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ORIGINAL ARTICLE

Large-Scale EGFR Mutation Testing in Clinical Practice: Analysis of a Series of 18,920 Non-Small Cell Lung Cancer Cases

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Abstract

We make use of a very large dataset of non-small cell lung cancer specimens to examine the molecular epidemiology of EGFR mutations, particularly with respect to rare and compound mutations, and to non-adenocarcinoma histological subtypes. We also demonstrate the feasibility of large-scale EGFR mutation screening using the full range of specimens encountered in routine practice. We retrospectively reviewed 18,920 unselected EGFR mutation results from our centre between July 2009 and October 2016, using Qiagen's therascreen EGFR RGQ PCR Kit. Mutation rates were correlated with patient demographics and tumour histology. Our testing success rate was 93.9%, with similar success rates using histological and cytological specimens. Rare, potentially-targetable mutations accounted for 9.5% of all mutations detected. We identified a 2.5% mutation rate in tumours diagnosed as squamous cell carcinomas. There was a trend towards increasing EGFR mutation rates with increasing age, and while Del19 was the commonest mutation in the young, L858R predominated in the elderly. We found that EGFR mutation heterogeneity is rare within tumours and between primary and metastatic deposits. Our data demonstrate that large-scale, reflex EGFR mutation testing is feasible and affordable in the context of a publicly-funded health system. Furthermore, we have shown that the use of techniques sensitive only to classical mutations and selection of patients on the grounds of age, sex and histology denies patients access to potentially beneficial TKI therapy.

Keywords EGFR · Lung cancer · Molecular pathology · TKI

Introduction

The goal of molecular pathology services should be to ensure that all patients who stand to benefit from targeted cancer therapies be given the opportunity to receive them. With the number of targetable molecular alterations increasing, especially in non-small cell lung cancer (NSCLC), attainment of this goal is posing considerable challenges to molecular pathology services: large numbers of molecular tests must be performed using small and often poor-quality samples, within a short turnaround time, with high success rates, delivering meaningful and actionable results, all at an affordable price.

EGFR mutation testing is mandatory prior to prescription of tyrosine kinase inhibitors (TKIs) in NSCLC. Four such drugs are currently available and – unlike aggressive chemotherapy regimens – can be prescribed even to performance status 4 patients with often dramatic results. In this way, this treatment modality represents a paradigm shift in the treatment of NSCLC whose prognosis has hitherto been dismal.

Ours is a referral centre which has been performing EGFR mutation testing on NSCLC samples from across the UK and from several sites in Europe since 2009. We test any type of formalin-fixed, paraffin-embedded tissue,

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including cytological specimens; we test all types of NSCLC. In most cases, we also test specimens for ALK translocation, ROS1 translocation and PD-L1 expression. As a result, we have amassed an extremely large EGFR mutation status dataset of 18,966 unselected NSCLC specimens. Here we present these data in terms of the epidemiology of EGFR mutations and of the feasibility of such testing.

Material and Methods

EGFR Mutation Testing

In all cases, DNA was first extracted from sections of formalin-fixed tissue embedded in paraffin blocks. This included both histological specimens and clots formed from cytological specimens. In a few instances where no tissue remained after sections were cut for morphological and immunohistochemical examination, DNA was extracted from stained sections. Testing was performed with real-time PCR, using Qiagen's therascreen EGFR RGQ PCR Kit, which detects 19 different exon 19 deletions (Del19), T790 M, L858R, L861Q, G719X, S768I and 3 different exon 20 insertions (Ins20).

Demographic and Histological Data

Patient age and sex, and tumour histology, were provided by the referring centre, and were available in the vast majority of cases.

Inclusion Criteria

Eighteen thousand nine hundred sixty-six NSCLC specimens underwent testing between July 2009 and October 2016. Data were prospectively collected for all cases. All cases were retrospectively reviewed. Forty-six cases were excluded from the analysis (see Table, Supplemental Data 1, which details the reasons for exclusion of these cases).

Eighteen thousand nine hundred twenty cases were included in the analysis. One thousand forteen cases represented duplicate tests on the same tumours. In these cases, only the result of the first successful test was included in the main analysis; subsequent results were analysed separately. Twenty-one tumours (from 10 patients) included in the analysis were believed on clinical grounds to be metachronous: nine patients had two tumours, and one had three tumours. Seventy-six tumours (from 36 patients) were believed to be synchronous; 33 patients had two tumours, two had three tumours and one had four tumours.

The baseline characteristics of successfully-tested samples are presented in Table 1.

Statistical Analysis

IBM SPSS Statistics Version 24 was used for all statistical analyses. We used chi-squared tests (and Fisher's exact tests, where needed, indicated with an asterisk) to examine the relationship between mutation status, patient age and sex, and histological tumour type. Two-tailed t-tests were used to compare mean ages between patient groups with different mutation statuses. In all cases, *P*-values less than 0.05 were considered statistically significant.

Results

Overall, 17,782 (93.8%) tests were successfully completed. The full range of specimen types tested, with their individual success rates, is presented in Table 2.

EGFR Mutation Rate

Of the 17,046 distinct tumours successfully tested for EGFR mutation status, 1737 (10.2%) bore a mutation and 15,307 (89.8%) were wild-type. The EGFR mutation rates by demographic and histological characteristics are summarised in Table, Supplemental Data 2. Female patients were more likely to bear a mutation (13.7 vs 6.6%, respectively; $p < 0.001$). There was a significant increase in the EGFR mutation rate with increasing age ($p < 0.001$). Adenocarcinomas were more frequently mutated than adenosquamous and squamous cell carcinomas (11.8 vs 6.2 vs 2.5%, respectively). Among adenocarcinomas, mucinous tumours were significantly more likely to bear mutations (31.3%; $p < 0.001$), followed by lepidic growth pattern tumours (18.5%; $p = 0.020$). There was no difference in mutation rate between primary and metastatic tumour samples (10.2 vs 10.4%).

Rare EGFR Mutations

A total of 1657 tumours bore a singlet EGFR mutation; of these, 1417 (85.5%) harboured a classical mutation (Del19 or L858R), and 240 (14.5%) harboured a rare mutation. There was no significant relationship between patient age or sex, and rare mutation rate (Table 3). A trend towards higher rates of rare mutations was observed with increasing age, but owing to small numbers of patients older than 90 years, this did not achieve statistical significance. A higher incidence of rare mutations was observed in squamous cell carcinomas than in adenocarcinomas, but this difference also fell short of statistical significance (16.7 vs 25.0%; $p = 0.447$).

Table 1 Baseline characteristics of the cases included for analysis

Sex, No. (%)	Male	8462 (49.6)
	Female	8510 (49.9)
	Unknown	74 (0.4)
Mean age (SD)		68.2 (10.5)
Age group, No, (%)	≤ 50 years	939 (5.5)
	51–60 years	2666 (15.6)
	61–70 years	6013 (35.3)
	71–80 years	5489 (32.2)
	81–90 years	1856 (10.9)
	> 90 years	72 (0.4)
	Unknown	11 (0.1)
Histological tumour type	Adenocarcinoma	11,720 (68.8)
	Adenosquamous carcinoma	226 (1.3)
	Squamous cell carcinoma	485 (2.8)
	Large cell carcinoma	70 (0.4)
	Large cell neuroendocrine carcinoma	33 (0.2)
	Sarcomatoid carcinoma	27 (0.2)
	Non-small cell lung cancer, NOS	4485 (26.3)
Adenocarcinoma histological subtypes	Lepidic	131 (1.1)
	Papillary	48 (0.4)
	Acinar	24 (0.2)
	Mucinous	16 (0.1)
	Adenocarcinoma, NOS	11,501 (98.1)

Table 2 Success rates of EGFR testing by specimen type

		Number of specimens (%)	Successful tests (%)
Histological specimens	All	11,047 (69.7)	10,377 (93.9)
	Lung biopsies	6362 (68.2)	5989 (94.1)
	Lung resections	800 (8.6)	791 (98.9)
	Lymph node biopsies	561 (6.0)	521 (92.9)
	Pleural biopsies	410 (4.4)	403 (98.3)
	Bone biopsies	372 (4.0)	304 (81.7)
	Chest wall biopsies	80 (0.9)	73 (91.3)
	Brain biopsies	56 (0.6)	55 (98.2)
	Mediastinal biopsies	45 (0.5)	44 (97.8)
	Skin biopsies	41 (0.4)	38 (92.7)
	Breast biopsies	25 (0.3)	23 (92.0)
	Bone resections	21 (0.2)	10 (47.6)
	Others	187 (1.7)	84 (44.9)
Cytological specimens	All	4807 (30.3)	4439 (92.3)
	Lymph node aspirate	2223 (48.0)	2058 (92.6)
	Pleural fluid	1524 (32.9)	1441 (94.6)
	Bronchial brushing	269 (5.8)	236 (87.7)
	Lung aspirate	232 (5.0)	213 (91.8)
	Bronchial washing	195 (4.2)	168 (86.2)
	Pericardial fluid	111 (2.4)	105 (94.6)
	Sputum	15 (0.3)	9 (60.0)
	Bone aspirate	8 (0.2)	8 (100)
	Adrenal aspirate	7 (0.2)	6 (85.7)
	Others	46 (1.0)	32 (70.0)

Table 3 Patient and tumour characteristics of classical EGFR mutation and rare EGFR mutation cases

		Number of patients ($N = 1708$)	Classical EGFR mutation ($n = 1417$)	Rare EGFR mutation ($n = 291$)	*P*-value
Sex, No. (%)	Male	547	461 (84.3)	86 (15.7)	0.295
	Female	1148	944 (82.2)	204 (17.8)	
Age (years)	Mean (SD)		68.4 (11.6)	70.1 (11.2)	
Age group, No. (%)	≤ 50 years	119	102 (85.7)	17 (14.3)	0.137*
	51–60 years	266	228 (85.7)	38 (14.3)	
	61–70 years	527	426 (80.8)	101 (19.2)	
	71–80 years	532	451 (84.8)	81 (15.2)	
	81–90 years	249	199 (79.9)	50 (20.1)	
	> 90 years	13	9 (69.2)	4 (30.8)	
Histological tumour type	Adenocarcinoma	1352	1126 (83.3)	226 (16.7)	0.057*
	Adenosquamous carcinoma	14	14 (100)	0	
	Squamous cell carcinoma	12	9 (75.0)	3 (25.0)	
	Large cell carcinoma	2	1 (50.0)	1 (50)	
	Sarcomatoid carcinoma	1	0	1 (100)	

Compound EGFR Mutations

Seventy-nine compound mutations were detected in the tumours analysed (4.6% of all mutated tumours, 0.5% of all tumours tested). The 11 distinct combinations of mutation identified are listed in Table, Supplemental Data 3; there were no significant differences in patient age or sex between the different combinations.

Figure 1 shows the relative incidence of each mutation occurring as a singlet versus a compound mutation. Del19 and L858R were significantly more likely to occur as singlets, while T790M, S768I and G719X were more likely to occur as parts of compound mutations.

No significant relationship was identified between patient or tumour characteristics and the rate of compound mutations (see Table, Supplemental Data 4, which shows the demographic and histological features associated with compound and singlet mutations).

The two mutations which were most frequently found as parts of compound mutations were G719X and S768I. For each of these mutations, patient and tumour characteristics were compared between cases of the singlet mutation and cases of the compound mutation and no significant differences were found (see Table, Supplemental Data 5, which compares the features of the singlet and compound mutations).

Individual EGFR Mutations

The full spectrum of singlet mutations is presented in Table 4. All mutations were commoner in females. Del19 and L858R showed a distinct relationship with patient age; in younger patients, Del19 was the commonest mutation, whereas L858R was the predominant mutation in older patients (Fig. 2). L861Q also showed a significant increase in incidence with age, becoming the joint-second commonest mutation in patients older than 90 years. G719X was overrepresented in squamous cell carcinomas, in which it represented 16.7% of all mutations detected; owing to small numbers of mutated squamous cell carcinomas, however, this difference did not achieve statistical significance.

Mutational Heterogeneity

In all tumours thought to be metachronous on clinical grounds, initial and subsequent tumours shared the same mutation status: nine pairs were wild-type and one pair bore

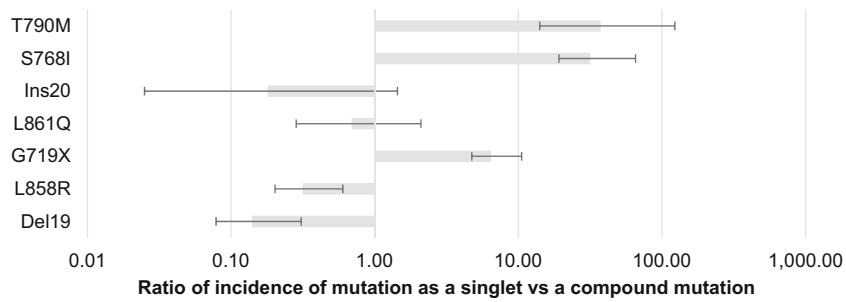

Fig. 1 The ratio of the incidence of each mutation as a singlet compared to its incidence as part of a compound mutation

Table 4 The spectrum of EGFR mutations by patient and tumour characteristics

		Del19, No. (%)	L858R, No. (%)	G719X, No. (%)	L861Q, No. (%)	Ins20, No. (%)	S768I, No. (%)	T790M, No. (%)
Number of cases		831	615	135	81	59	73	23
Sex, No. (%)	Male	276 (33.5)	194 (31.9)	41 (30.4)	25 (30.9)	20 (34.5)	16 (21.9)	7 (30.4)
	Female	549 (66.5)	415 (68.1)	94 (69.6)	56 (69.1)	38 (65.5)	57 (78.1)	16 (69.6)
Age (years)	Mean (SD)	67.9 (11.8)	70.3 (11.2)	69.2 (10.7)	73.2 (9.6)	68.7 (12.8)	68.4 (11.5)	61.8 (9.9)
Age group, No. (%)	≤ 50 years	77 (61.1)	27 (21.4)	8 (6.3)	2 (1.6)	5 (4.0)	5 (4.0)	2 (1.6)
	51–60 years	152 (54.1)	80 (28.5)	18 (6.4)	5 (1.8)	10 (3.6)	13 (4.6)	3 (1.1)
	61–70 years	253 (44.2)	184 (32.2)	58 (10.1)	24 (4.2)	14 (2.4)	28 (4.9)	11 (1.9)
	71–80 years	251 (44.6)	209 (37.1)	33 (5.9)	28 (5.0)	19 (3.4)	17 (3.0)	6 (1.1)
	81–90 years	94 (36.2)	108 (41.5)	17 (6.5)	19 (7.3)	11 (4.2)	10 (3.8)	1 (0.4)
	> 90 years	3 (23.1)	6 (46.2)	1 (7.7)	3 (23.1)	0	0	0
Histological tumour type	Adenocarcinoma	661 (45.9)	489 (34.0)	102 (7.1)	63 (4.4)	47 (3.3)	58 (4.0)	20 (1.4)
	Adenosquamous carcinoma	7 (50.0)	7 (50.0)	0	0	0	0	0
	Squamous cell carcinoma	7 (58.3)	2 (16.7)	2 (16.7)	0	1 (8.3)	0	0
	Large cell carcinoma	1 (50.0)	0	0	0	1 (50.0)	0	0
	Sarcomatoid carcinoma	0	0	0	1 (100)	0	0	0

Del19. In ten of the 36 patients with synchronous tumours, there was a disparity in mutation status between the tumours; nine patients harboured wild-type/Del19 combinations and one patient a wild-type/L858R combination; in the remaining patients, all tumours were wild-type (25 patients) or harboured L858R (1 patient).

In total, 536 patients underwent multiple rounds of testing of the same tumour: in 154 cases (28.7%), testing was performed on both a primary tumour and metastatic deposit; in the remaining 382 cases (71.3%), multiple tests were performed on the primary tumour. In 5 cases (0.9%), there was a disparity in the results of the different rounds of testing, as detailed in Table 5. In all cases, the discrepancy arose as a result of failure to detect a mutation either at the first or second test, rather than detection of an alternative mutation in either test.

Discussion

Our dataset is important because it gives insights into the molecular epidemiology of EGFR mutations, and because it demonstrates that large-scale EGFR mutation screening is feasible.

We identified a very low rate of EGFR mutation in squamous cell carcinomas. Squamous cell carcinoma of the lung is known to bear a heavy mutational load, in which EGFR mutations feature infrequently [1–3]. On this basis, the utility of testing squamous cell carcinomas for EGFR mutation status has been questioned. However, two lines of reasoning militate against this view. Firstly, it has been shown that EGFR mutations in squamous cell carcinomas predict response to TKI therapy, albeit far less reliably than in adenocarcinomas [4–6]. Secondly, with the majority of lung cancer diagnoses being made on the basis of small biopsy or cytological specimens, it is entirely possible that a tumour labelled squamous cell carcinoma may actually be an adenosquamous carcinoma. It has been shown that the EGFR mutation rate in adenosquamous carcinomas is similar to that in adenocarcinomas [7, 8], that the same mutations are found in both components of the tumour [9, 10], and that EGFR mutations predict TKI response in these tumours [6]. Thus, even if it were the case that bona fide squamous cell carcinomas never bear EGFR mutations (or that they are not targetable in squamous cell carcinomas), mutation testing of biopsy and cytological specimens would nonetheless be justified.

Although data were limited, we identified a significant difference in EGFR mutation rate between subtypes of adenocarcinoma. We found that EGFR mutations were most frequent in mucinous adenocarcinomas, followed by lepidic, acinar and papillary growth patterns. This is very surprising, given that virtually all published studies have identified extremely low mutation rates in mucinous adenocarcinomas [11–13]. It is

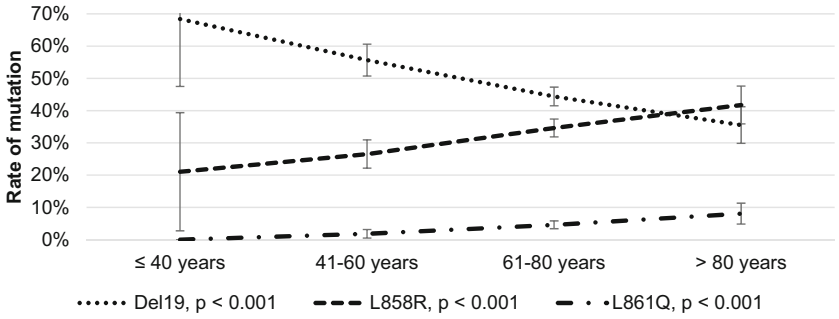

Fig. 2 The rates of Del19, L858R and L861Q by patient age group

probable that this discrepancy arises partly from the fact that histological subtype was known only for a small number of cases, and partly from the fact that most samples were received from multiple external centres with the possibility that histological subtypes were incorrectly transcribed onto the request form.

We found a complex relationship between EGFR mutation prevalence and age. Overall, we found that older patients were more likely to harbour mutations. Evidence on this matter is conflicting, with some studies reporting a decreasing mutation rate with age [14], some reporting no difference [15], and others agreeing with our findings [16, 17]. Furthermore, we found that, while Del19 is the predominant mutation in patients aged younger than 80 years, L858R is the commonest mutation in older patients. This finding has been reported by several other studies [16, 18, 19], and has significance, because it is known that L858R is generally associated with poorer TKI response than Del19 [20–22]. Nonetheless, there is evidence that older patients bearing EGFR mutations derive benefit from TKI therapy [23–25]. Taken together, this reinforces the importance of extending EGFR mutation testing to include elderly patients.

We identified a very low rate of discordant results both within single tumours and between different tumour deposits. The issue of heterogeneity of EGFR mutations is controversial. In general, studies examining mutation status of different areas of a primary tumour have found very little heterogeneity with respect to EGFR mutation status [26–30], supporting the hypothesis that EGFR mutations are initiating events in oncogenesis. Higher rates of discordance have been identified between primary and metastatic tumour deposits [31], although rates are still generally low. The significance of this is difficult to assess, given that discrepant results may result from use of low-sensitivity assays, small samples, and low-quality tumour tissue; indeed, this is likely to have been the cause of the discrepancies identified in our series. In any case, our data suggest that EGFR mutation heterogeneity is an uncommon phenomenon.

Besides all this, our data demonstrate that the goal of giving every NSCLC patient the opportunity of receiving targeted molecular therapy is – far from being a vain hope – an eminently attainable target. In pursuit of this, three issues must be considered.

Firstly, it is an unhappy irony that frail patients who potentially stand to gain the most from TKI therapy are those from whom often the poorest specimens (often cytological) are acquired; it is therefore incumbent upon molecular pathology services to ensure that all measures are taken to allow these tissue samples to be tested. EGFR mutation testing is validated on any formalin-fixed tissue. There is no intrinsic property of cytological specimens which precludes molecular testing; indeed, we have demonstrated that if cytological samples are used to produce formalin-fixed clots, mutation testing can be performed with success rates approaching those of biopsies. Analysis is even possible in cases where no tissue remains in blocks after cutting sections for morphological and immunohistochemical examination; testing can successfully be performed using stained sections, as was the case in a number of specimens in our dataset. We therefore

Table 5 Details of cases in which the first and subsequent rounds of testing of the same tumour yielded differing results

Patient	Interval between tests (months)	First result	First tissue tested	Second result	Second tissue tested
1	0	G719X	Bronchial washing	Wild-type	Bronchial biopsy
2	0	Wild-type	Brain resection	L858R	Lung biopsy
3	1	Wild-type	Pleural fluid	L858R	Pleural fluid
4	3	L858R	Bronchial biopsy	Wild-type	Bronchial biopsy
5	2	L858R	Lung biopsy	Wild-type	Lung biopsy

advocate the maxim that if there is sufficient material for a diagnosis of NSCLC to be made, there is sufficient tissue for EGFR mutation testing to be attempted.

Secondly, selection of an appropriate testing technique is of the upmost importance. With tumour specimens requiring testing for an ever-growing array of molecular targets, there has been a vogue in the recent past to favour multiplex testing using next-generation sequencing (NGS). There is no doubt that NGS has an extremely important role to play in research and in the examination of genes where mutations are not limited to particular hotspots. However, NGS's requirement for large amounts of high-quality DNA, its slower turnaround, and its requirement for considerable expertise and effort in data analysis rather limit its use in EGFR mutation testing, where speed and parsimony are paramount and where exhaustive examination for any and all mutations is not clinically required. Our approach, then, is to use multiple platforms to test for each actionable molecular alteration simultaneously, minimising tissue and time requirements to a level which is realistic in clinical practice. In a sense, then, technologies ought to be selected in such a way as to meet clinical demands, rather than clinical demands being met insofar as is possible using the desired technology.

Finally, in routine clinical practice, a balance is to be struck between testing for too many and too few molecular alterations. At one extreme, an exhaustive analysis of every mutation in the EGFR gene is of little use to patients; little or no data exist relating to the efficacy of TKI therapy in such circumstances, and so provision of such information places clinicians and patients alike in the invidious position of basing therapeutic decisions on no evidence. On the other hand we found that, rather than being rare, non-classical EGFR mutations accounted for 14.5% of all mutations which we detected; of these, there is some evidence of TKI efficacy in all but Ins20 and T790M, meaning that 9.5% of all mutated tumours bore a non-classical, potentially TKI-sensitive mutation. To neglect to test for such mutations on the grounds that they are unjustifiably common would have denied almost three hundred of our patients access to potentially transformative therapy. Continuous reappraisal of the evidence relating to specific EGFR mutations and of the mutation sensitivity of technologies in use is essential to delivering an effective service.

Conclusion

We present data from a very large series of NSCLC specimens tested for EGFR mutation status. Two important conclusions can be drawn from our data. Firstly, EGFR mutations are found in all groups of patients and in almost all histological tumour types; this strongly argues against exclusion criteria for EGFR mutation testing among NSCLC specimens. Secondly and perhaps more importantly, with the appropriate logistics and judicious selection of analytical techniques, large-scale reflex testing of small and low-quality NSCLC samples for EGFR mutation status (alongside ALK, ROS1 and PD-L1) is feasible and affordable within the setting of a publicly-funded health system.

The goal of molecular pathology should be to ensure that any patient who stands to benefit from targeted therapy should be given the opportunity to receive such treatment. Our experience shows that this goal is well within reach.

Compliance with Ethical Standards

Conflict of Interest Matthew Evans: no conflict of interest.
Brendan O'Sullivan: no conflict of interest.
Matthew Smith: no conflict of interest.
Frances Hughes: no conflict of interest.
Tina Mullis: no conflict of interest.
Nicola Trim: no conflict of interest.
Philippe Taniere: no conflict of interest

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