EGFR LI and Ki-67 LI are independent prognostic parameters influencing survivals of surgically treated squamous cell lung cancer patients

J. NIEMIEC[1], L. KOLODZIEJSKI[2], S. DYCZEK[3]

[1]Laboratory of Radiation Biology, e-mail: joannna@eikon.pl, Centre of Oncology, Kraków, Poland; [2]Department of Oncological Surgery, and [3]Diagnostic Radiology Department, Centre of Oncology, Krakow, Poland

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In literature there are still opinion differences concerning the prognostic significance of epidermal growth factor receptor (EGFR) expression and proliferative potential in patients with non small cell lung cancer (NSCLC). This prompted us to study those parameters.

The Ki-67 labeling index (Ki-67 LI), EGFR labeling index (EGFR LI), and mitotic index (MI) were analyzed in the group of 78 consecutive, surgically treated squamous cell lung cancer (SqCLC) patients. The expression of Ki-67 and EGFR protein was visualized on formalin fixed, paraffin embedded sections using immunohistochemistry (IHC). Mitotic index was assessed on formalin fixed, paraffin embedded sections, stained with hematoxylin and eosin using morphological criteria.

Mean values of Ki-67 LI and MI were higher for G2+G3 tumors than for G1 tumors. EGFR LI was higher for G1+G2 than for G3 tumors, and for pT3 than for pT1+pT2 tumors. Patients having tumors with Ki-67 ≤28% or 13% ≥EGFR LI + EGFR LI 80% survived significantly shorter than those having tumors with Ki-67 LI >28% or <13% ≤GFR LI 80%.

In multivariate analysis, 13% EGFR LI 80% and Ki-67 28% were independent negative prognostic parameters influencing survivals of SqCLC patients.

Key words: Ki-67 labeling index, EGFR labeling index, mitotic index, squamous cell lung cancer, prognostic factor

In lung cancers, like in most solid tumors, high proliferative index is one of the first phenotypic changes that appear as manifestation of accumulated genetic lesions. On the other hand, specific signaling pathways are responsible for changes in proliferative potential of airway epithelium [14]. One of signaling pathways that are best characterized is that of the group of epidermal growth factor receptor (EGFR) membrane proteins [14]. Facts mentioned above suggest that, in lung cancer, among parameters with prognostic significance [19], both proliferation and EGFR expression might be of prognostic significance.

In lung cancer prognostic significance of proliferative potential has been tested using many methods (mitosis count, AgNOR count, bromodeoxyuridine labeling index, S phase fraction, proliferative index, potential doubling time). The results however are still conflicting. Some authors found longer survival in patients with high proliferative potential [17], some found inverted correlation [40] or found no relation between proliferative potential and patients' survival [4]. Similar discrepancies between the results are characteristic for EGFR expression in lung cancer [6, 38, 35]. The disagreement mentioned above might be caused by histological heterogeneity of analyzed NSCLC groups and by the lack of standard methods assessing EGFR expression.

Those facts prompted us to evaluate prognostic significance of Ki-67 labeling index (Ki-67 LI), mitotic index (MI) and EGFR LI in SqCLC patients.

Material and methods

Patients. Seventy-eight consecutive patients (71 men and 7 women) with SqCLC underwent radical surgery between 1986–1999. The mean age of patients was 58.9±0.9, and varied from 41 to 73 years. Before surgery the patients did not receive radio- or chemotherapy. According to commonly accepted criteria [2, 30] the same surgeon operated all patients. Thirty-nine patients underwent lobectomy, and 39 pneumonectomy, assessed as sufficiently radical by the surgeon and

the pathologist [2, 30]. Mediastinal lymphandectomy was performed only in suspected cases (enlargement of mediastinal lymph nodes or histologicaly-confirmed metastases in "frozen section") – in the others only sampling was performed [31, 32]. Nineteen patients after radical surgery were subjected to adjuvant radiotherapy and two, to chemotherapy. The clinical (TNM) and pathological (pTNM) stages were established according to TNM UICC 1997 criteria [29, 42]. Table 1 and Table 2 summarize the stage and grading of analyzed tumors. Each patient was followed-up from surgery till June 26, 2002 (for the purpose of this study follow-up was finished at 5th year after surgery). From thirty cancer deaths: 7 (23.3%) were caused by loco regional cancer recurrence and 23 (76.7%) by distant metastases. From 48 alive patients, forty-seven live without progression of malignant disease and 1 with metastases of SqCLC. The study has been approved by the Ethical Committee by the Centre of Oncology.

Material. Shortly after excision, the fresh specimens (about 0.5 cm^2) were fixed in 10% neutral buffered formalin and embedded in paraffin. The slides were examined by a pathologist in order to establish histology and grading.

Staining procedures. Sections were cut at 4 μm, mounted on Super Frost^{R}Plus (Menzel-Gläser, Germany) slides, and then deparaffinized and hydrated through a series of xylens and alcohols.

Immunohistochemical visualization of Ki-67 antigen. Hydrogen peroxide in 70% methanol was used to block the activity of endogenous peroxidases. For antigen retrieval heating the slides in a microwave oven in 0.01 M citrate buffer (pH 6.0), was performed (four, 5-minute cycles – 800 W). Nonspecific binding of immunoglobulins was blocked by 20% swine serum (normal) (DAKO Ltd.). The slides were incubated overnight at 4 °C with Ki-67 antibody (rabbit anti-human Ki-67 antigen, DAKO, Ltd.) that was diluted 1/200 in TBS. Ki-67 antibody was localized during two, one-hour incubations at room temperature. The first one with biotinylated swine anti-rabbit immunoglobulin (DAKO, Ltd) diluted 1/300 in TBS, while the second with streptavidin-POD (peroxidase) conjugate (Boehringer Mannheim, Ltd.) diluted 1/800 in TBS. Peroxidase was visualized using 0.01% 3.3-diaminobenzidine tetrahydrochloride and 0.015% hydrogen peroxide. Next, slides were counterstained with hematoxylin. For negative control, TBS was substituted for the primary antibody.

Immunohistochemical visualization of EGFR. The sections were digested for 15 minutes at room temperature with DAKO^{R}Ready-to-use proteinase K (DAKO Ltd.). Then, the sections were rinsed in Tris-Buffered Saline (TBS) at pH 7.4. EGFR was visualized using monoclonal mouse anti-human EGFR, clone H11 (DAKO Ltd.), (dilution 1/200) and DAKO EnVision^{TM} + system. Sections were counterstained with hematoxylin. For negative control, TBS was substituted for the primary antibody. Positive controls were performed on SqCLC sections known to exhibit membranous overexpression of EGFR.

Histology. Tumor samples were fixed in buffered formalin routinely processed and embedded in paraffin. For assessment of MI, hematoxylin-eosin-staining was performed.

Evaluation of Ki-67 LI. Only tumor cells with positive nuclear staining for Ki-67 were counted (Fig. 1A). Cells were scored at 400 magnification. Between 500 and 1000 cells from each slide were counted in 5–6 fields. The Ki-67 LI was calculated as the percentage of Ki-67 labeled cells.

Evaluation of EGFR LI. Assessment of EGFR expression was based on EGFR protein staining (weak or strong) in membranes of tumor cells (Fig. 1B). Positive control slide showing strong membranous staining was used as a reference sample. EGFR LI was assessed in ten randomly selected fields (at 400x magnification), and computed as a number of cells with positive membranous staining for EGFR divided by the number of all cells that were counted. EGFR LI was expressed as a percentage.

Evaluation of mitotic index. Hematoxylin-eosin-stained slides were scored for mitosis. Mitosis was quantified by morphology alone using the criteria common for mitosis. Cells were scored at 1000x magnification. Ten randomely selected fields (over 1000 cells) were scored for malignant cells, with intact nuclei, undergoing mitosis. Mitosis was ex-

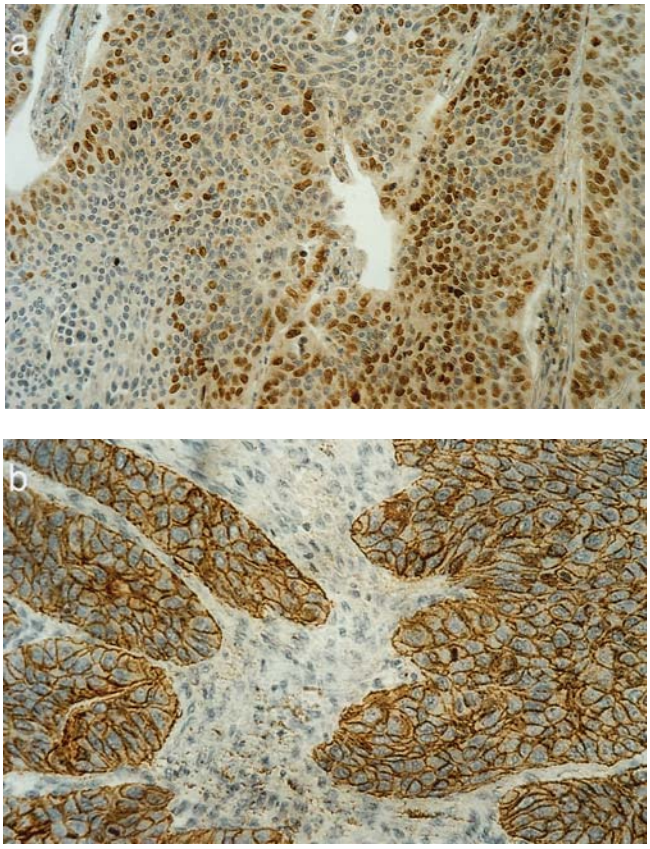

Figure 1. Results of immunocytochemistry: a, nuclear staining in Ki-67-positive tumor cells; b, membranous staining in EGFR-positive tumor cells.

pressed as percentage of mitotic cells. The same person who was unaware of the patient data did all countings.

Statistical analysis. Descriptive statistics were used to determine mean values of Ki-67 LI, EGFR LI and MI and standard errors of means (SE). Mann-Witney U test was used to establish the statistical significance of differences between means. Associations between categorical variables were analyzed using Pearson chi-square test. In all statistical procedures, α=0.05 was considered significant. Disease-specific survival (the patients whose cause of death was not malignant were treated as alive) was analyzed. The probability of survival was calculated using Kaplan-Meyer method [24]. Univariate analysis was done using log-rank test. Because mean or median values for Ki-67 LI and EGFR LI were not important, the optimal cut-off points, ('minimal' p values) were chosen by log-rank test. The joint effects of remaining covariates, were analyzed using Cox proportional hazard model and stepwise regression procedure [5].

Results

Mean values of Ki-67 LI, MI were: 32.4±1.1, 0.31±0.03 respectively, the median values were 31.7 and 0.24% respectively. The EGFR LI ranged from 0 to 92.4% with a mean value of 29.7%±3.4.

Correlation was found between Ki-67 LI and mitotic index (p=0.042). However there was no correlation between EGFR LI and Ki-67LI or MI. There was no association between TNM, pTNM, age, gender, and biological parameters that were measured. Relations between clinicopathological and biological parameters are summarized in Table 1. Significant correlation between age and EGFR LI was found (p=0.008) – older patients had lower EGFR LI.

The Kaplan-Meier estimated 5-year disease specific survival was 55%. In univariate analysis, 5-year survival was significantly longer for patients with tumors with Ki-67 LI >28% than those with Ki-67 LI ≤28% (p=0.0195), (Fig. 2A, Tab. 2).

Thirty-four patients with EGFR LI ≤13% survived shorter than 44 with EGFR LI >13% (0.0102). Moreover, 71 patients with EGFR LI ≤80% survived shorter than 7 with EGFR LI >80% (p=0.0143). So, the shorter survival is observed both, for group with low EGFR LI (≤13%) and for group with high EGFR LI (>80%). This indicates that 13% and 80% might be two cut-off points. On the basis of this observation we created three groups: EGFR LI ≤13% (34 patients); 13% <EGRF LI ≤80% (37 patients) and EGFR LI >80% (7 patients). Patients with EGFR LI ranging between 13% and 80% survived significantly longer than with EGFR LI lower than 13% or higher than 80% (p=0.0003, p=0.0005, respectively). There was no difference in length of survival between patients with EGFR LI ≤13% and those with EGFR LI >80% (p=0.3). This prompted us to join the two groups into one: 13% ≥EGFR LI ∪ EGFR LI >80% (Fig. 2B, Tab. 2). In further analysis we used two groups: 13% ≥EGFR LI ∪ EGFR LI >80% (42 patients) vs. 13 <EGFR LI ≤80% (36 patients), (p=0.0001).

Patients with pN1+pN2 tumors survived significantly shorter than with pN0 tumors (p=0.0126), (Fig. 3A, Tab. 2). Pathological stage was the next factor that significantly influenced patients' survival (p=0.0117), (Fig. 3B, Tab. 2). However TNM stage (Tab. 2), pathological grade, MI had no significant impact on survival.

Table 1. Correlations between clinicopathological parameters and Ki-67 LI, EGFR LI, MI in SqCLC patients

Parameter	n	Ki-67 LI (%) mean ± SE	EGFR LI (%) mean ± SE	MI (%) mean ± SE
T				
T1+T2	2+66	32.2 ± 1.1	30.3 ± 3.6	0.30 ± 0.03
T3	10	34.0 ± 5,1	20.6 ± 7.9	0.45 ± 0.08[a]
pT				
pT1+pT2	1+62	32.0 ± 1.1	27.0 ± 3.7	0.30 ± 0.03
pT3	15	33.9 ± 3.2	41.0 ± 7.5[b]	0.37 ± 0.07
Grade				
G1	14	25.0 ± 2.5	40.6 ± 8.5	0.18 ± 0.05
G2 + G3	25+38	34.0 ± 1.6[c]	27.7 ± 3.6	0.34 ± 0.03[c]
Grade				
G1 + G2	14+25	31.1 ± 1.8	39.7 ± 4.9	0.25 ± 0.03
G3[*]	38	33.6 ± 1.3	20.2 ± 4.2[d]	0.38 ± 0.05[d]

[*]in one case grade was not assessed; [a]T1+T2 vs. T3, p=0.0393 (for MI); [b]pT1+pT2 vs. pT3, p=0.0432 (for EGFRLI); [c]G1 vs. G2+G3, p=0.0012 (for Ki-67); p=0.0223 (for MI); [d]G1+G2 and G3, p=0.0010 (for EGFR LI); p=0.0411 (for MI).

Table 2. Univariate analysis for SqCLC patients treated with surgery. Data for 5-year disease specific survival

Parameter	n	The Kaplan-Meier estimated 5-yr survival (%)	median survival (months)	(log-rank test) p value
Ki–67 LI				
Ki–67>28%	54	64	–	
Ki–67≤28%	24	32	15	0.0195
EGFR LI				
13%<EGFR LI≤80%	36	82	–	
13%≥ LI ∪ LI>80%	42	33	16	0.0001
TNM				
I + II	40+26	54	–	
III	12	55	–	NS
pN				
pN0	38	70	–	
pN1 + pN2	33+7	42	23	0.0126
pTNM stage				
I + II	32+31	62	–	
III	15	29	14	0.0128
G				
1 + 2	39	51	–	
3	38	58	–	NS

NS – not significant

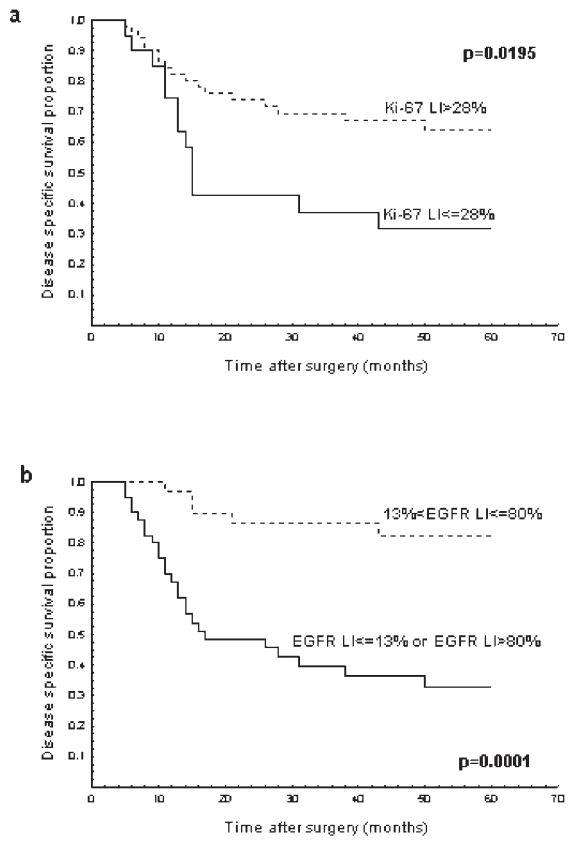

Figure 2. Correlation between biological parameters and patients' survival after surgery: a, correlation between Ki-67 LI and patients' survival; b, correlation between EGFR LI and patients' survival.

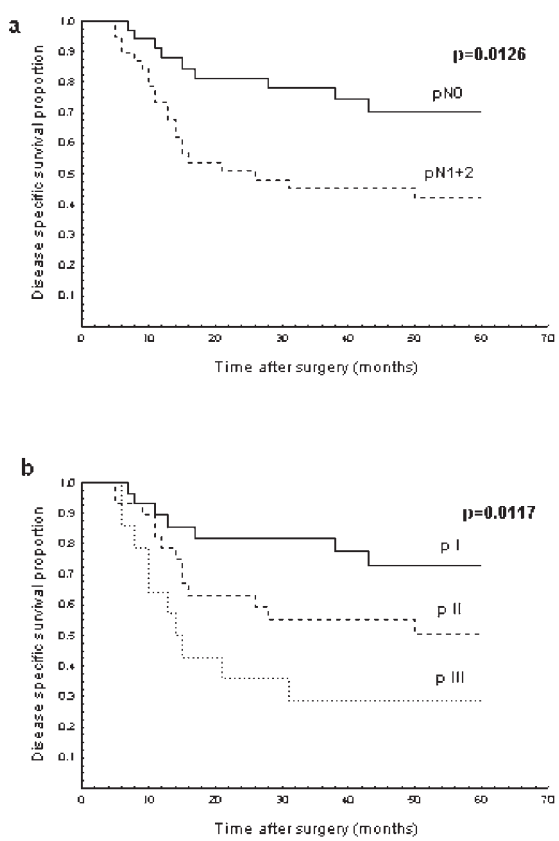

Figure 3. Correlation between clinical parameters and patients' survival after surgery: a, correlation between pN and patients' survival; b, correlation between pTNM and patients' survival.

In Cox multivariate analysis, Ki-67 LI and EGFR LI were significant for disease specific survival (Tab. 3).

Discussion

The main goal of this study was estimation of prognostic significance of Ki-67 LI, MI and EGFR LI in the group of 78 consecutive surgically treated SqCLC.

In present study, mean Ki-67Li was 32.4±1.1% (median was 31.7± 9.8%). Those results are comparable with other authors' findings. In FONTANINI [13] study (in the group of squamous cell lung cancers) mean Ki-67 LI was 38.3±17.3%, while in O'NEILL [34] it was 31.8%. In CAGINI [4] study (in the group of NSCLC) mean Ki-67 LI was 25±19%, while in HOMMURA [22] it was 36.7%. In our study, median MI was 0.24%, while in KOMAKI's at al [26] 0.4%.

The mean value of EGFR LI, found in this study was 29.7% ±3.4 while in FONTANINI [12] study it was 42.1±26.2. Other authors did not show EGFR expression as an absolute percentage. They classified tumors into groups with different EGFR protein expressions [6, 7, 9, 35, 38] or analyzed staining intensity only [18].

Table 3. Final results of Cox multivariate analysis. Disease specific survival for 78 SqCLC treated with radical surgery

	Final results	
Parameter	RR	(Cox proportional hazards) p value
EGFR LI		
13%<EGFR LI≤80%	1	
13%≥LI ∪ LI>80%	6.7	0.0002
Ki-67 LI		
Ki-67 LI>28%	1	
Ki-67 LI≤28%	2.4	0.0414
pTNM		
I + II	1	
III	2.3	0.0630

The significant correlation between Ki-67 LI and MI was found also by other authors [21]. In presented data and other author' studies [12] the correlation between proliferation and EGFR expression was not found.

Presented results confirmed that, moderately and poorly differentiated tumors have higher Ki-67 LI (MI) than well-

differentiated ones [40, 22]. However, in some studies [25, 39] such relation was not found.

In presented study significantly higher MI in T3 tumors than in T1+T2 tumors was found. SHIBA [40] and HOMMURA [22] showed association between T, pT (respectively), and Ki-67 LI, while FONTANINI [13] found no association between T and Ki-67 LI. Association between Ki-67 LI and: gender [40, 22], age [22], T parameter [40, 22], stage [40, 22] observed by other investigators, was not confirmed in presented study.

Significantly higher EGFR LI values were found in well and moderately differentiated than in poorly differentiated tumors. These results are in agreement with DAZZI [9] who observed strong EGFR positivity more often in well differentiated tumors than in less and undifferentiated ones. Other authors found inverted correlation [7] or did not find any correlation between grade and EGFR expression [3, 12, 36, 37, 43].

Significantly higher EGFR expression in pT3 group than in pT1+pT2 tumors that was found in presented study, was not confirmed by other investigators [3, 10, 12, 16]. Lower EGFR expression was noted in the group of older patients, whereas COX et al [7] found inverted relation, and other authors did not find any association between age and EGFR expression [16, 36, 37].

Presented data and other authors' results indicate that there is no significant relationship between EGFR level and gender, TNM, pTNM [3, 7, 10, 36, 37]. However, FUJINO [16] and VEALE [43] found significantly lower EGFR expression in early stage cancers (pI+pII, I+II respectively) than in advanced stage cancers (pIII+pIV, III).

Patients with Ki-67 LI>28% survived significantly longer than those with Ki-67 LI≤28%, that is consistent with results of KOMAKI [26] who, in univariate analysis, in the group of SqCLC found longer survival for patients with high Ki-67 LI. Association, between high proliferation rate, assessed using BrdUrd LI, and longer survival was also found by GASINSKA [17]. Some authors found inverted correlations in the group of NSCLC [22, 40, 28, 21, 20, 39, 41], in adenocarcinomas of the lung [23], or in non small, non squamous cell lung cancer [22]. Only in a few studies, from the cited above, prognostic significance of Ki-67 LI was confirmed by multivariate analysis [22, 40, 28, 21]. No association between survival and Ki-67 LI was found in the group of NSCLC [4, 8] or SqCLC [22]. Differences between these results may be caused by various antibodies that were used, methods of Ki-67 LI assessment, and different histological type, stage and grade of analyzed tumors.

We have found that patients having tumors with EGFR LI between 13% and 80% survived significantly longer than those with EGFR LI less than 13% and more than 80%. In most studies, EGFR expression was not correlated with patients' survival [3, 6, 7, 11, 15, 18, 36, 37]. However, some authors found EGFR overexpression as a positive (in univariate analysis only) [38] and some as a negative [35, 44, 45] factor influencing patients' survival. Findings mentioned above may confirm, to some extent, our results, as we found that both very high (EGFR LI>80%) and low (EGFR LI ≤13%) EGFR expressions had negative impact on patients' survival.

What is interesting when other authors' categorizations was applied for EGFR LI (assessed by us), we were able to confirm that: longer survival was characteristic for patients with intermediate EGFR expression (EGFR LI=20–50% or 50–80), shorter for patients with very low (0–20%) or very high expression (>80%), (data not shown). However, better discrimination between groups (lower p value) was observed when optimal cut-off points were applied for EGFR LI instead of cut-off points proposed by other authors. This suggests that optimal cut-off point method is more sensitive one, and, in some cases, should be used for categorization of continues variables like EGFR LI. In our opinion, assessment of EGFR expression after IHC staining, should be done using very sensitive method (for example number of positively stained cells in 10 fields), and results should be categorized using optimal cut-off point method. Application of less sensitive methods might cause oversight of groups with very low or very high EGFR expression. In results presented in our previous study, oversight of the group of tumors with very high EGFR expression and poor prognosis occurred, when method based on percentage of positively stained field's or staining intensity was applied [33]. Moreover, when we applied method based on staining intensity we were not in a position to find correlation between EGFR expression and patients' prognosis. It should be noted that, more sensitive than IHC methods, like PCR [3] or radioligand binding assay [44] could be used for assessment of EGFR expression however, no prognostic significance was found when PCR was applied [3]. On the other hand IHC, as not expensive and simple method seems to be the most appropriate for clinical use.

In our and other authors results [1], MI was not associated with the prognosis of patients with lung cancers. In other authors' studies, high MI was associated both with longer [25, 26] or with shorter patients' survival [27].

The discrepancies between results of different authors suggest that the problem of prognostic significance of proliferation and EGFR expression is far from being resolved.

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