Epidermal Growth Factor Receptor Expression in Cervical Intraepithelial Neoplasia and Its Modulation during an α-Difluoromethylornithine Chemoprevention Trial

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ABSTRACT

Chemoprevention trials designed to prevent progression to invasive cervical cancer will benefit from the identification of biomarkers that assess the risk of developing tumors, predict likelihood of response to treatment, and measure biological response to intervention. The purpose of this study was to examine expression of epidermal growth factor receptor (EGFR) as a marker for progression of cervical intraepithelial neoplasia (CIN) and as a surrogate end point biomarker in a chemoprevention trial with α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase. To evaluate quantitative and spatial changes in EGFR expression during cervical tumorigenesis, paraffin sections from 42 archival cervical cone biopsies, each containing multiple stages of CIN, were immunohistochemically stained for EGFR, and the level and spatial expression of EGFR were quantitated by image analysis. In the progression from normal epithelium to CIN 1 to CIN 2 to CIN 3 to invasive cancer, EGFR expression showed two types of changes. Normal control epithelium showed EGFR expression predominantly confined to the basal layer, while histologically normal epithelium in specimens containing CIN showed relatively increased EGFR expression in the basal layer and the extension of EGFR expression away from the basal layer. The total EGFR relative staining intensity (RSI) of epithelium increased with the degree of CIN, predominantly due to a progressive expansion of EGFR-expressing cells away from the basal layer rather than an increase in the level of EGFR expression per cell. To determine whether EGFR expression would be modulated by a 1-month chemopreventive intervention with DFMO, pretreatment and posttreatment cervical biopsy specimens from 25 patients (22 evaluable) were examined for EGFR expression. Although the overall levels of EGFR expression were not modulated in either histological responders or nonresponders, responders showed a prominent down-regulation of EGFR expression away from the basal layer after DFMO treatment. Interestingly, pretreatment EGFR expression levels predicted for DFMO response [i.e., eight responses (72.7%) for 11 cases with RSI levels below 0.35 versus one response (9.1%) for 11 cases with RSI levels above 0.35 (P < 0.01)]. These results suggest that CIN progression is associated with a spatial dysregulation of EGFR expression that can be reversed by DFMO treatment, especially in patients whose pretreatment CIN 3 lesions exhibit relatively low EGFR expression.

INTRODUCTION

Cervical cancer remains an important health problem among women worldwide (1). The development of novel cancer-preventive strategies will benefit from an intimate knowledge of the clinical and molecular basis of cervical tumorigenesis (2). The progression of CIN³ to invasive cancer is thought to represent a multistep process that involves an accumulation of genetic changes in tissue (3) that are then translated into phenotypic changes associated with dysregulation of cell growth and differentiation. Markers of these genetic and phenotypic changes can then theoretically be used as indicators of the degree of progression in premalignant lesions (e.g., risk markers) as well as SEBs of response to chemopreventive intervention (4).

EGFR is one member of a growth factor receptor family that has been shown to play an important role in the growth and differentiation of cells in culture as well as in tissues (5). When dysregulated in expression or activity, EGFR is thought to contribute to the growth of preneoplastic and neoplastic lesions through ligand-dependent and ligand-independent mechanisms (5-7). In turn, the increase of EGFR-associated tyrosine kinase activity is thought to regulate downstream pathways (8-10) that exert pleiotropic functions in cells depending on their tissue origin and state of differentiation (5). In this regard, EGFR expression has been found to be higher in a variety of cancer and precancer lesions than the surrounding normal tissue (11-14).

The abbreviations used are: CIN, cervical intraepithelial neoplasia; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; EGFR, epidermal growth factor receptor; RSI, relative staining intensity; SD, standard deviation; SEB, surrogate end point biomarker.
To examine whether the degree of EGFR overexpression might be a useful marker of progression in cervical precancer lesions, EGFR expression has been studied semiquantitatively in CIN lesions; EGFR expression was shown to be higher in these lesions than in normal epithelium (13–16). However, these semiquantitative methods have not been able to demonstrate differences in EGFR expression among different grades of CIN (13, 14). In addition, previous studies have not addressed the question of changes in the spatial distribution of EGFR expression in dysregulated intraepithelial neoplasia to better understand its functional role in CIN progression. Thus, the first goal of this study was to characterize the spatial and quantitative patterns of EGFR expression in tissues spanning the transition from normal cervical epithelium through various stages of CIN to invasive cervical cancer.

A better understanding of the tumorigenesis process could lead to the identification of targets for chemopreventive or therapeutic intervention (17–19). Because dysregulated proliferation is one common phenotypic change associated with tumor development, one approach for preventive intervention has been to slow cell growth. ODC, a key element of polyamine synthesis, is one of the enzymes known to be up-regulated in response to growth factor stimulation pathways, and its aberrant expression has been shown to have transforming potential (20). Thus, it has been suggested that inhibition of ODC might have potential as a preventive strategy (18, 19). DFMO, an irreversible inhibitor of ODC, has been shown to exhibit antitumor and antimetastatic activity in many carcinogen-treated animal models and is currently being tested as a chemopreventive agent for specific human cancer and precancer lesions (17–21).

Recently, a chemoprevention trial using DFMO for the treatment of individuals with CIN 3 lesions was carried out at The University of Texas M. D. Anderson Cancer Center to determine the minimal DFMO dose needed to see a biological response (21). Biopsies were obtained before and at the end of treatment (30 daily doses of DFMO) and subjected to biomarker analysis. The purpose of the second part of this study was to determine whether EGFR levels would be modulated in vivo during DFMO treatment of CIN 3 lesions and whether the degree of modulation would be associated with clinical response. The results reported herein indicate that lesions with relatively high pretreatment levels of EGFR are relatively resistant to DFMO treatment. Moreover, DFMO treatment does not seem to modulate EGFR expression even in responding patients, suggesting that DFMO acts downstream of EGFR in the regulation of proliferation in vivo.

MATERIALS AND METHODS

Selection of Samples. For studies designed to quantify spatial changes in EGFR expression during the transition from normal epithelium to CIN lesions to invasive cervical tumors, 41 formalin-fixed, paraffin-embedded tissue specimens containing CIN lesions were obtained from the Department of Pathology at the University of Texas M. D. Anderson Cancer Center. These specimens, obtained from patients treated between 1988 and 1994, were selected because they contained multiple degrees of dysplasia within the same tissue section (e.g., two or three different grades of CIN lesions with or without adjacent normal tissue). Of the 41 specimens evaluated, 36 included histologically normal adjacent mucosa, 21 CIN 1, 23 CIN 2, and 36 CIN 3. Fifteen of the 41 specimens exhibited all three grades of CIN as well as adjacent normal epithelium in the same specimen. Eight of the 41 specimens also contained invasive carcinoma. In addition, nine normal cervical tissue specimens, obtained from hysterectomies performed because of noncancerous or precancerous conditions, served as “normal” controls.

For studies designed to evaluate the modulation of EGFR expression during DFMO treatment, uterine cervical biopsy specimens were obtained from 25 patients with CIN 3 lesions both before and after 4 weeks of oral administration of DFMO (0.0625–1.0 g/m²/day). The pretreatment biopsies were obtained during colposcopy and the posttreatment samples were cone biopsies. In 3 of these 25 cases, biopsies were invaluable for EGFR expression measurements because of truncation of the superficial epithelial layers. Thus, 22 cases were evaluable for pre- and posttreatment comparisons.

All specimens were fixed in formalin and embedded in paraffin. Four-micrometer-thick histopathological sections were used for these analyses. One set of sections from each specimen was stained with H&E for pathological diagnosis, and the adjacent sections were used for immunohistochemical detection of EGFR expression and quantitation by image analysis. A common positive control section for EGFR expression was placed on each slide alongside the tissue section to control for variations in immunohistochemical staining efficiencies. The positive control was prepared by embedding pellets of squamous head and neck cancer 886 cells in paraffin as described previously (11). H&E-stained histological slides were reviewed and mapped by two pathologists (A. M. and I. V. B.) to identify regions of interest. The locations of epithelial regions exhibiting CIN 1–3 with or without adjacent normal cervical epithelium were identified according to criteria described previously (22).

Immunohistochemical Analysis. Anti-EGFR monoclonal antibody (Clone E30) was obtained from BioGenex, Inc. (San Ramon, CA) and used to immunohistochemically detect EGFR using a modified avidin-biotin immunoperoxidase method as described previously (11). Briefly, after deparaffinization by xylene and rehydration with graded alcohols, endogenous peroxidase activity was blocked by incubating slides in methanol with 3% H₂O₂ for 10 min. After washing in PBS, the slides were incubated with nonimmune horse serum to decrease the background signal, rinsed in PBS, and incubated with a 1:3 dilution of prediluted anti-EGFR mouse monoclonal antibody for 2 h at 37°C per the manufacturer’s recommendation. The slides were then washed with PBS, incubated with biotinylated secondary antibody for 45 min at room temperature, and incubated with biotin-avidin peroxidase conjugate (ABC Kit, Vector Laboratories, Burlingame, CA) at a dilution of 1:50 for 30 min at room temperature. After washing in PBS, the EGFR antigen was visualized with a 0.1% 3,3′-diaminobenzidine solution (Sigma Chemical Co., St. Louis, MO) in PBS and H₂O₂ (0.01%). The slides were not counterstained to enable quantitation of EGFR expression by image analysis.

Image Analysis. The intensity of EGFR expression was determined using the Magiscan Image Analysis System (Joyce-Loeb, Ltd., Dukessway, England) attached to a Nikon light
microscope with a computer-controlled stage. The relative degree of EGFR expression was quantitated separately in each epithelial layer. The first step of analysis involved visual localization of the representative CIN lesions or adjacent normal epithelium using the adjacent H&E-stained slides as templates. Basal, parabasal, intermediate, and superficial regions of the epithelium were separately circled with a light pen, and each circled region was measured for its integrated absorbance and area (in pixels). The specific intensity of each region was calculated as the total integrated absorbance divided by the area of the region, minus the contribution of integrated absorbance of an equivalent area of background. To overcome the variations in degree of staining from slide to slide, and to be able to compare one slide with another, all values for the specimens were normalized to those for the 886 cell sections that were placed on each slide and thus stained under identical conditions. Thus the normalized value of EGFR expression, or the RSI of EGFR expression, represents a measure of the relative level of EGFR expression (relative to that of 886 cells). This evaluation is represented by the following equation:

\[
\text{RSI of EGFR} = \left( \frac{\text{OI} - (\text{KB} \times \text{OA})}{\text{OA}} \right)_{\text{sample/mean}} \left[ \frac{\text{OI} - (\text{KC} \times \text{OA})}{\text{OA}} \right]_{886 \text{ cells}}
\]

where OI is the integrated signal over an integrated area (OA), KB is the absorbance of the background of tissue per unit area, and KC is the absorbance of the background of 886 cells per unit area.

Because the x and y coordinates of each detected region were collected along with the absorbance values, topological maps of the spatial distribution of EGFR expression were generated using a relative pseudo color scale.

**Statistical Analysis.** Statistical analysis included unpaired t-test and \( \chi^2 \) analyses for significance (where applicable). The statistical package STAT (Stata Corp., College Station, TX) and the Magiscan Image Analysis System statistical program were used for these analyses.

**RESULTS**

**Changes in EGFR Expression with CIN: Analysis of Archival Specimens.** Immunohistochemical evidence of EGFR expression was easily detected in the normal cervical epithelium controls. Expression was predominantly detected in the basal layer (Fig. 1A). In contrast, in histologically normal epithelium adjacent to CIN lesions, while EGFR expression was present in the basal layer, it also seemed to extend into the parabasal cell layers and tapered off in the intermediate layers (Fig. 1B). As the histological grade increased from CIN 1 to CIN 3, EGFR expression seemed to gradually extend further away from the basal layer into the more superficial layers (Fig. 1B and C). The histological classification of CIN 3 lesions described here includes both severe dysplasia and carcinoma in situ (23). Nevertheless, carcinoma in situ lesions (i.e., abnormal immature cells occupying the whole epithelium) appeared to express the highest levels of EGFR, and this level of expression was detected throughout the whole epithelium (Fig. 1D).

To better estimate the immunohistochemically detected levels of EGFR expression, image analysis of the stained slides was carried out using the Magiscan Image Analysis System and the mean RSIs (relative to control 886 cells) determined for the histological areas of interest (Table 1). Although control normal epithelium and normal epithelium adjacent to CIN lesions seemed histologically similar, normal epithelium adjacent to CIN lesions showed more than twice the overall levels of EGFR expression than did normal control epithelium (RSIs, 0.19 and 0.08, respectively; \( P < 0.01 \)). As shown in Table 1 and Fig. 2A and B, this apparent increase in overall EGFR expression was due to two types of changes: (a) there was a modest increase in the level of EGFR expression in the basal cell component; and (b) there was an expansion of cells with relatively high levels of EGFR expression away from the basal layer into the parabasal layers.

This trend toward an expansion of cells with increased EGFR expression into the epithelium continued with progression from normal to CIN 1 to CIN 2 to CIN 3 (Table 1). On the other hand, although only eight archival specimens contained invasive disease, their overall EGFR expression levels were lower than that observed in CIN 3 lesions (i.e., median RSI of 26.5, range 12–48). Although the relative level of EGFR expression remained somewhat constant in the basal layer in CIN lesions, intermediate and superficial epithelial layers showed progressively higher EGFR expression with increasing CIN grade. There were more than 10-fold increases in the level of EGFR expression in the intermediate and superficial layers as lesions progressed from normal epithelium to CIN 3. Moreover, the degree of tapering of EGFR expression away from the basal layer was less pronounced in more advanced CIN lesions (Fig. 2C and D). That is, the slopes of the regression lines continuously decreased from adjacent normal epithelium to CIN 1, to CIN 2, and to CIN 3 (Fig. 3).

At first glance, it would seem that the increases in EGFR expression in the intermediate and superficial layers during histological progression might simply reflect the expansion of cells with basal-like qualities. However, as shown in Table 1, increases in EGFR expression seemed to precede the histological appearance of basal-like cells in various areas of the epithelium. For example, the parabasal layers of normal epithelium adjacent to CIN lesions already showed elevated EGFR expression before the appearance of an expanded basal layer. Similarly, the intermediate and superficial layers of CIN 1 lesions showed elevated EGFR expression before the appearance of basal-like cells. This trend continued for CIN 2 lesions. Thus, the measurement of EGFR expression levels may provide additional information to that obtained by histological analysis in describing the progression of CIN lesions.

**Changes in EGFR Expression with DFMO Treatment: Analysis of a Clinical Trial.** Thirty patients with CIN 3 were treated in a clinical Phase I trial with DFMO at five dose levels, ranging from 0.0625 to 1.0 g/m²/day for 1 month. As described elsewhere (24), half of the patients showed histological response by the end of the month-long treatment. Pre- and posttreatment cervical biopsies from 25 of the 30 patients were available for analysis of changes in EGFR expression; samples from 22 patients were evaluable. Of these, 9 patients had histological complete or partial responses and 13 patients showed no evidence of histological response.
EGFR RSI was measured on the most histologically advanced lesions of each pre- and posttreatment specimen in accordance with the maps created by the two pathologists on the H&E-stained slides. Because CIN 3 lesions showed elevated EGFR expression throughout the epithelium, quantitative assessments were first made by including all layers of the epithelium together. As shown in Fig. 4, the initial EGFR RSI values exhibited a wide range of values (median, 0.35; range, 0.04–0.58). These values were somewhat lower than those observed for CIN 3 lesions in the archival specimens (median, 0.43;
Fig. 2  Semiquantitative spatial representation of EGFR expression in the same tissue sections illustrated in Fig. 1. The levels of EGFR expression were normalized to common internal standards and are presented using a relative pseudo color scale (bottom). Note the gradual extension of EGFR expression into the more superficial layers with evidence of histological progression.

range, 0.16–0.97), perhaps because of the inclusion of patients with invasive carcinoma and carcinoma in situ in the archival series or because of the limited sizes of the pretreatment biopsies.

DFMO treatment did not seem to significantly modulate EGFR expression levels in these patients with CIN 3 disease (Fig. 4). For example, the mean RSI value (± SE) before DFMO treatment for the group as a whole was 0.32 ± 0.07; the mean RSI value after treatment was 0.35 ± 0.08. When EGFR modulation was compared in responders and nonresponders, the trend was somewhat surprising if it were postulated that successful DFMO treatment would result in a down-regulation of EGFR expression. As might be expected, there was no significant change in overall EGFR expression during treatment in the nonresponders (pretreatment mean, RSI, 0.49 ± 0.05; posttreatment mean, RSI, 0.43 ± 0.08; P = 0.06). Similarly, overall EGFR expression in the responders seemed to remain constant (pretreatment mean, RSI, 0.26 ± 0.05; posttreatment mean, RSI, 0.29 ± 0.05; P = 0.11).

On the other hand, the responders and nonresponders showed different spatial patterns of EGFR expression throughout the epithelium after DFMO treatment. As shown in Fig. 5, when the levels of EGFR expression were measured layer by layer, the responders exhibited a change in the slope of EGFR expression through the epithelium (pretreatment slope, -0.166; posttreatment slope, -0.422). This change in slope of EGFR expression was not seen in the nonresponders (pretreatment slope, -0.174; posttreatment slope, -0.169). Interestingly, because the total EGFR levels in the responders remained the same after treatment whereas the negative slope of expression increased, this meant that the levels of EGFR expression in the basal layer increased after treatment in the responding cases.
Fig. 3 Comparative EGFR expression levels as a function of epithelial layer with histological progression from control normal epithelium to normal epithelium adjacent to CIN lesions, and different grades of CIN lesions. Note the change in slopes of EGFR expression from basal to superficial layers with histological progression.

To better understand the nature and role of dysregulated EGFR expression during cervical tumorigenesis, archival cone biopsies were specifically chosen if multiple stages of histological progression were present within the same specimen. The advantage of this approach is that trends in changes in EGFR expression for the overall group can be tested within individual cases. The results described here demonstrate that EGFR expression is sequentially dysregulated during cervical tumorigenesis, and this result confirms previously published results by others in which EGFR expression levels were described semi-quantitatively for the whole epithelium (13–16).

The present report also adds new insights into the process of EGFR dysregulation during cervical neoplasia. For example, the EGFR expression dysregulation process seems to involve two separate types of events. First, the level of EGFR expression in the basal layer in histologically normal epithelium adjacent to CIN lesions seems to be twice that observed in control normal epithelium. Thus, one component of EGFR overexpression, i.e., increased expression per cell, seems to precede histological evidence of altered epithelium. Although the mechanism underlying the up-regulation of EGFR expression in normal epithelium adjacent to CIN is not understood, several possibilities exist. Because these studies were carried out using archival material selected for the presence of more advanced lesions, one possibility is that paracrine factors from the adjacent CIN lesions influence the regulation of EGFR expression in normal epithelium. An alternative explanation is that up-regulation of EGFR expression in normal epithelium adjacent to CIN reflects an early step in the tumorigenesis process. For example, some data suggest that HPV-associated gene products (i.e., E5 protein) up-regulate EGFR expression (25). Examination of these possibilities will be facilitated by the spatial EGFR expression maps generated by this study.
A second hallmark of a useful biomarker is that it might help in assessing risk of progression. The results reported here and elsewhere (26–28) show that the degree of EGFR expression dysregulation increases as tissues progress toward invasive cancer. However, even within histologically similar lesions, the levels of EGFR expression were found to have a broad range. The archival specimens used in this study were chosen because they contained multiple stages of CIN within the same specimen. Thus, the finding of higher EGFR expression in normal epithelium adjacent to CIN lesions than in normal epithelium not in the field of CIN may suggest that EGFR expression might be a potential marker of progression risk. Although the number of samples studied in this report were not sufficient to adequately address this question, EGFR expression in lesions of similar histological stage seemed to be higher in cases in which more advanced lesions (e.g., invasive cancer) were present in the same specimen (data not shown). Similar types of observations have been found with other biomarkers for the cervix as well as for other epithelial sites. Thus, the role of the use of EGFR expression as a biomarker of risk of progression deserves careful retrospective and prospective exploration.

The studies reported here suggest that the total levels of EGFR expression in target cervix tissue were not significantly modulated by DFMO treatment. However, although the overall level of EGFR expression did not serve as a useful intermediate marker of response, it did provide some useful information about the mechanisms of DFMO response. Measurements of proliferation changes after DFMO treatment using the same clinical specimens demonstrated that DFMO treatment resulted in a decrease in the proliferation index (i.e., decreased fractions of proliferating cell nuclear antigen- and MPM-2-stained cells and decreased overall DNA content of the lesions; 22, 29, 30). Moreover, the degree of proliferative down-regulation was greater in the responders than in the nonresponders. Thus, because down-regulation of EGFR expression was not associated with response, this would suggest that DFMO acts downstream of the regulation of EGFR expression. It has been reported previously that ODC transfection into NIH/3T3 cells increases EGFR tyrosine kinase activity (31, 32). Thus, one possible explanation is that DFMO, by inhibiting ODC, decreases the tyrosine kinase activity of EGFR, which results in decreased proliferation in the epithelium.

Although DFMO treatment did not seem to alter the total levels of EGFR expression in epithelial tissue, it did alter the spatial pattern of EGFR expression in the responding cases. Thus, although the apparent levels of EGFR expression seemed to increase in the basal and parabasal cells of responders after DFMO treatment, the levels of EGFR expression in the intermediate and superficial layers were markedly decreased in association with response to treatment. The mechanistic basis for this observation is not known. However, it was reported recently that transfection-mediated overexpression of ODC results in a decrease of EGFR expression in NIH/3T3 cells (31). Thus, it is conceivable that, conversely, inhibition of ODC by DFMO might lead to an increase in EGFR levels in the basal component but may reduce EGFR transcription in the superficial layers.

The potential mechanism of response of CIN 3 lesions to DFMO is not well-understood. One possibility is that although DFMO did not alter EGFR expression, it might have affected
EGFR activity, modulated a downstream signal transduction pathway, or more directly interfered with DNA synthesis. The tyrosine kinase activity of EGFR is thought to be important for its downstream functions (8–10), and its activity can be modulated by both ligand-dependent and ligand-independent mechanisms (5, 6). In turn, activation of the EGFR tyrosine kinase pathway has been shown to result in an increase in ODC activity and consequent cell proliferation (33, 34). Because DFMO is an inhibitor of ODC activity (17), it is suspected that in this setting, DFMO is eliciting its clinical activity downstream of EGFR function. In support of this notion, Brzozowski et al. (33) showed that DFMO administration could interfere with the healing activity of epidermal growth factor (i.e., promotion of DNA synthesis) in stress-induced lesions in rats. Nevertheless, in the clinical setting reported here, the fact that EGFR expression remained elevated despite clinical and histological evidence of response would suggest that the dysregulated cells are still present in the tissue after partial or complete response. This possibility would argue against the mechanism of response involving elimination of the more advanced components of the lesions and preferential regrowth of normal epithelium. Rather, it would suggest that DFMO affects the phenotype of the cells in the tissue. The resultant implication is that the chemopreventive intervention should be continued beyond the time of response.

The results reported here also indicated that high pretreatment EGFR expression was associated with decreased response to DFMO treatment. Again, the basis of this finding is not well-understood. Because EGFR expression dysregulation seems to increase with the progression of CIN lesions, this might suggest that the CIN 3 lesions showing the highest EGFR expression represent more biologically advanced lesions that might be less responsive to single-agent chemopreventive strategies. A similar finding was reported for the treatment of oral leukoplakia; lesions with inappropriately high levels of p53 expression showed resistance to treatment with 13-cis-retinoic acid (35). This finding led to the exploration of the use of combinations of chemopreventive interventions in the treatment of more advanced lesions, and early clinical results indicate provocative activity (36). Thus, in the setting of chemoprevention trials in individuals with CIN 3 lesions, the studies reported here might suggest that individuals with high EGFR expression in their lesions might be considered for clinical trials using combinations of chemopreventive agents. Because high EGFR expression is associated with lessonsen response, one potential strategy might be to combine DFMO with an agent that targets EGFR expression or activity itself (37, 38).

In summary, the results of this study suggest that EGFR expression is dysregulated during histological progression in cervical tissues in two ways: (a) as an up-regulation of expression per cell; and (b) as an expansion of EGFR-expressing cells in the epithelium. The results also indicate that the measurement of EGFR expression may be useful as an indicator of the degree of progression along the CIN pathway and possibly as a marker of risk of invasive cervix cancer. Future studies are needed to further explore the mechanisms and functional consequences of dysregulated EGFR expression in cervical lesions, with the hope of identifying suitable targets as well as suitable individuals for particular strategies of chemopreventive intervention.

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