in tissue sections from 21 of 56 (37.5%) BTCC tissues: 7 cases were scored as +,

Table 2. Correlation between EGFR and HER-2 in BTCC.

| EGFR | No. of patients | HER-2 | | rs | P |
|----------|-----------------|----------|----------|-------|-------|
| | | Negative | Positive | | |
| Negative | 25 | 21 | 4 | 0.458 | 0.000 |
| Positive | 31 | 14 | 17 | | |
| Total | 56 | 35 | 21 | | |

Table 3. Associations between EGFR/HER-2 expression in bladder cancer and clinical pathological parameters.

| Variable | No. of patients | EGFR expression | | P | HER-2 expression | | P |
|-----------------------|-----------------|-----------------|----------|--------|------------------|----------|--------|
| | | Negative | Positive | | Negative | Positive | |
| Sex | | | | | | | |
| Male | 38 | 17 | 21 | 0.984 | 22 | 16 | 0.310 |
| Female | 18 | 8 | 10 | | 13 | 5 | |
| Age | | | | | | | |
| <60 | 21 | 10 | 11 | 0.729 | 11 | 10 | 0.233 |
| ≥60 | 35 | 15 | 20 | | 24 | 11 | |
| Tumor size, cm | | | | | | | |
| <3 cm | 28 | 14 | 14 | 0.420 | 17 | 11 | 0.783 |
| ≥3 cm | 28 | 11 | 117 | | 18 | 10 | |
| Single or multiple | | | | | | | |
| Single | 25 | 11 | 14 | 0.931 | 16 | 9 | 0.835 |
| Multiple | 31 | 14 | 17 | | 19 | 12 | |
| First or recurrent | | | | | | | |
| First | 24 | 17 | 7 | 0.001* | 20 | 4 | 0.005* |
| Recurrent | 32 | 8 | 24 | | 15 | 17 | |
| Tumor grade | | | | | | | |
| G1–G2 | 23 | 16 | 7 | 0.002* | 17 | 5 | 0.068 |
| G3 | 33 | 9 | 24 | | 18 | 16 | |
| Tumor stage | | | | | | | |
| T1–T2 | 22 | 14 | 8 | 0.021* | 19 | 3 | 0.003* |
| T3–T4 | 34 | 11 | 23 | | 16 | 18 | |
| Lymph node metastasis | | | | | | | |
| Negative | 36 | 18 | 18 | 0.279 | 20 | 16 | 0.150 |
| Positive | 20 | 7 | 13 | | 15 | 5 | |

* P<0.05.

11 cases were scored as ++, and 3 cases were scored as +++. In contrast, immunohistochemical expression of HER-2 was not absent in 10 cancer-free controls. The expression of HER-2

in BTCC was markedly higher than that in non-cancer bladder tissues (P<0.05; Table 1). We subsequently compared the immunohistochemistry staining results of EGFR and HER-2.

Table 4. Associations between expression of EGFR and different clinical stages and pathologic grades.

| Variable | EGFR | | | | Total | P |
|-------------|------|---|----|-----|-------|-------|
| | - | + | ++ | +++ | | |
| Tumor stage | | | | | | |
| T1 | 8 | 1 | 1 | 0 | 10 | |
| T2 | 7 | 3 | 2 | 0 | 12 | |
| T3 | 4 | 4 | 5 | 2 | 15 | |
| T4 | 6 | 3 | 6 | 4 | 19 | <0.05 |
| Tumor grade | | | | | | |
| G1 | 10 | 2 | 0 | 0 | 12 | |
| G2 | 6 | 3 | 2 | 0 | 11 | |
| G3 | 9 | 6 | 12 | 6 | 33 | <0.05 |

Table 5. Associations between expression of HER-2 and different clinical stages.

| Variable | HER-2 | | | | Total | P |
|-------------|-------|---|----|-----|-------|-------|
| | - | + | ++ | +++ | | |
| Tumor stage | | | | | | |
| T1 | 8 | 1 | 1 | 0 | 10 | |
| T2 | 11 | 0 | 1 | 0 | 12 | |
| T3 | 10 | 1 | 2 | 1 | 15 | |
| T4 | 5 | 5 | 7 | 2 | 19 | <0.05 |

A positive correlation was observed between expression of EGFR and HER-2 in the BTCC tissues ($P<0.01$, $r=0.458$, Table 2). $P<0.01$, $r=0.458$.

The association between EGFR/HER-2 expression in BTCC and clinical pathological parameters

As shown in Table 3, EGFR expression was significantly associated with tumor first occurrence or recurrence, pathological T stage, and pathological grade (all $P<0.05$); for example, the positive rate of EGFR expression in BTCC tissue was 75.0% (24/32) in the recurrent group, but 29.2% (7/24) in the primary group; 72.7% (24/33) in the high-grade group (G3), but 30.4% (7/23) in the low-grade group (G1–G2); 67.6% (23/34) in the high-stage group (T3–T4), but 36.4% (8/22) in the low-stage group (T1–T2). Similar results were obtained for HER-2 expression, which was obviously linked with tumor first occurrence or recurrence and pathological T stage (all $P<0.05$); for instance, the positive rate of HER-2 expression in BTCC tissue was 53.1% (17/32) in the recurrent group but 16.7% (4/24) in the primary group, and 52.9% (18/34) in the high-stage group (T3–T4) but 13.6% (3/22) in the low-stage group (T1–T2).

The expression level of EGFR was significantly higher in the recurrent group, higher-stage group, and higher-grade group than in the primary group, lower-stage group, and lower-grade group. There was no significantly correlation between EGFR expression and other 5 clinical parameters (sex, age, tumor size, single or multiple, and lymph node metastasis). The expression level of HER-2 was obviously higher in the high-stage group and recurrent group than in the low-stage group and primary group. There was no significant correlation between HER-2 expression and the other 6 clinical parameters.

The specific expression of EGFR in different clinical stages and pathologic grades

As observed from the results of the analysis above, a statistically significant correlation was also present between EGFR expression and clinical stages and pathologic grades. As shown in Table 4, the expression level of EGFR increased with higher clinical stages and pathologic grades of BTCC ($P<0.05$).

The specific expression of HER-2 in different clinical stages

As shown in Table 5, the expression level of HER-2 increased with higher clinical stages of BTCC ($P<0.05$). A statistically significant correlation was present between HER-2 expression and clinical stages ($P<0.05$).

Discussion

The human epidermal growth factor receptor (EGFR) family comprises 4 members – EGFR, HER-2, HER-3, and HER-4 – which is a transmembrane glycoprotein, and structurally they have 3 important parts: an extracellular ligand-binding domain, a hydrophobic transmembrane region, and an intracellular kinase domain [21]. These tyrosine kinases are activated by the binding of epidermal growth factor (EGF) to its extracellular ligand-binding domain. Normally, the 2 receptors undergo dimerization after the initial binding of EGF to EGFR, leading to autophosphorylation of the dimer and finally activation of it [22]. The activated receptor then recruits proteins that phosphorylate and activate Ras protein, which can then turn on the switch of the MAPK/ERK complex [23], and transduce a mitogenic signal to downstream signaling pathways, which play an important role in cell proliferation, apoptosis, differentiation, and angiogenesis [24–26]. EGFR is a tyrosine kinase transmembrane receptor mainly expressed on epithelial cells, and it is involved in carcinogenesis by regulating cell mortality, apoptosis, cancer invasion, and metastasis [27]. Expression and overexpression of the EGFR have been demonstrated in human solid tumors, such as in cancers of the bladder, breast, colorectal, gastric, NSCLC, prostate, and ovary [28]. The expression of HER-2 in normal tissues is minimal in the bile, duct, gastrointestinal tract, liver, skin, genital organs, and urinary tract [29]. HER-2 overexpression can induce oncogenic transformation and is related to a poor prognosis [16].

EGFR and HER-2 have many homologies in structure and share many biochemical characteristics [30]. EGFR and HER-2 proteins are the primary signal proteins involved in the pathway of signal transduction cascade in metastatic and invasive tumors [24]. It has been reported that expression of EGFR and HER-2 promotes cell migration, which plays an important role in cellular metastasis [31]. Experimental evidence has also indicated that the overexpression or amplification of EGFR and HER-2 in bladder carcinoma is associated with higher tumor grade/stage and poorer prognosis [32–34].

The present study found 55.4% positivity for EGFR and 37.5% positivity for HER-2 (Table 1); there was a positive correlation between expression of EGFR and HER-2 in BTCC tissues (Table 2). These findings are consistent with other reports showing high EGFR and HER-2 expression in tumor tissues,

in contrast to the low levels in the non-cancer tissues. More than 50% of human BTCC have a high EGFR expression, and the level of expression was related to stage and grade of BTCC, as well as patient survival [35]. Sriplakich et al. [36] found that 58% of tumors were EGFR-positive in 100 BTCC patients, and, most importantly, the level of EGFR expression was higher in the T2-4 tumors. Moreover, Neal et al. [34] showed a 36% positivity of EGFR in both superficial and invasive bladder carcinoma. A study by Cardillo et al. [37] reported a higher rate (75%) of EGFR expression on 56 invasive bladder carcinoma tissues. However, the positive expression rate of HER-2 ranges from 7% [38] to 71% [39], due to different experimental methods and materials with a wide range of variation. On the other hand, because of the utility of different reagents, particularly due to the diversity of sensitivity of the same antibody from different manufacturers, the criteria for evaluation make it difficult to achieve consistent levels of HER-2. Jimenez et al. [40] found that 28% of a total of 80 patients with muscle-invasive urothelium carcinoma expressed HER-2. Another study demonstrated 41% positivity of HER-2 in T2 invasive bladder cancer tissues, and found that the amount of HER-2 in the high-grade tumors was higher than that in the low-grade ones [41]. Caner et al. [42] and Tsai et al. [43] showed that positivity for HER-2 was 61% and 58%, respectively, in bladder carcinoma tissues. However, little research on EGFR and HER-2 has been carried out in bladder carcinoma. Kiyoshima et al. [44] reported that EGFR and HER-2 were positive in 63% and 22% of the patients, respectively, among 67 patients with BTCC. In the contrast, another study showed 23% positivity for EGFR and 60% positivity for HER-2 [45]. Interestingly, some results revealed that simultaneous expression of EGFR and HER-2 occurred in carcinomas of the urinary bladder [46,47], and increased expression level of these 2 proteins were related to the histologic grades and the stages of the disease, and denotes aggressive biologic behavior. Consistently, we observed a positive correlation between expression of EGFR and HER-2 in the BTCC tissues (Table 2), and it may be available to use EGFR and HER-2 as drug targets in treatment of bladder cancer.

Our study data also indicated that EGFR and HER-2 are expressed at a significantly higher level on BTCC cells than on non-cancer cells. Jianhong Li et al. [48] found that the expression of EGFR was a closely related to the stages of disease and survival in patients with urothelial carcinoma by observing 248 cases of bladder cancer. Neal et al. [34] indicated that strong staining for the EGFR was found in 48% of tumors and was associated with high-stage cancers by immunohistochemistry staining of 101 bladder cancer cases. However, the expression rate of EGFR has a positive association with multiple factors, such as time to recurrence, recurrence rate, and tumor progression in patients with pTa and pT1 tumors. Benjamin et al. [49] performed a study on the correlation between EGFR expression and prognosis of bladder cancer with postoperative long-term follow-up of 212 bladder cancer patients,

which demonstrated that the survival rate of EGFR-negative patients after surgery was significantly higher than for those who were EGFR-positive. Furthermore, in our study, we found that the expression level of EGFR was significantly higher in the recurrent group, high-stage group, and high-grade than in the primary group, low-stage group, and low-grade group (Table 3). Furthermore, the expression level of EGFR increased with higher clinical stage and pathologic grades of BTCC (Table 4).

Studies on the expression of HER-2 in bladder carcinoma have shown large differences in the association between HER-2 and tumor stage or grade. Shawky Holah et al. [50] analyzed the HER-2 overexpression in 59 patients with urothelial carcinoma by immunohistochemistry, and found HER-2 overexpression was obviously correlated with different tumor grades, but found no association between HER-2 overexpression and tumor stage. Similarly, Jamal et al. [51] showed that HER-2 has no obvious correlation with tumor stage or grade. In our study, the expression level of HER-2 was obvious higher in the high-stage group and recurrent group than in the low-stage group and primary group (Table 3). The expression level of HER-2 increased with higher clinical stage of BTCC, but there was no

association between HER-2 expression and the tumor grade (Table 5). According to the above results, EGFR/HER-2 could be a valuable biomarker for progression of bladder cancer, and can contribute to identifying patients who are at increased risk of disease progression and recurrence. In addition, Pontus Eriksson et al. [52] found that HER-2 amplifications and overexpression may be 2 fundamentally different molecular subtypes – Urothelial-like (Uro) or the Genomically Unstable (GU) subtypes – and that EGFR expression is associated with the SCC-like subtype. Hence, recruiting bladder cancer patients for HER2-directed therapy almost certainly has to consider the molecular context (i.e., molecular subtypes) into consideration. Proper tumor classification is likely to be critical for developing new HER2- and EGFR-based treatment regimens.

Conclusions

Our findings demonstrate that there is higher EGFR and HER-2 expression in BTCC tissues. Evaluation of EGFR and HER-2 expression can help define the role of these new targeted therapies in treatment of BTCC.

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