EGFR mutant-specific immunohistochemistry has high specificity and sensitivity for detecting targeted activating *EGFR* mutations in lung adenocarcinoma

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ABSTRACT

Aim We assessed the diagnostic accuracy of epidermal growth factor receptor (*EGFR*) mutant-specific antibodies for detecting two common activating *EGFR* mutations.

Methods Immunohistochemical expression of mutation-specific antibodies against *EGFR* exon 19 deletion E746-A750 ((c.2235_2249del15 or c.2236_2250del15, p. Glu746_Ala750del) and exon 21 L858R point mutation (c.2573T>G, p.Leu858Arg) were assessed in a cohort of 204 resected early stage node negative lung adenocarcinomas, and protein expression was compared with DNA analysis results from mass spectrometry analysis.

Results Of seven cases with L858R point mutation, six were positive by immunohistochemistry (IHC). There were three false positive cases using L858R IHC (sensitivity 85.7%, specificity 98.5%, positive predictive value 66.7%, negative predictive value 99.5%). All seven E746-A750 exon 19 deletions identified by mutation analysis were positive by IHC. Four additional cases were positive for exon 19 IHC but negative by mutation analysis. The sensitivity of exon 19 IHC for E746-A750 was 100%, specificity 98.0%, positive predictive value 63.6% and negative predictive value 100%.

Conclusions Mutant-specific EGFR IHC has good specificity and sensitivity for identifying targeted activating *EGFR* mutations. Although inferior to molecular genetic analysis of the *EGFR* gene, IHC is highly specific and sensitive for the targeted *EGFR* mutations. The antibodies are likely to be of clinical value in cases where limited tumour material is available, or in situations where molecular genetic analysis is not readily available.

INTRODUCTION

Identification of tumours harbouring sensitising epidermal growth factor receptor (*EGFR*) mutations is important in selecting patients likely to respond to EGFR-tyrosine kinase inhibitor (TKI) therapy. Activating mutations in *EGFR* occur in exons 18–21 in lung adenocarcinomas with in-frame deletions in exon 19 (most frequently E746-A750, c.2235_2249del15, p. Glu746_Ala750del, COSM6223 or c.2236_2250del15, COSM6625) and the L858R missense mutation in exon 21 (c.2573T>G, p.Leu858Arg, COSM6224) being the commonest, accounting for approximately 80–90% of cases in most published studies.[1 2] Many molecular tests are available for *EGFR* mutation detection, but they are less widely available and generally have longer turnaround times than immunohistochemistry (IHC). Many molecular tests are also relatively expensive and require larger amounts of tumour tissue than IHC. We assessed the accuracy of IHC for detecting *EGFR* mutations in a cohort of 204 resected early stage node negative lung adenocarcinomas using mutant-specific antibodies against two of the commonest EGFR mutations: the L858R point mutation (p.Leu858Arg) in exon 21 and the 15 base pair deletion E746-A750 (p.Glu746_Ala750del) in exon 19.

MATERIALS AND METHODS

Patient population

A retrospective cohort of 204 consecutive patients who underwent surgical resection for early stage node negative lung adenocarcinoma (pathological stage IB AJCC 6th edition TNM staging) between January 1990 and May 2008 were identified from the files of Royal Prince Alfred Hospital and Concord Repatriation General Hospital. No patients received adjuvant or neoadjuvant treatment. Male patients made up 59% (120) of the patients, and females 41% (84). The median age at diagnosis was 69 years (range 40–87 years). The tumour pathology was reviewed by a pathologist (WC) who marked representative areas and confirmed tumours as adenocarcinomas according to the World Health Organisation 2004 classification.[3] Ethical approval for the study was obtained from the Human Research Ethics Review Committees of Royal Prince Alfred Hospital (X10-0278; HREC/10/RPAH/491) and Concord Repatriation General Hospital (CH62/6/2004-116).

Tissue microarray construction

Two representative formalin fixed paraffin embedded (FFPE) tumour blocks from each case were retrieved from pathology archives. Tissue microarrays (TMAs) were constructed using two 1 mm cores of tumour from representative areas of each block marked by a pulmonary pathologist (WC) as previously described.[4 5] Cores of normal bronchial/bronchiolar epithelium and peripheral lung parenchyma from each patient were also included.

Molecular-based mutation analysis

Two 1 mm cores of FFPE tissue were obtained for DNA extraction from the same tissue blocks used for TMA construction. Cores were taken from areas selected by a pathologist to ensure sufficient

tumour DNA was obtained for analysis. NucleoSpin FFPE DNA Kit (Machery Negel) was used for DNA extraction according to the manufacturer's instruction, with overnight proteinase digestion. NanoDrop ND-1000 Spectrophotometer was used to assess the quality and quantity of the DNA. *EGFR* mutations were analysed using the OncoCarta Panel v1.0 Kit and analysed based on the matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF) technology on the Sequenom MassArray platform (Sequenom, San Diego, California, USA). Detailed results of mutation analysis of this cohort has been previously reported.[2] In the mass spectrometry analysis, both boundaries of E746-A750 (c.2235_2249del15 and c.22362250del15) were covered with two probes. However, the DNA testing does not necessarily cover all exon 19 deletions, particularly less common ones, and the available probes in OncoCarta Panel V.1.0 may not cover both boundaries of the other identified deletions. Fragment analysis was used to confirm the deletion sizes detected in the mass spectrometry analysis, and to exclude deletions in exon 19 IHC positive cases with no mutations identified by mass spectrometry. In brief, *EGFR* exon 19 was amplified with the forward (5′-CCAGAAGGTGAGAAAGTTAAAAT-3′) and reverse (5′-FAM-ACCCCCACACAGCAAAGCAG-3′) primers.[6] The amplicon sizes (119 bp with or without the deletion) were confirmed by 3130xl capillary electrophoresis.[7] Mutation analysis and IHC was performed at an Australian National Association of Testing Authorities (NATA) accredited laboratory to the ISO15189 standard, which is comparable with the Clinical Laboratory Improvement Amendments (CLIA) in the USA.

Immunohistochemistry for mutation analysis

EGFR mutation status was assessed using commercial monoclonal mutant-specific antibodies to the *EGFR* exon 21 L858R point mutation (1 : 50 dilution, clone 43B2, Cell Signalling Technology, Danvers, Massachusetts, USA) and exon 19 deletion E746-A750 (1 : 100 dilution, clone 6B6, Cell Signalling Technology). IHC was undertaken using a Leica BOND-MAX automated immunostainer. Antigen retrieval by heat-induced epitope retrieval at 97°C for 30 min using an alkaline retrieval solution. A polymer-based detection system was used. Cores of lung adenocarcinoma previously confirmed to harbour *EGFR* exon 19 deletion and L858R were used as positive controls.

IHC staining was independently assessed by two pathologists (WC/AG) who were blinded to the molecular results, and any differences were resolved by consensus. Percent of positively staining cells and intensity of staining was assessed. Intensity was scored as 0, 1+, 2+, or 3+ for nil, mild, moderate and marked positive staining. Cases with 1+ intensity staining or higher in at least 10% of cells were considered positive as this was the scoring method used in most previous studies.[8–10] Results were obtained in all cases. The concordance of immunohistochemical scoring between the two pathologists was high for both L858R ($r=0.74$, $p<0.001$) and exon 19 deletion IHC ($r=0.74$, $p<0.001$).

RESULTS

EGFR mutations were identified in 30 (14.7%) patients including 25 mutations known to be sensitive to EGFR TKIs (table 1). Fragment analysis identified one additional E746-A750 exon 19 deletion than identified by mass spectrometry alone. Mutations in exons 19 and 21 accounted for 84.0% of cases with sensitising *EGFR* mutations. There were 7 L858R (p.Leu858Arg) mutations and 13 exon 19 deletions, 7 (53.8%) of which were the E746-A750 (p.Glu746_Ala750del) exon 19 deletion (table 1). The *EGFR* mutations targeted by the two mutant-specific antibodies accounted for 56.0% of the 25 cases with *EGFR* mutations sensitive to TKIs.

Table 1 Clinical characteristics and *EGFR* status by mutation analysis

	n Patients (%)
Total	204
Age	
Median	69 years
Range	40–87 years
Gender	
Male	120 (58.8)
Female	84 (41.2)
EGFR mutation analysis*	
E746-A750 exon 19 del	7 (3.4)
Other exon 19 del†	6 (2.9)
L858R	7 (3.4)
Other EGFR mutations‡	10 (4.9)
Wild type EGFR	174 (85.3)

*34 EGFR mutations were identified in 30 patients.
†p.Leu747_Ser752>S, (c.2240_2257del18, COSM12370 (both directions)), p.Glu746_Thr751>V (c.2237_2252>T, COSM12386), p.Glu746_Thr751>VA, (c.2237_2251>TGG, COSM53205), p.Leu747_Thr751del (c.2239_2253del15, COSM6254 (forward direction only)), p.Leu747_Ser752del (c.2239_2256del18, COSM6255 (both directions)), p.Leu747_Ser752>Q, (c.2239_2258>CA, COSM12387.
‡exon 18 point mutations, exon 20 point mutations and insertions, and one exon 21 L861Q (2).

The mutant-specific antibodies showed cytoplasmic staining in positive cases. There was no positive staining in normal tissues. Six of seven tumours with L858R point mutation by mutation analysis were positive by IHC (figure 1, table 2). Interestingly, the case that was falsely negative by IHC harboured a double *EGFR* mutation (combined L858R and p.H773_V774insNPH in exon 20). There were no other L858R or E746-A750del cases that harboured multiple *EGFR* mutations. There were three false positive cases using L858R IHC. The sensitivity of mutation-specific *EGFR* L858R antibody was 85.7% (95% CI 48.7 to 97.4%), specificity 98.5% (95% CI 95.6 to 99.5%), positive predictive value 66.7% (95% CI 35.4

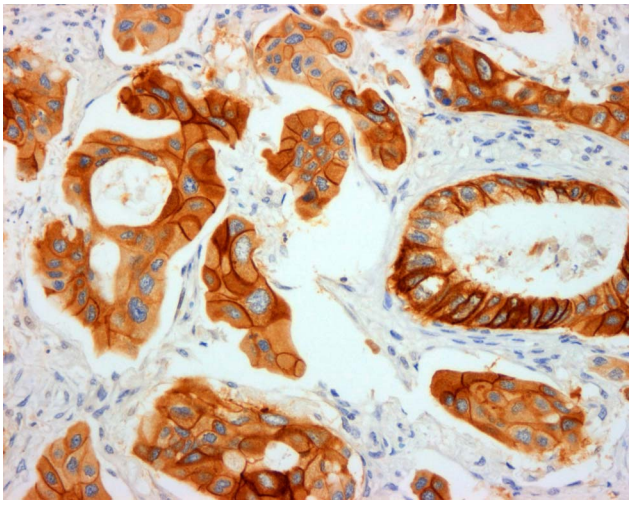

Figure 1 Adenocarcinoma with positive staining for EGFR exon 21 L858R mutation-specific antibody (×200). Mass spectrometry demonstrated L858R mutation in this case.

Table 2 *EGFR* exon 21 L858R detected by IHC versus mutation analysis

	Mass spectrometry mutation status		
Exon 21 L858R (p.Leu858Arg) mutation	**Positive**	**Negative**	**Total**
IHC+	6	3	*9*
IHC−	1	194	*195*
Total	*7*	*197*	*204*

IHC, immunohistochemistry.

to 87.9%) and negative predictive value 99.5% (95% CI 97.2 to 99.9%).

All seven E746-A750 exon 19 deletions identified by mutation analysis were positive by IHC (figures 2 and 3) (table 3). Four additional cases were positive for exon 19 IHC and, interestingly, one of these harboured p.Leu747_Ser752>Q exon 19 18 base pair deletion. The other three cases lacked any exon 19 deletion by mutation analysis. The sensitivity of mutation-specific *EGFR* E746-A750 exon 19 deletion antibody was 100% (95% CI 64.6 to 100%), specificity 98.0% (95% CI 94.9 to 99.2%), positive predictive value 63.6% (95% CI 35.4 to 84.8%) and negative predictive value 100% (95% CI 98.0 to 100%) for detecting the targeted mutation. IHC staining for L858R and exon 19 deletion was mutually exclusive.

The overall sensitivity of the two antibodies for detecting both targeted mutations was 92.9% (95% CI 68.5 to 98.7%) and specificity 96.3% (95% CI 92.6 to 98.2). However, the sensitivity of the exon 19 E746-A750 antibody for detecting any of the 13 exon 19 deletions in the study was only 61.5% (95% CI 35.1 to 88.0%). The sensitivity of both antibodies for detecting any of the 25 sensitising *EGFR* mutations in the population was also 56.0% (95% CI 36.5 to 75.5%).

DISCUSSION

In our cohort of 204 lung adenocarcinomas, we found antibodies directed against the two commonest *EGFR* mutations had high specificity (≥98.0%) and high negative predictive values (99.5% for L858R and 100% for E746-A750) for their targeted mutations, as well as moderate to high sensitivity (85.7% for L858R and 100% for E746-A750). The sensitivity of the antibodies has varied considerably in previous studies with some reporting fairly high sensitivities ranging from 63% to 100% for the exon 19 deletion,[8 10–17] while others have found sensitivity of only 40%[9] and 42.2%.[18] Similarly for L858R, while most have reported high sensitivity ranging from 75% to 100%,[10 12–18] two studies reported low sensitivities of 36%[9] and 40%.[11] By contrast, specificity has been consistently high ranging from 91% to 100% for the exon 19 deletion, and 77% to 100% for L858R,[8–18] suggesting false positives are relatively rarely encountered. While studies to date have used the same antibody clones as used in this study, other clones have recently become commercially available.

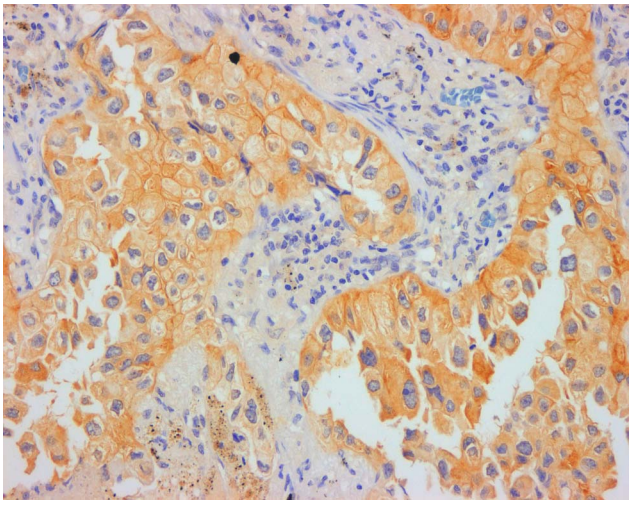

Figure 2 Adenocarcinoma with positive staining *for EGFR* exon 19 deletion mutation-specific antibody (×200). This tumour was confirmed to have *EGFR* E746-A750 exon 19 deletion by mutation analysis.

A number of methodological differences in these prior studies may account for the variation in reported accuracy of the antibodies. As in our study, most reports have based results on the intensity of staining, with some considering 2+ or stronger staining as positive,[11 12 15 16 19] and others considering 1+ or more staining as positive.[8–10] Others have used the product of the percentage of positively staining cells and the staining intensity,[13 18] or alternative methods, such as combining IHC score with total EGFR expression.[17] TMAs were used in our study as well as a number of previous studies,[8–10 12 13 18] while others have used whole sections[17] or small biopsy and cytological specimens.[14 19] Additionally, different molecular genetic assays were used as the gold standard for detecting mutations including direct sequencing,[8–10 13 15–17] peptide nucleic acid-locked nucleic acid PCR clamp assay,[14] fragment length analysis,[12] mass spectrometry[12] and Therascreen,[11] and the sensitivity of these methods varies considerably, making it difficult to directly compare studies.

A limitation of any approach to *EGFR* mutation testing that uses targeted assays of only some of the commoner *EGFR* mutations is that rarer sensitising mutations in *EGFR* cannot be identified. The two commonest activating mutations in *EGFR* in lung cancers are the L858R point mutation in exon 21, and E746-A750 in exon 19, and in many studies these two types of mutations account for about 90% of all identified mutations.[20] In our study, mutations in these two exons accounted for 84.0% of cases with sensitising *EGFR* mutations, and the specific mutations covered by the two antibodies only accounted for 56.0% of cases. The overall sensitivity of the two antibodies for identifying any sensitising mutations in the entire cohort was 56.0% and use of the two antibodies alone would be insufficient for routine clinical application. Kato *et al*,[13] similarly, found the overall sensitivity of mutant-specific IHC for detecting *EGFR* mutations to be fairly low (43.9%) when all *EGFR* mutations were taken into account.

Apart from the 15bp/5AA E746-A750 deletion in *EGFR*, other exon 19 deletions occur in lung cancer resulting in slightly different epitopes with deletions of 3–8 amino acids.[1 2] In our study, the 15 base pair E746-A750 deletion accounted for 53.8% of exon 19 deletions in *EGFR*. Mutation analysis identified six other cases of exon 19 deletions, and only one of these was detected by the E746-A750 antibody (p.Leu747_Ser752>Q). In concordance with our results, most other authors have found the antibody is generally inadequate at identifying variant exon 19 deletions.[11 15 17 21]

Interestingly, in our cohort, the only false negative case using L858R IHC had combined L858R and p.H773_V774insNPH mutations in exon 20. This false negative could be explained by protein structure alterations since the additional exon 20 insertion is adjacent to the p.Leu858Arg target in the 3-dimensional

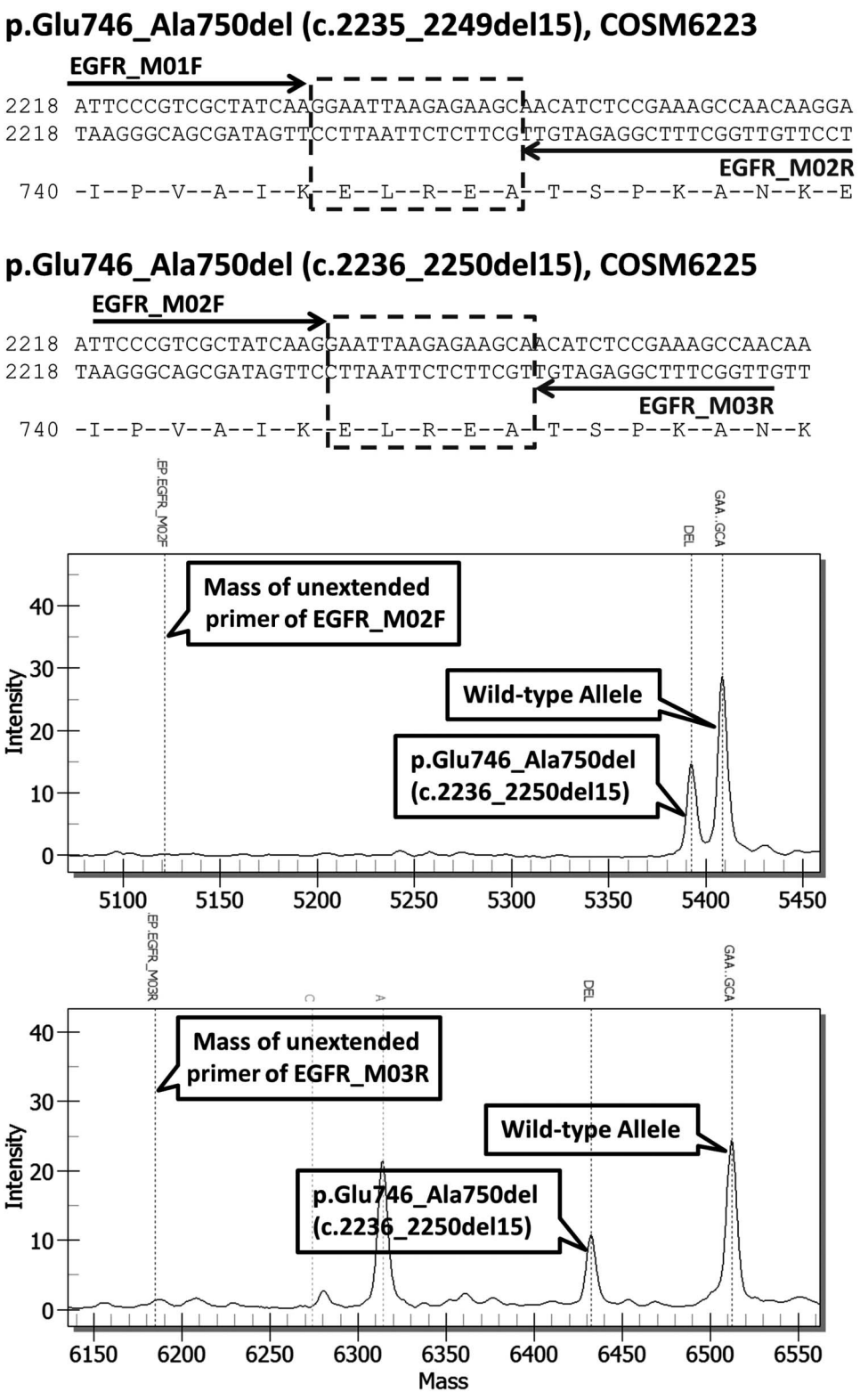

Figure 3 *EGFR* E746-A750 exon 19 deletion (p.Glu746_Ala750) from Sequenom MassARRAY.

structure[22] and could interfere with antibody access or change the epitope of the p.Leu858Arg mutant.

Despite the limited range of possible mutations in lung cancer covered by the two antibodies, IHC has several advantages over molecular genetic assays. IHC is a widely used routine test that is relatively fast and cheap, is frequently automated, and can be performed on cases with small numbers or low proportions of tumour cells that can be problematic for molecular genetic assays requiring DNA extraction. This could particularly be useful in cases with insufficient tumour cells for molecular assays including some cytological specimens or small biopsies with scant tumour cells that are not uncommon in lung cancer specimens. *EGFR* mutant-specific antibodies have performed well in samples that tend to be challenging for molecular genetic assays, such as cytological specimens[14 19] and small biopsies, including decalcified bone biopsies.[19] However, while

Table 3 *EGFR* exon 19 E746-A750 deletion detected by IHC versus mutation analysis.

	Mass spectrometry mutation status		
Exon 19 deletion E746-A750 (p. Glu746_Ala750del)	**Positive**	**Negative**	**Total**
IHC+	7	4	*11*
IHC−	0	193	*193*
Total	*7*	*197*	*204*

IHC, immunohistochemistry.

there are many practical advantages to using IHC, there is only limited evidence for the ability of mutant-specific IHC to predict response to EGFR-TKI treatment.[17 18]

Although inferior to molecular genetic analysis of the *EGFR* gene in terms of comprehensive coverage and accurate description of the underlying changes, IHC is highly specific and moderately sensitive for the targeted *EGFR* mutations with negative predictive values of at least 99.5% in our study. The antibodies are likely to be of clinical value in selected situations, such as where limited tumour material is available, or in situations where molecular genetic analysis is not readily available.

Take-home messages

- EGFR mutant-specific immunohistochemistry is highly specific and sensitive for the targeted *EGFR* mutations with negative predictive values of at least 99.5% in our study.
- Although inferior to molecular genetic analysis of the *EGFR* gene in terms of comprehensive coverage and accurate description of the underlying changes, the antibodies are likely to be of clinical value in selected situations, such as where limited tumour material is available, or in situations where molecular genetic analysis is not readily available.

Contributors This is the original work of all authors, and all authors have contributed significantly to the study conception and design or analysis and interpretation of data. All authors have made a substantial contribution to drafting and critically revising the manuscript. No one else who fulfils criteria for authorship has not been included.

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Competing interests SOT has received honoraria for participating in an Advisory Board for Roche. None declared for other authors.

Ethics approval Ethical approval for the study was obtained from the Human Research Ethics Review Committees of Royal Prince Alfred Hospital (X10-0278; HREC/10/RPAH/491) and Concord Repatriation General Hospital (CH62/6/2004-116).

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