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p53 and EGFR gene mutations in malignant tumors of the lung

Relevance: Increased incidence of lung cancer globally and in Kazakhstan, lack of screening in hereditary cases, high mortality, and low survival of patients necessitate the study of the molecular genetic causes of the disease. At present, gene mutation studies for lung cancer diagnostics are expanding. However, many gene mutations revealed remain undercovered in the scientific literature, and there is not enough data on their prognostic and diagnostic value.

The purpose of the study was to discover the specifics of the p53 gene mutations and reveal the EGFR exon 19 deletions and exon 21 L858R mutations in malignant tumors of the lung of various histogenesis.

Methods: The mutations were studied in tumors (200 samples) and adjacent tissue (200 samples) of patients with lung cancer (squamous cell carcinoma (SCC) and adenocarcinoma (ADC) of the lung) by polymerase chain reaction (PCR), electrophoresis, and EcoR1- and Pst1-restriction of samples after p53 gene fragments and cDNA amplification and mRNA revertase treatment. Another 263 lung cancer samples were evaluated by real-time PCR for EGFR exon 19 deletions and EGFR exon 21 L858R mutations.

Results: The p53 gene was not expressed in 50% of SCC and adenocarcinoma of the lung samples. Restriction revealed p53 mRNA mutations in 100% of SCC and 75% of ADC samples. p53 exon-intron 5-6 was mutated in 50% of ADC and 70% of SCC samples, exon-intron 7-9 – in 60% of SCC cases. EGFR exons 19 and 21 mutations found in 65 of 263 lung tumor samples were associated with increased sensitivity to EGFR tyrosine kinase inhibitors.

Conclusion: The p53 gene mutations revealed in most samples of SCC and ADC of the lung could be used to diagnose lung cancer and predict its severity. The identified EGFR mutations allow predicting the effectiveness of targeted therapy.

Keywords: mutations, deletions, p53 gene, p53 mRNA, EGFR gene, restriction, squamous cell carcinoma (SCC) of the lung, adenocarcinoma (ADC) of the lung.

Introduction: Despite an active development and introduction of new technologies in diagnostics and treatment of malignant neoplasms (MNs) [1-5], lung cancer remains the most common cancer localization globally and continuously ranks first in cancer mortality causes. In recent decades, the increase in global incidence is about 1.5% annually. Up to 95.6% of lung cancer patients die from this disease. At that, lung cancer mortality is five times higher in men than in women [1]. The development of MN, including lung cancer, is associated with many damages in the genes, chromosomal regions, signaling pathways [4]. Thus, in lung cancer, abnormal expression or dysfunction of tumor suppressor genes were registered in the p53, p16, Rb, FHIT, RASSF1A, SEMA3B, PTEN, hOGG1, and BAP1 genes [5].

In small-cell lung cancer (SCLC), chromosomal deletions, truncations, and missense mutations in genes encoding p53 and the RB gene are most common and observed in 90% of cases [6-9]. The tumor growth suppressor p53 is mutated in more than 90% of SCLC and more than 50% of non-small-cell lung cancer (NSCLC) cases. Some researchers have

found in NSCLC an increased activity of the EGFR receptor and a whole cascade of mutations triggered by the activation of this receptor [10, 11].

Detecting a genetic mutation and finding an agent active against this mutation allows targeting the tumor. The epidermal growth factor receptor (EGFR) is the best example of finding genetic mutations in lung cancer, followed by developing relevant biological agents [10]. Changes in EGFR were found in approximately 10% of NSCLC patients. Treatment of these patients with tyrosine kinase inhibitors (TKI) like gefitinib and erlotinib has a high clinic efficiency.

The purpose of the study was to discover the specifics of the p53 gene mutations and reveal the EGFR exon 19 deletions and exon 21 L858R mutations in malignant tumors of the lung of various histogenesis.

Materials and Methods:

The object of the study was the cancerous and adjacent normal tissues in the postoperative samples of patients. We studied the mutational damages in the p53 gene (TGS) in the tumor and adjacent tissue and the deletions and mutations in the EGFR

gene in malignant tumors of the lung of various histogenesis.

DNA was isolated using the Bioline ISOLATE II Genomic DNA Kit.

The *p53* exon-introns 5-6 and 7-9 were amplified by polymerase chain reaction (PCR) in the presence of primers specific to these sites and selected using the "PRIMER" software. The revertase reaction was carried out according to the instructions for the "REVERTA-L-100" AmpliSens reagent kit. 10 µL (1 mL) of isolated RNA was added to 10 µL of the reaction mixture containing hexanucleotide primers, RNA eluent, deoxynucleotide triphosphates (dNTP), a ribonuclease inhibitor, and 200 units of reverse transcriptase. The resulting reaction mixture was incubated at 37°C for 30 minutes. The first synthesized cDNA chain was then added to the mixture to copy (amplify) the DNA fragments by PCR.

At the second stage, PCR products of DNA and cDNA fragments were subjected to site-specific endonucleolysis using EcoR1- and Pst1-restrictases. The restriction was performed in an incubation medium containing 5 µL of the PCR reaction product, 1.25 µL of buffer "H" from the restriction reaction kit, 1 unit of the restriction enzyme in 1 µL. The total sample volume was brought to 12.5 µL with water, incubated at 37°C for one hour, then cooled in ice and dispersed in 2% agarose gel electrophoresis. Electrophoregrams were analyzed using the "Gel-analysis" program. In total, we examined 200 tumor samples and 200 adjacent tissue samples from postoperative material of patients with squamous cell carcinoma (SCC) of the lung and adenocarcinoma (ADC) of the lung.

Another 263 lung tumor samples underwent real-time PCR to evaluate the L858R mutation in exon 21 and deletions in exon 19 in the *EGFR* gene. Tumor DNA was isolated from freshly frozen tissue or tissue fixed with formalin and encased in paraffin.

PCR followed the instructions for the real-time reagent kit-PCR-EGFR-2M.

We used the Student's *t*-test and calculated the significance level *P* and the standard deviation (SD) between the series. The Pearson correlation coefficient, the reliability of the correlation coefficient r_{xy} , *t*-criterion, and error-free forecast probability (*p*) were calculated according to the published methodology [12, 13].

All procedures followed the ethical standards of the local ethics committee of the Kazakh Institute of Oncology and Radiology and the Declaration of Helsinki. Informed consent to participate in research studies was obtained from all patients. The manuscript contains no identifying patient information.

Results: Studies on mutations in the *p53* and *EGFR* genes, which control one of the main pathways for activating cell growth genes, were aimed at recording the loss of various fragments of the *p53* gene and their effect on the *p53* gene expression in SCC and ADC of the lung, as well as the *EGFR* gene mutations sensitive to TKI in various malignant lung tumors.

The *p53* fragments with exon-intron 5-6 and 7-9 were found in all the studied samples of ADC of the lung (in 5 series of 20 samples each) and adjacent tissue (in 5 series of 20 samples each). *p53* mRNA was expressed in half of the studied ADC samples, and 15 of 20 studied adjacent tissue samples. After treatment with EcoR1- and Pst1-restrictases, the number of samples containing the *p53* exon-intron 5-6 PCR product decreased by 50% among ADC samples and 25% among adjacent tissue samples (Table 1). These structural and functional differences between ADC and adjacent tissue indicated cell polymorphism in both cancerous and adjacent tissues and were most likely associated with the *p53* exon-intron 5-6 mutations and deletions.

Table 1 – Average number and share of mutations in adenocarcinoma of the lung and adjacent tissue samples, before and after restriction

Gene fragment, mRNA	Number and share of samples with a PCR product of a gene fragment or mRNA					
	p53 (exon-intron 5-6)	Restricted ADC ^a	Tissue adjacent to ADC ^b	Restricted tissue adjacent to ADC ^b	The share of ADC mutant samples, %	The share of mutant samples of tissue adjacent to ADC, %
p53 (exon-intron 7-9)	20	10.0±5.0 (50%)	20	15.0±4.32 (75%)	50	25
p53 mRNA	20	20 (100%)	20	20 (100%)	0	0
mRNA p53	10.0±5.0 (50%)	5.0±3.54 (25%)	15.0±4.32 (75%)	10.0±4.08 (50%)	75	50

Notes: ADC – adenocarcinoma of the lung

^a Average number of cancerous samples with PCR product in 5 groups of 20 samples each (n=100)

^b Average number of adjacent tissue samples with PCR product in 5 groups of 20 samples each (n=100)

PCR product of the *p53* gene with exon-intron 5-6 and 7-9 was half less common in SCC samples than in adjacent tissues. However, the *p53* gene was not expressed in all SCC samples and 85.7% of adjacent tissue samples after treatment with EcoR1- and Pst1-restrictases (Ta-

ble 2). The *p53* gene silencing in both cancerous and adjacent normal tissue could be due to the mutations in the *p53* gene exon-intron 5-6 and 7-9 and possibly with changes in regulatory sites that could be methylated or damaged, as evidenced by restriction enzyme treatment.

Table 2 – Average number and share of mutations in squamous cell carcinoma of the lung and adjacent tissue samples, before and after restriction

Gene fragment, mRNA	Number and share of samples with a PCR product of a gene fragment or mRNA					
	p53 (exon-intron 5-6)	Restricted SCC samples ^a	Tissue adjacent to SCC ^b	Restricted tissue adjacent to SCC ^b	The share of SCC mutant samples, %	The share of mutant samples of tissue adjacent SCC, %
p53 (exon-intron 7-9)	11.40±3.71	5.7±2.84 (28.5%)	17.14±2.64	11.44±3.3 (57.2%)	71.5	42.8
p53 mRNA	12.00±4.38	8.0±3.26 (40%)	20	20 (100%)	60	0
mRNA p53	11.40±4.38	0	5.72±3.42	2.86±2.02 (14.3%)	100	85.7

Notes: SCC – squamous cell carcinoma of the lung

^a Average number of cancerous samples with PCR product in 5 groups of 20 samples each (n=100)

^b Average number of adjacent tissue samples with PCR product in 5 groups of 20 samples each (n=100)

Our research revealed mutations preventing the amplification of the *p53* exon-intron 5-6 and the mutations in the *p53* exon-intron 7-9. Such mutations formed the truncated and mutated mRNAs and were

not found in 100% of SCC and 85% of adjacent tissue samples, as well as in 75% of ADC and 50% of adjacent tissue samples after treatment with EcoR1- and Pst1-restrictases (Figure 1).

Notes: LSCC – SCC of the lung, LADC – ADC of the lung

Figure 1 – Frequency of mutations in the *p53* exon-introns 5-6 and 7-9 and *p53* mRNA in SCC and ADC of the lung and adjacent tissue before and after restriction

Inequal mutation spectrum in different studied sites of the *p53* gene indicates the possibility of predicting targeted therapy depending on the areas of damage in the cell signaling system of SCC, ADC, and

adjacent tissue.

The mutations in the *p53* exon-introns 5-6 and 7-9 revealed in 100% of SCC samples suppress the gene function. In adjacent tissue, the mutations

were found in exon-intron 5-6 only in 43% of samples. Probably, those mutations did not significantly change the phenotype of tissue cells. However, they affected the *p53* function in 85.5% of adjacent tissue samples.

The statistical processing (calculating the correlation coefficient and r_{xy} , t -criterion, and the error-free forecast probability (p)) followed the published guidelines [12, 13]. Calculation results are described below and presented in Table 3.

The traits were compared by constructing rows, denoting them through the X for the gene contained in the ADCL sample and Y for the gene contained in the adjacent lung tissue sample.

The correlation coefficient was calculated as follows:

- Determining the M_x average in the "x" variant row and M_y average in the "y" variant row by formulas: $M_x = \sum x/n$ (Column 1, Table 1), $M_y = \sum y/n$ (Column 2, Table 3).

- Finding the deviations d_x and d_y for each variant from the average amount for the "x" and "y" rows by formulas: $d_x = x - M_x$ (Column 3, Table 1), $d_y = y - M_y$ (Column 4, Table 3).

- Multiplying, then summing up the deviations d_x and d_y : $\sum d_x \times d_y$ (Column 5, Table 3).

- Squaring each deviation (d_x and d_y), then summing up the squares by row "x" and row "y" by formulas: $\sum d_x^2$ (Column 6, Table 1), $\sum d_y^2$ (Column 7, Table 3).

- Multiplying the sums of squares ($\sum d_x^2 \times \sum d_y^2$) and taking the square root of the product as follows:

$$\sqrt{(\sum d_x^2 \times \sum d_y^2)}; \quad (1)$$

$$r_{xy} = \frac{\sum (d_x \times d_y)}{\sqrt{(\sum d_x^2 \times \sum d_y^2)}}; \quad (2)$$

Finding the correlation coefficient error (mr_{xy}) and the t -criterion by formulas:

$$mr_{xy} = \pm \sqrt{\frac{1-r_{xy}^2}{n-2}}; \quad (3)$$

$$t = \frac{r_{xy}}{mr_{xy}}; \quad (4)$$

The t -criterion of 2.424871 corresponds to the probability of an error-free forecast of $p > 95.5\%$.

Thus, according to the calculations, the gene was less frequent in ADC samples compared to the adjacent tissues samples with reliability of $r_{xy} = +0.813733$, $p > 95.5\%$.

Table 3 – Example of calculation of the correlation coefficient and r_{xy} , t -criterion and error-free forecast probability (p)

ADC of the lung, gene (x)	Tissue adjacent to ADC of the lung, gene (y)	d_x	d_y	$d_x \times d_y$	d_x^2	d_y^2
1	2	3	4	5	6	7
15	17	5	5	25	25	25
13	13	3	1	3	9	1
10	14	0	2	0	0	4
8	7	-2	-5	10	4	25
4	9	-6	-3	18	36	9
$M_x = \sum x/n$	$M_y = \sum y/n$	$d_x = x - M_x$	$d_y = y - M_y$	$\sum d_x \times d_y$	$\sum d_x^2$	$\sum d_y^2$
10	12			56	74	64

The calculations yielded the following results: the correlation coefficient reliability – 0.813733, the correlation coefficient error – 0.335578, and t -criterion – 2.424871.

The t -criterion of 2.424871 corresponded to the probability of error-free forecast of $p > 95.5\%$. This evidenced a reduced frequency of the gene samples in ADC of the lung compared to the adjacent tissue with the reliability of $r_{xy} = +0.813733$, $p > 95.5\%$.

In total, 65 out of 263 (24.7%) lung tumor samples had deletions in *EGFR* exon 19 and mutations in *EGFR* exon 21. In ADCs of different localization in the lungs with metastases ($n=173$), 32 (18.5%) samples had deletions in exon 19, and 14 (8%) had mis-

sense mutations with a leucine-arginine substitution (L858R) in exon 21 (Figure 2).

In SCC ($n=34$), three samples (8.8%) had deletions in exon 19, while L858R exon 21 substitutions were not detected. In bronchoalveolar cancer ($n=7$), six samples had a deletion in exon 19, and only one had a mutation in exon 21. In mixed cancer ($n=5$), a deletion was detected in exon 19. Exon 19 deletions were found in single samples of ADC metastases, non-small cell carcinoma, and small cell carcinoma; leucine-arginine L858R substitutions in exon 21 were found in single samples of undifferentiated cancer, small cell carcinoma, and bronchioloalveolar cancer.

Figure 2 – Distribution of EGFR del 19 exon and L858R 21 exon mutations in various histological forms of lung cancer: squamous cell cancer, adenocarcinoma, undifferentiated carcinoma, adenocarcinoma metastases (Mts.), non-small-cell carcinoma, bronchioloalveolar cancer, mixed cancer

No mutations of interest were found in 198 samples of lung neoplasms. This does not exclude the possibility of other mutations with deletions, missense mutations, an increase in the number of copies of the *EGFR* gene, and the *p53* mutations that can block the signal transmission pathways and stimulate cell division.

Discussion: The *p53* gene damage found in the SCC of the lung was missing in the ADC of the lung and tissue adjacent to SCC and ADC. However, *p53* was silent in half of SCC and ADC samples and 25-50% of ADC-adjacent and 70-80% of SCC-adjacent tissue, evidencing the dysregulation of cell growth, differentiation, and apoptosis.

Such a reduced content of *p53* mRNA PCR product in adjacent tissue and the absence of samples containing *p53* mRNA PCR product in SCC and ADC samples suggests aggressive and invasive cell growth, although it does not alter the typical morphology of these tissues.

The difference in ADC and SCC mutations could determine their histological and morphological features and serve to develop approaches to SCC and ADC diagnostics and treatment.

EGFR exons 19 and 21 mutations found in 24.7% of lung tumor samples were associated with increased sensitivity to *EGFR* TKIs. Thus, nearly 25% of lung neoplasms are sensitive to *EGFR* TKIs, including gefitinib and erlotinib [14, 15]. At that, lack of positive response to targeted therapy is likely due to mutations in *p53* exon-introns 5-6 or in other signal pathways.

Dahabreh et al. [16] and Hirsch et al. [17] suggested that the increased number of *EGFR* copies could be associated with the tumor progression and predict the response to TKIs in NSCLC. The activating *K-ras* mutation is a strong predictor of resistance to *EGFR* inhibitors. Therefore, TKIs should only be administered in tumors with wild-type *K-ras* [18].

Several studies reported a close correlation between the *EGFR* protein expression, the number of *EGFR* copies, and *EGFR* mutations in NSCLC [19, 20]. Although sensitivity to TKIs was more correlated with the *EGFR* copy number than with *EGFR* mutations, the latter could predict the response to TKIs [20]. The IHC-assessed *EGFR* protein expression strongly correlates with the FISH-assessed *EGFR* copy number [21].

The *p53* mutations revealed in most SCC and ADC samples could be used to diagnose and predict lung cancer severity; *EGFR* mutations can predict the response to targeted therapy. This is especially important for early diagnosis and the beginning of treatment of emergence and development of malignant tumors since the cancer treatment starting time directly impacts the survival rate [22].

Conclusion: Thus, the *p53* gene was silent in 50% of the studied SCC and ADC samples. However, the restriction revealed *p53* mRNA mutations in 100% of the studied SCC samples and 75% of ADC samples. The mutations in exons 19 and 21 of the *EGFR* gene were found in 25% of lung cancer cases.

The mutations we detected in most SCC and ADC samples of the lung allow using similar research of cell growth and division control genes to diagnose and predict the severity of the disease and the effectiveness of targeted therapy.

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ТҰЖЫРЫМ

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Өкпенің қатерлі ісіктеріндегі p53 және EGFR генінің мутациялық зақымдануы

Өзектілігі: әлемде, оның ішінде Қазақстанда өкпе обырымен сырқаттанушылықтың көбеюі, тұқымқуалаушылық формаларында скринингтің болмауы, өлім-жітімнің жоғары көрсеткіштері және науқастардың өмір сүруінің төмендігі аурудың молекулалық-генетикалық негізін зерттеу қажеттілігін негіздейді. Қазіргі уақытта өкпенің қатерлі ісігін диагностикалау үшін гендердің мутациялық зақымдануын зерттеудің кеңейтудің нақты тенденциясы байқалады. Алайда, көптеген генетикалық мутациялар ғылыми әдебиеттерде жеткілікті түрде сипатталмаған, сондықтан олардың көпшілігі үшін олардың болжамды және диагностикалық құндылығы туралы мәліметтер жоқ.

Зерттеудің мақсаты: p53 гендік мутациясының ерекшелігін анықтау және 19 экзондағы жоылууды және әртүрлі гистогенездің қатерлі өкпе ісіктеріндегі 21 EGFR экзондағы L858R мутациясын анықтау.

Зерттеу әдістері: өкпе қатерлі ісігі (өкпенің жалпақ жасушалы қатерлі ісігі (ӨЖЖҚІ) және өкпе аденокарциномасы (ӨАДҚ) бар науқастардың ісіктері (200 үлгі) мен іргелес тіндеріндегі мутацияны диагностикалау p53 генінің фрагменттерін күшейткеннен кейін және p53 cDNA - дан кейін, мРНҚ-ның кері реакциясынан кейін, полимеразды тізбекті реакция (ПТР), электрофорез және есор1-және Pst1-рестриктазды өңдеу арқылы жүргізілді. Сондай-ақ, өкпе қатерлі ісігінің 263 үлгісінде нақты уақыт режимінде ПТР көмегімен 19 экзондағы жою және 21 EGFR экзондағы L858R мутациясы бағаланды.

Зерттеу нәтижелері: ӨЖЖҚІ және ӨАДҚ үлгілерінің жартысында p53 генінің өрнегі анықталған жоқ. Үлгілерді рестриктазалармен өңдеу нәтижесінде зерттелетін ӨЖЖҚІ үлгілерінде 100% және ӨАДҚ үлгілерінде 75% p53 мРНҚ мутацияларының болуы анықталды. ӨЖЖҚІ және ӨАДҚ үлгілерінде p53 генінің 5-6 экзон-интрондық фрагментінде, тиісінше 50% және 70% мутациялар анықталды. ӨЖЖҚІ үлгілерінің 60%-ында p53 генінің 7-9 экзон-интрондық фрагментінде мутациялар анықталды. Өкпе ісіктерінің 263 үлгісінде EGFR тирозин киназының ингибиторларына сезімталдықты арттыратын эпидермальды өсу факторы (EGFR) рецептор генінің 19 және 21 экзондағы мутациялар анықталды.

Қорытынды: ӨЖЖҚІ және ӨАДҚ үлгілерінің көпшілігінде анықталған p53 ген мутациясы аурудың ауырлығын диагностикалау және болжау үшін осындай зерттеуді қолдануға мүмкіндік береді. Анықталған EGFR мутациялары мақсатты терапияның тиімділігін болжауға мүмкіндік береді.

Түйінді сөздер: мутациялар, делециялар, p53 гені, p53 мРНҚ, EGFR гені, шектеу, өкпенің жалпақ жасушалық карциномасы (ӨЖЖҚІ), өкпе аденокарциномасы (ӨАДҚ).

АННОТАЦИЯ

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Мутационные повреждения генов p53 и EGFR в злокачественных опухолях легких

Актуальность: Увеличение заболеваемости раком легкого в мире, в том числе в Казахстане, отсутствие скрининга при наследственных формах, высокие показатели смертности и низкая выживаемость больных обуславливают необходимость изучения молекулярно-генетической основы заболевания. В настоящее время прослеживается четкая тенденция к расширению исследований мутационных повреждений генов для диагностики рака легких. Однако многие встречающиеся генетические мутации в достаточной степени не описаны в научной литературе, к тому же для многих из них отсутствуют данные об их прогностической и диагностической ценности.

Цель исследования: Определить специфику генных мутаций p53 и выявить делеции в 19 экзоне и мутации L858R в 21 экзоне EGFR в злокачественных опухолях легких различного гистогенеза.

Методы: Диагностику мутаций в опухолях (200 образцов) и прилегающих тканях (200 образцов) больных раком легкого (плоскоклеточный рак легкого (ПКРЛ) и аденокарцинома легкого (АДКЛ)) проводили после амплификации фрагментов гена p53 и кДНК p53, после ревертазной реакции мРНҚ, с помощью полимеразной цепной реакции (ПЦР), электрофореза и Ecor1- и Pst1-рестриктазной обработки образцов. Также, в 263 образцах рака легкого оценивали делеции в 19 экзоне и мутации L858R в 21 экзоне EGFR с помощью ПЦР в режиме реального времени.

Результаты исследований: В половине образцов ПКРЛ и АДКЛ не выявлена экспрессия гена p53. В результате обработки образцов рестриктазами выявлено наличие мутаций мРНҚ p53 в 100% исследуемых образцах ПКРЛ и в 75% образцах - АДКЛ. Выявлены мутации в 5-6 экзон-интронном фрагменте гена p53 в образцах АДКЛ и ПКРЛ, в 50% и в 70%, соответственно. В 60% образцов ПКРЛ выявлены мутации в 7-9 экзон-интронном фрагменте гена p53. В 65 из 263 образцов опухолей легких выявлены мутации в 19 и 21 экзонах гена рецептора эпидермального фактора роста (EGFR), которые повышают чувствительность к ингибиторам тирозинкиназы EGFR.

Заключение: Выявленные мутации гена p53 в большинстве образцов ПКРЛ и АДКЛ позволяют использовать подобное исследование для диагностики и прогнозирования тяжести заболевания. Выявленные мутации EGFR позволяют прогнозировать эффективность таргетной терапии.

Ключевые слова: мутации, делеции, ген p53, мРНҚ p53, ген EGFR, рестрикция, плоскоклеточный рак легкого (ПКРЛ), аденокарцинома легкого (АДКЛ).

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