





Immunohistochemical assessment of *HRAS* Q61R mutations in breast adenomyoepitheliomas

Fresia Pareja,¹  Michael S Toss,² Felipe C Geyer,¹  Edaise M da Silva,¹ Mahsa Vahdatinia,¹ Ana Paula M Sebastiao,¹ Pier Selenica,¹ Austin Szatrowski,¹ Marcia Edelweiss,¹ Hannah Y Wen,¹ Raluca Mihai,² Zsuzsanna Varga,³  Maria P Foschini,⁴ Brian P Rubin,⁵ Ian O Ellis,² Sarat Chandarlapaty,⁶ Achim A Jungbluth,¹ Edi Brogi,¹ Britta Weigelt,¹ Jorge S Reis-Filho¹  & Emad A Rakha²

¹Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA, ²Department of Histopathology, Nottingham University Hospitals NHS Trust, Nottingham, UK, ³Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland, ⁴Department of Biomedical and Neuromotor Sciences, Section of Anatomical Pathology, Bellaria Hospital, University of Bologna, Bologna, Italy, ⁵Department of Pathology, Cleveland Clinic, Cleveland, OH, USA, and ⁶Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

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Aims: Breast adenomyoepitheliomas (AMEs) are uncommon tumours. Most oestrogen receptor (ER)-positive AMEs have mutations in phosphoinositide 3-kinase (PI3K) pathway genes, whereas ER-negative AMEs usually harbour concurrent mutations affecting the *HRAS* Q61 hotspot and PI3K pathway genes. Here, we sought to determine the sensitivity and specificity of RAS Q61R immunohistochemical (IHC) analysis for detection of *HRAS* Q61R mutations in AMEs.

Methods and results: Twenty-six AMEs (14 ER-positive; 12 ER-negative) previously subjected to massively parallel sequencing ($n = 21$) or Sanger sequencing ($n = 5$) of the *HRAS* Q61 hotspot locus were included in this study. All AMEs were subjected to IHC analysis with a monoclonal (SP174) RAS Q61R-specific antibody, in addition to detailed histopathological analysis. Nine ER-negative AMEs

harboured *HRAS* mutations, including Q61R ($n = 7$) and Q61K ($n = 2$) mutations. Five of seven (71%) AMEs with *HRAS* Q61R mutations were immunohistochemically positive, whereas none of the AMEs lacking *HRAS* Q61R mutations ($n = 17$) were immunoreactive. RAS Q61R immunoreactivity was restricted to the myoepithelium in 80% (4/5) of cases, whereas one case showed immunoreactivity in both the epithelial component and the myoepithelial component. RAS Q61R immunohistochemically positive AMEs were associated with infiltrative borders ($P < 0.001$), necrosis ($P < 0.01$) and mitotic index in the epithelial ($P < 0.05$) and myoepithelial ($P < 0.01$) components. RAS Q61R IHC assessment did not reveal Q61K mutations (0/2).

Conclusions: IHC analysis of RAS Q61R shows high specificity (100%) and moderate sensitivity (71%) for detection of *HRAS* Q61R mutations in breast AMEs,

Address for correspondence: J S Reis-Filho, Department of Pathology, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA. e-mail: Jorge.Reis-Filho@icr.ac.uk

E A Rakha, Department of Histopathology, Nottingham University Hospitals NHS Trust, Nottingham NG5 1PB, UK. e-mail: Emad.Rakha@nottingham.ac.uk

and appears not to detect *HRAS* Q61K mutations. IHC analysis of *RAS* Q61R may constitute a useful

technique in the diagnostic workup of ER-negative AMEs.

Keywords: adenomyoepithelioma, breast, *HRAS*, immunohistochemistry

Introduction

Breast adenomyoepitheliomas (AMEs) constitute a heterogeneous group of lesions with dual epithelial and myoepithelial cell differentiation, classically composed of glandular epithelial structures surrounded by myoepithelial cell proliferation.^{1–3} Although most lesions are benign, a spectrum of lesions pertaining to this category have been described, ranging from purely benign to frankly malignant.^{2,4–6} Moreover, a subset of AMEs show overlapping morphology and immunophenotype with other lesions. At one end of the spectrum, AMEs overlap with intraductal papillomas with myoepithelial cell hyperplasia, whereas, at the other end of the spectrum, malignant AME can mimic metaplastic breast carcinoma, or what is called malignant myoepithelial carcinoma,^{7–10} making a definite diagnosis of AME difficult to formulate at times.¹¹ The World Health Organization (WHO) guidelines describe malignant forms of AME, and some cases have been reported in the literature,^{5,8} but clear criteria for distinguishing this entity from spindle cell metaplastic carcinoma, apart from the identification of a benign AME component in the tumour, remain to be defined.^{1,2} It is also our observation (E.A.R.) that, in a proportion of cases, the concordance between the morphology and the immunoprofile of myoepithelial and epithelial cell components is low, making an accurate diagnosis and the distinction of each component a challenging task in such cases. The latter feature is important in cases showing atypia, as the criteria for defining atypia are different between epithelial and myoepithelial cell components. The lack of definite diagnostic and molecular features of AME, with their ambiguous nature and histogenesis, leads to challenges in patient management and outcome prediction.

The repertoire of genetic alterations affecting breast cancers has now been well characterised, and includes recurrent mutations affecting *TP53*, *PIK3CA*, *PTEN*, and *GATA3*.^{12–14} In the large pool of breast carcinomas, and other rare tumours that originate in the breast, highly recurrent somatic gene alterations are not uncommon.^{15,16} We have recently shown that AMEs are underpinned by characteristic genetic alterations, which vary according to their oestrogen receptor (ER) status.¹⁷ ER-positive AMEs are

associated with mutually exclusive *PIK3CA* or *AKT1* hotspot mutations, whereas up to 60% of ER-negative AMEs harbour concurrent *HRAS* Q61 hotspot mutations and mutations affecting either *PIK3CA* or *PIK3R1*.¹⁷ On the basis of the results of this study¹⁷ and a comparison with the repertoire of somatic mutations in common forms of breast cancer by The Cancer Genome Atlas¹² and The International Cancer Genome Consortium,¹⁴ we concluded that *HRAS* Q61 hotspot mutations are vanishingly rare in common forms of breast cancer, and that their presence in conjunction with phosphoinositide 3-kinase (PI3K)–AKT pathway activation probably constitutes the driver genetic events in the development of AME.

There is an increasing interest in the application of immunohistochemistry for the detection of specific hotspot mutations, particularly those that could be targetable, such as *BRAF* V600E mutations¹⁸ in melanoma,¹⁹ colorectal carcinoma,²⁰ and papillary thyroid carcinoma,²¹ among others.

In this study we sought to determine the sensitivity and specificity of *RAS* Q61R immunohistochemical analysis for the detection of previously confirmed *HRAS* Q61R mutations in a series of AMEs. We also investigated whether specific histological differences between *RAS* Q61R immunohistochemically positive and immunohistochemically negative AMEs can be identified.

Materials and methods

CASES AND DNA SEQUENCING DATA

In this study, we included 26 breast AMEs with available material from the work by Geyer *et al.*¹⁷ Representative formalin-fixed paraffin-embedded (FFPE) histological blocks of breast AMEs included in this study were retrieved from the author's institutions. Approval from the Institutional Review Board and the local research committees was obtained, and patient consent was obtained in accordance with the approved protocols. All cases were centrally reviewed by five pathologists with expertise in breast pathology (F.C.G., M.E., I.O.E., E.A.R., and J.S.R.F.) for diagnosis confirmation according to the WHO criteria.¹ Assessment of various histological characteristics was conducted by three pathologists (F.P., F.C.G., and

A.P.M.S.) and included growth pattern (tubular or papillary); tumour border (encapsulated, multinodular, or infiltrative); epithelial and myoepithelial nuclear grade, which was evaluated according to the Nottingham grading system for breast cancer;²² epithelial and myoepithelial mitotic rate, defined as the number of mitotic figures per mm²; and, the presence or absence of necrosis. Whole-exome sequencing (WES) ($n = 9$), Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT)²³ targeted sequencing ($n = 12$) and Sanger sequencing ($n = 5$) data for assessment of the mutational status of the *HRAS* Q61 hotspot locus were retrieved from Geyer *et al.*¹⁷ IHC staining for p63 and ER was conducted in the study by Geyer *et al.*²⁴ ER status was assessed by the use of IHC analysis according to the American Society of Clinical Oncology/College of American Pathologists guidelines,²⁵ with 1% of positive tumour nuclei as the cut-off for ER positivity. Hotspot mutations were annotated according to Chang *et al.*²⁶

SANGER SEQUENCING

Areas with overgrowth of epithelial or myoepithelial cells of three AMEs were selected on the basis of p63 expression and morphology. The epithelium and myoepithelium of only the selected areas were separately microdissected from 8- μ m-thick FFPE histological sections under a stereomicroscope (Olympus SZ61; Center Valley, PA, USA), following careful histological review and use of the corresponding p63 IHC stains to highlight the myoepithelium as reference. DNA was extracted with the DNAeasy Blood and Tissue Kit (Qiagen; Waltham, MA, USA), according to the manufacturer's instructions. The presence of mutations affecting the *HRAS* Q61 hotspot locus was assessed by the use of Sanger sequencing. In brief, polymerase chain reaction (PCR) amplification was conducted with the AmpliTaq Gold 360 Master Mix Kit (Life Technologies; Carlsbad, CA, USA), as previously described.¹⁶ Following purification with exoSAP-IT, PCR products were subjected to Sanger sequencing with previously validated primers¹⁷ encompassing the *HRAS* Q61 hotspot locus (Table S1). Sequence electropherograms corresponding to the forward and reverse strands were manually analysed.

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IHC analysis was conducted with the monoclonal antibody SP174 (ab227658; Abcam, Cambridge, MA,

USA), which is an antibody generated against Q61R mutant NRAS that also recognises Q61R mutant HRAS and KRAS,²⁷ owing to the homology of the various RAS proteins.²⁸ The analyses were performed on a Leica Bond-3 automated stainer platform (Leica, Buffalo Grove, IL, USA). In brief, 4- μ m-thick deparaffinised FFPE tissue sections were incubated with primary antibody at a dilution of 1:100, after a heat-based antigen retrieval step employing a high-pH buffer solution (ER2; Leica). As a secondary reagent, a polymeric kit (Refine; Leica) was employed. Appropriate positive and negative controls were included in each run. The evaluation of the IHC expression of mutant RAS Q61R was performed by three pathologists (F.P., F.C.G., and A.P.M.S.) blinded to the results of the sequencing analysis.

STATISTICAL ANALYSIS

Statistical analyses were performed with R v3.1.2. Fisher's exact test was used for comparisons between categorical variables. All tests were two-sided, and *P*-values of <0.05 were considered to be statistically significant.

Results

Our study included 26 AMEs previously reported in Geyer *et al.*,¹⁷ 12 of which were ER-negative (Table 1; Table S2). Nine and 12 AMEs had been previously subjected to WES or MSK-IMPACT sequencing, respectively.¹⁷ In addition, the presence of mutations affecting the *HRAS* Q61 hotspot locus had been previously investigated with Sanger sequencing in five AMEs (Table S2).¹⁷ Seventy-five per cent (9/12) of the ER-negative AMEs harboured *HRAS* Q61 hotspot mutations, including Q61R ($n = 7$) and Q61K ($n = 2$) mutations (Figure 1). None of the ER-positive AMEs ($n = 14$) were found to harbour *HRAS* Q61 hotspot mutations (Figure 1).

We sought to determine whether *HRAS* Q61R mutations could be detected by the use of IHC analysis. We subjected all 26 cases to IHC analysis with an antibody that detects Q61R mutant RAS (i.e. mutant NRAS, KRAS, or HRAS).²⁷ Seventy-one per cent (5/7) of AMEs harbouring *HRAS* Q61R mutations were immunoreactive against these antibodies, showing diffuse cytoplasmic and/or membranous staining, as previously reported in different tumour types,^{29,30} and consistent with the reported localisation of RAS isoforms in the plasma membrane and cytoplasmic organelles^{31–33} (Figures 1 and 2A–D). Notably, the

Table 1. Clinicopathological features of the breast adenomyoepitheliomas studied according to RAS Q61R immunoreactivity

	RAS Q61R IHC analysis		
Clinicopathological feature	Positive (N = 5), % (n)	Negative (N = 21), % (n)	P-value*
ER			
Positive	0.0 (0)	66.7 (14)	0.012
Negative	100.0 (5)	33.3 (7)	
Growth pattern			
Tubular	60.0 (3)	76.2 (16)	0.5875
Papillary	40.0 (2)	23.8 (5)	
Tumour border			
Encapsulated	0.0 (0)	23.8 (5)	0.0003
Multinodular	0.0 (0)	66.7 (14)	
Infiltrative	100.0 (5)	9.5 (2)	
Nuclear grade (epithelium)			
Low	0.0 (0)	23.8 (5)	0.0816
Intermediate	0.0 (0)	38.1 (8)	
High	100.0 (5)	38.1 (8)	
Nuclear grade (myoepithelium)			
Low	0.0 (0)	4.8 (1)	0.467
Intermediate	20.0 (1)	47.6 (10)	
High	80.0 (4)	47.6 (10)	
Epithelial mitoses/mm ²			
≤0.8	40.0 (2)	81 (17)	0.0494
>0.8 but ≤2.1	40.0 (2)	19 (4)	
>2.1	20.0 (1)	0 (0)	
Myoepithelial mitoses/mm ²			
≤0.8	0.0 (0)	85.7 (18)	0.0013
>0.8 but ≤2.1	60.0 (3)	4.8 (1)	
>2.1	40.0 (2)	9.5 (2)	
Necrosis			
Absent	0.0 (0)	76.2 (16)	0.0038
Present	100.0 (5)	23.8 (5)	

ER, oestrogen receptor; IHC, immunohistochemical.

*Two-tailed Fisher's exact test.

two AMEs harbouring *HRAS* Q61K mutations were negative on IHC analysis (Figure 1). None of the 17 *HRAS* wild-type AMEs included in this study showed immunoreactivity on IHC analysis (Figures 1 and 2E). RAS Q61R immunoreactivity was found to be restricted to the myoepithelial component in four cases (Figure 2A,B), and present in both the epithelial component and the myoepithelial component in one case (AM52; Figure 2C,D). Taken together, these findings show that the IHC detection of *HRAS* Q61R mutations in ER-negative breast AMEs has moderate sensitivity (71%) and high specificity (100%), whereas, in ER-positive AMEs, this antibody was of limited use, as no cases were found to harbour *HRAS* Q61 hotspot mutations and/or RAS Q61R protein expression.

We next sought to determine whether *HRAS* Q61R hotspot mutations were present in the epithelium and the myoepithelium of AMEs, or whether they were restricted to either histological component. We conducted Sanger sequencing analysis of the *HRAS* Q61 hotspot locus in the separately microdissected epithelial and myoepithelial components of cases with available material (AM32, AM48, and AM52). Our analysis revealed the presence of *HRAS* Q61R hotspot mutations in both the epithelial and the myoepithelial components of all three adenomyoepitheliomas assessed (Figure 3A–F). These findings support the view that both the epithelium and the myoepithelium of adenomyoepitheliomas are neoplastic.

RAS Q61R immunohistochemically positive AMEs more frequently showed infiltrative tumour borders (100% versus 9.5%; $P < 0.001$; Table 1; Figure 4A), an association with necrosis ($P < 0.01$; Table 1; Figure 4B) and higher epithelial ($P < 0.05$) and myoepithelial ($P < 0.01$) mitotic indexes (Table 1; Figure 4C,D) than immunohistochemically negative cases. All of the RAS Q61R immunohistochemically positive AMEs were ER-negative (Table 1; Figure 4E, F), whereas the majority (67%) of the RAS Q61R immunohistochemically negative AMEs were ER-positive ($P < 0.05$; Table 1). We observed no association of immunoreactivity for RAS Q61R with growth pattern or epithelial or myoepithelial nuclear grade ($P > 0.05$; Table 1).

Discussion

In this study, we evaluated the sensitivity and specificity of IHC analysis for the detection of mutated RAS Q61R protein in a set of 26 previously

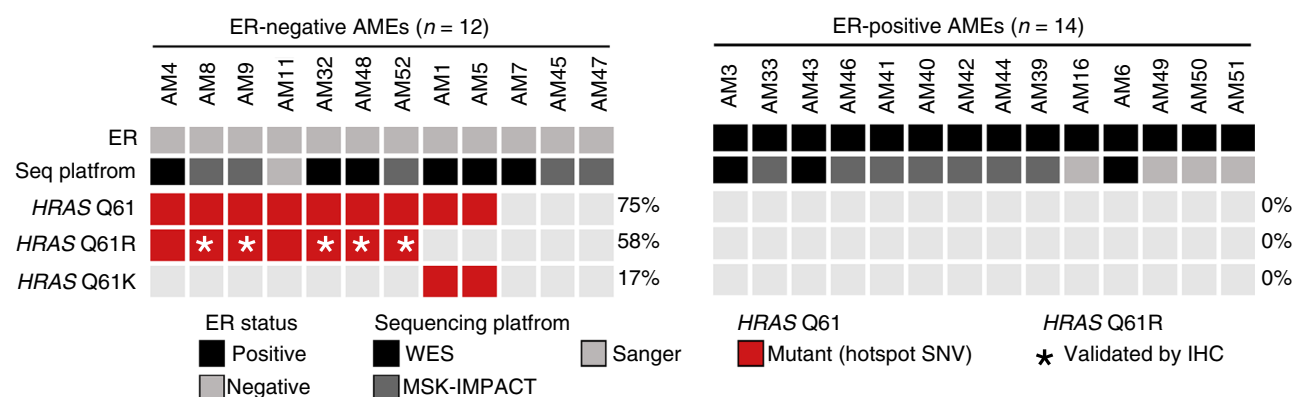


Figure 1. Oestrogen receptor-negative breast adenomyoepitheliomas harbour recurrent *HRAS* Q61R hotspot mutations, which are detectable by immunohistochemistry, co-occurring with mutations in phosphoinositide 3-kinase genes. The heatmap depicts somatic mutations affecting the *HRAS* Q61 hotspot locus and immunoreactivity for RAS Q61R in the 26 breast adenomyoepitheliomas included in this study. Cases are shown in columns, and genes are shown in rows. Mutations are colour-coded according to the legend. Oestrogen receptor status and the sequencing platform used are depicted in phenotype bars (top). [Colour figure can be viewed at wileyonlinelibrary.com]

sequenced AMEs,¹⁷ comprising 14 ER-positive AMEs and 12 ER-negative AMEs; of the 12 ER-negative AMEs, nine harboured mutations affecting the *HRAS* Q61 hotspot locus. Our findings show that IHC analysis has a high specificity and a moderate sensitivity for identifying *HRAS* Q61R-mutated AMEs among ER-negative cases. The two *HRAS* Q61K-mutated cases, however, were not detected by IHC analysis in this series. IHC evaluation showed no immunoreactivity in any of the 14 ER-positive AMEs, which lacked *HRAS* mutations, providing further support for the remarkable specificity of IHC evaluation for the detection of Q61R *HRAS* mutations. Interestingly, RAS Q61R immunoreactivity was observed to be restricted to the myoepithelial component in four cases, and present in both the epithelial component and the myoepithelial component in one case. Nonetheless, our Sanger sequencing analyses of separately microdissected epithelium and myoepithelium of three AMEs revealed the presence of *HRAS* Q61R mutations in both histological components. These findings are in agreement with our previous observations showing that the *HRAS* Q61R mutations identified in ER-negative AMEs were clonal.¹⁷ Hence, it is possible that the epithelial components of a subset of cases expressed a *HRAS* Q61R mutant, but at levels not detectable by IHC analysis. The molecular mechanisms underpinning the differences in expression and/or IHC detection of *HRAS* Q61R mutations in the epithelium and the myoepithelium of AMEs warrant further study.

Breast AMEs constitute a specific group of tumours both within the large pool of breast lesions, and within the wide spectrum of myoepithelial

lesions.³⁴ Although not all AMEs show *HRAS* mutations, and most of them are either negative or weakly positive for ERs,¹⁰ a strong correlation between *HRAS* mutation and the ER-negative subgroup has been previously reported by our group.¹⁷ In our study, of 12 ER-negative AMEs, nine harboured *HRAS* mutations affecting the Q61 hotspot locus, whereas the remaining three were wild-type for *HRAS*. We have also recently observed that a subset of AMEs lacking *HRAS* mutations might be underpinned by *HMGA2* rearrangements, suggesting that a subset of AMEs could be related to salivary gland pleomorphic adenomas.³⁵ The relevance of the genetic alterations underpinning AMEs is of practical importance when we are faced with differential diagnosis dilemmas, such as the discrimination of AMEs from other ER-negative breast tumours, including metaplastic carcinomas, adenoid cystic carcinomas, and pleomorphic adenomas, with which adenomyoepitheliomas may show morphological overlap.³⁶ The detection of *HRAS* Q61R mutations in AMEs with ambiguous morphology may help to settle such challenging cases. Nonetheless, given that RAS Q61R IHC assessment has moderate sensitivity for the detection of *HRAS* Q61R mutations, and does not detect *HRAS* Q61K mutations, and that a subset of approximately 30–40% of ER-negative AMEs lack *HRAS* mutations,¹⁷ the diagnosis of AME should not be ruled out in cases showing the typical histological features but lacking RAS Q61R expression on IHC analysis.

Although it is generally considered that triple-negative breast neoplasms are biologically more aggressive, this is not the case across the entire spectrum of

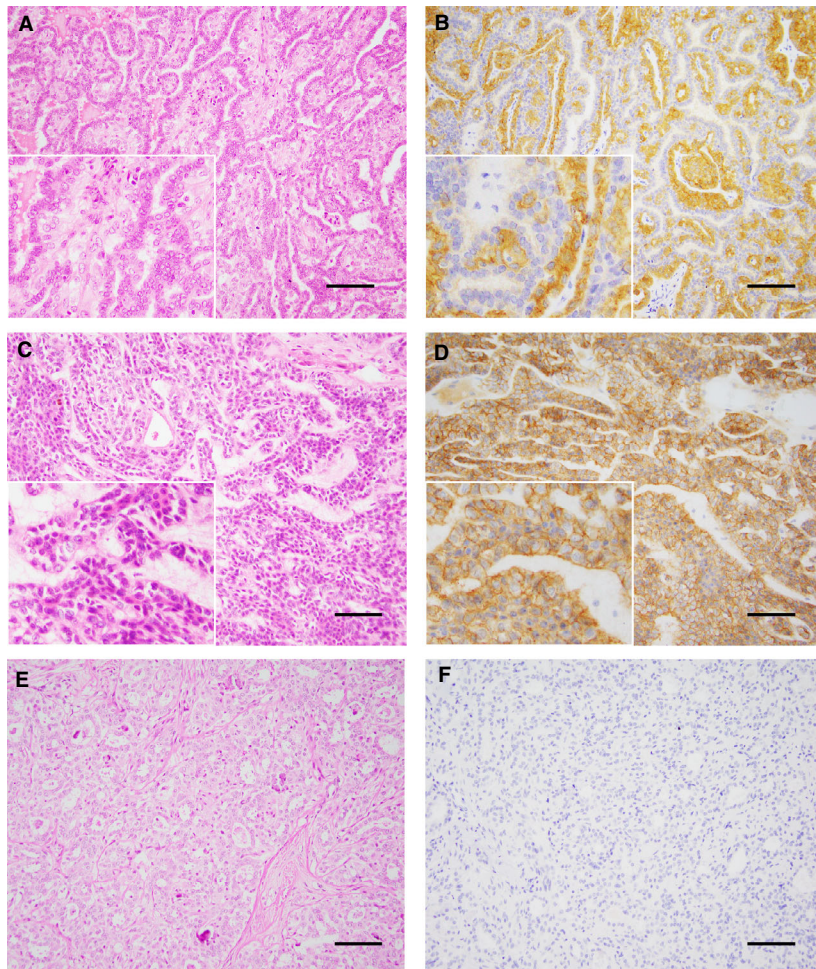
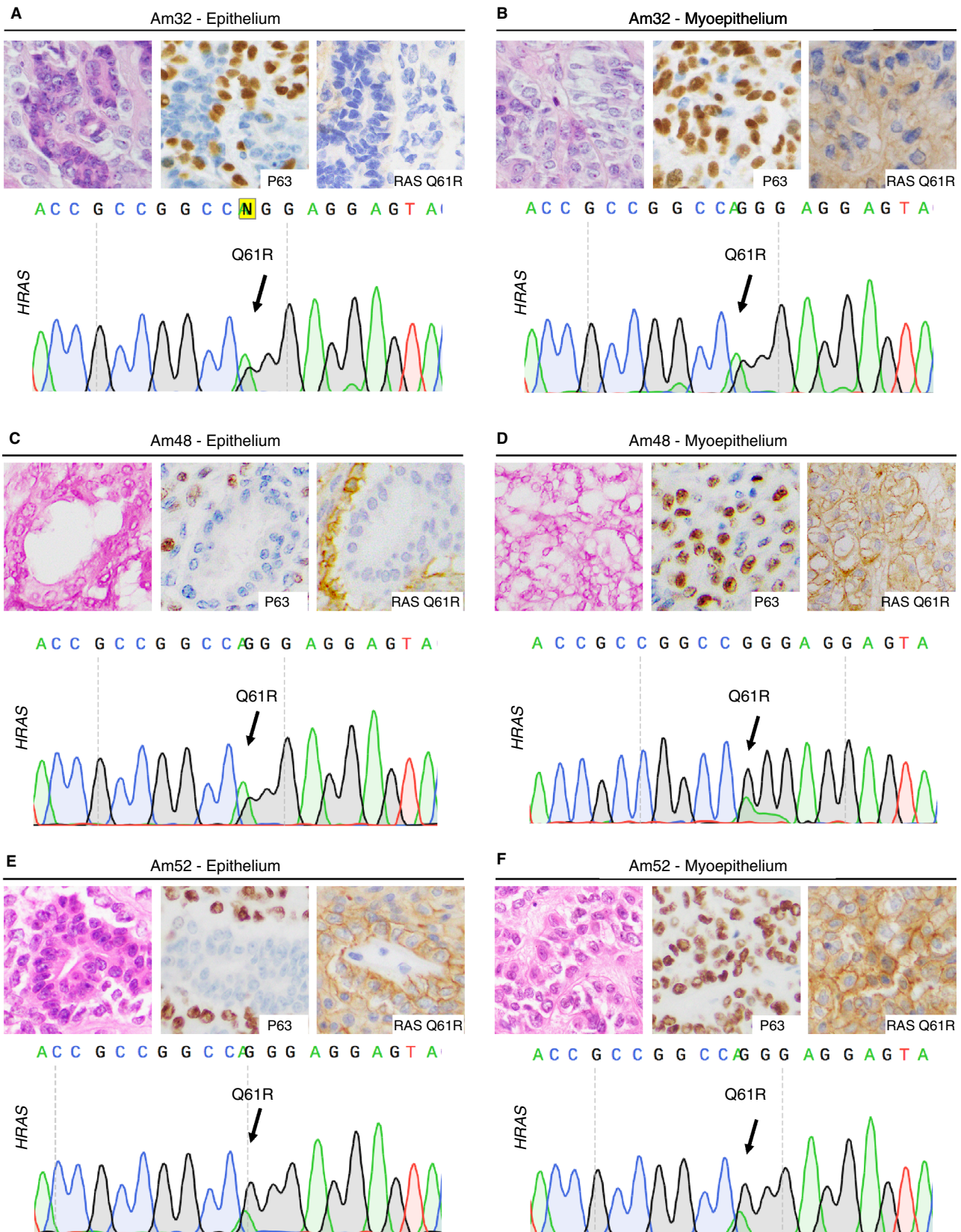


Figure 2. Detection of *HRAS* Q61R hotspot mutations in breast adenomyoepitheliomas by immunohistochemical analysis. **A,B**, Representative micrographs of (A) haematoxylin and eosin (H&E)-stained sections and (B) RAS Q61R immunohistochemical expression of AM8, showing immunoreactivity in the myoepithelial component. **C,D**, Representative micrographs of (C) H&E-stained sections and (D) RAS Q61R immunohistochemical expression of AM52, showing immunoreactivity in both the epithelial component and the myoepithelial component. **E,F**, Representative micrographs of (E) H&E-stained sections and (F) lack of RAS Q61R immunohistochemical expression of AM3. Scale bars: 50 μ m.

these tumours.³⁷ AMEs are considered to be among the least aggressive breast neoplasms, and are generally cured by local excision alone.¹ A spectrum of these lesions appears to exist, with some AMEs showing unpredictable behaviour. Nodal metastasis has been reported in AMEs, ranging from benign to atypical cases with no frank malignant histological features.^{4,5,14,38} There are no established criteria on

how to catalogue these lesions,¹ although general features, such as mitotic activity, necrosis, cellular pleomorphism, peripheral invasive borders, and overgrowth of myoepithelium, have been suggested.² In our study, of seven AMEs categorised as having infiltrative borders, five were immunoreactive for mutant RAS Q61R. A sixth case with infiltrative borders showed the Q61K mutation, making a total of six of

Figure 3. *HRAS* Q61R mutations are present in the epithelial and myoepithelial components of breast adenomyoepitheliomas. **A–F**, Representative micrographs of haematoxylin and eosin (H&E)-stained sections (left) and p63 (centre) and RAS Q61R (right) immunohistochemical expression in the epithelial and myoepithelial components of adenomyoepitheliomas AM32 (**A,B**), AM48 (**C,D**), and AM52 (**E,F**). Also shown are representative Sanger electropherograms of the *HRAS* Q61 hotspot loci of separately microdissected epithelial and myoepithelial components of AM32 (epithelium, **A**; myoepithelium, **B**), AM48 (epithelium, **C**; myoepithelium, **D**), and AM52 (epithelium, **E**; myoepithelium, **F**).



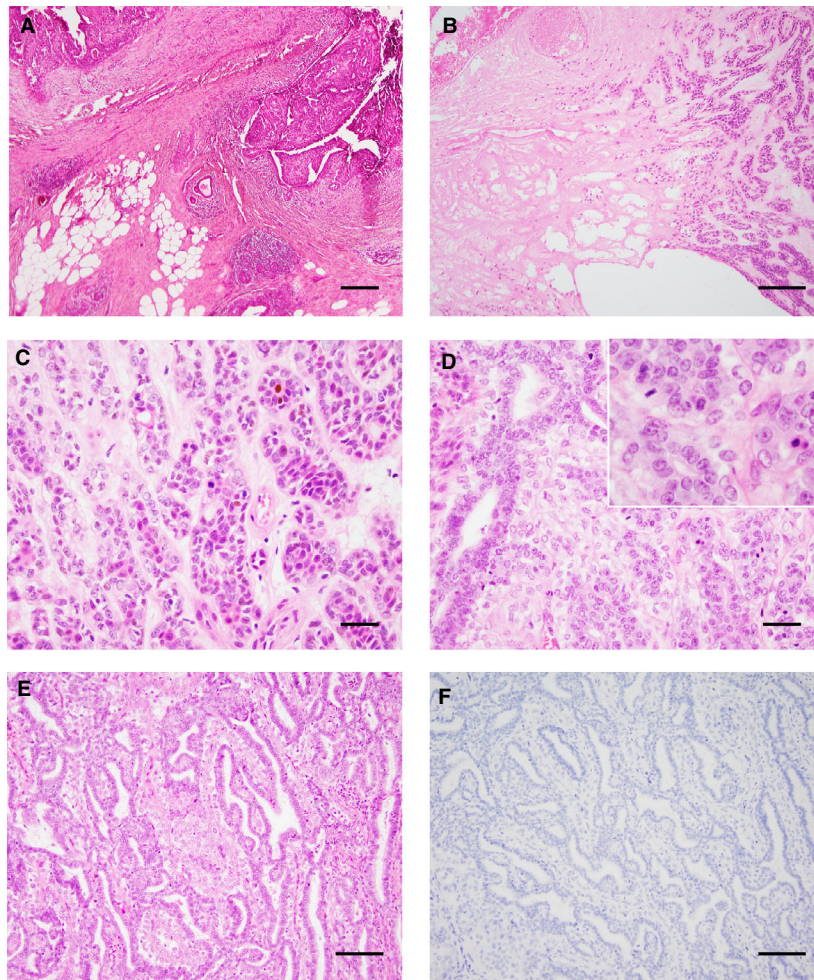


Figure 4. Histopathological characteristics of breast adenomyoepitheliomas immunoreactive for RAS Q61R. A–D, Representative micrographs of (A) haematoxylin and eosin (H&E)-stained sections of AM9 showing infiltrative borders, (B) AM52 showing areas of necrosis, (C) high epithelial and myoepithelial nuclear grade, and (D) frequent epithelial and myoepithelial mitotic figures. E,F, Representative micrographs of (E) H&E-stained sections and corresponding (F) oestrogen receptor (ER) immunostain of AM8. Scale bars: A, 200 μ m; B, 100 μ m; C,D, 20 μ m; and E,F, 50 μ m. [Colour figure can be viewed at wileyonlinelibrary.com]

seven cases with infiltrative borders associated with *HRAS* mutations. The presence of necrosis was also recorded in all five cases with IHC positivity for RAS Q61R, and RAS Q61R immunohistochemically positive AMEs had a higher mitotic index in both the epithelial component and the myoepithelial component than immunohistochemically negative AMEs. These findings corroborate the more aggressive histological features associated with lack of ER expression and the presence of *HRAS* Q61 hotspot mutations in AMEs.¹⁷

Limitations of the current study include the relatively low number of AMEs harbouring *HRAS* mutations, and the lack of follow-up information, owing to the multi-institutional nature of our cohort. Despite

these limitations, our study demonstrates that IHC analysis of *HRAS* Q61R shows high specificity and moderate sensitivity for the detection of *HRAS* Q61R mutations in breast AMEs, and it appears not to detect *HRAS* Q61K mutations. As it has previously been concluded that these mutations probably constitute founder genetic events in the development of ER-negative AMEs,¹⁷ detection of immunoreactivity for RAS Q61R mutations would aid in distinguishing these unusual breast lesions. Given the fact that, in the context of primary breast tumours, *HRAS* Q61R mutations appear to be restricted to ER-negative AMEs, IHC assessment of *HRAS* Q61R may represent a useful marker in the diagnostic workup of these lesions.

Conflicts of interest

J. S. Reis-Filho reports receiving personal/consultancy fees from Goldman Sachs and REPARE Therapeutics, membership of the scientific advisory boards of VolitionRx and Page.AI, and ad-hoc membership of the scientific advisory boards of Roche Tissue Diagnostics, Ventana Medical Systems, Novartis, Genentech, and InVivo, outside the scope of this study. All other authors declare no conflicts of interest.

Author contributions

J. S. Reis-Filho and E. A. Rakha conceived the study. Z. Varga, M. P. Foschini, B. P. Rubin, I. O. Ellis, E. Brogi and E. A. Rakha contributed with cases. F. Pareja, F. C. Geyer, A. P. M. Sebastiao, M. Edelweiss, I. O. Ellis, J. S. Reis-Filho and E. A. Rakha reviewed the cases. F. Pareja, F. C. Geyer, E. M. da Silva, M. Vahdatinia, A. P. M. Sebastiao, P. Selenica, A. Szatrowski, H. Y. Wen, R. Mihai and S. Chandarlapaty analysed and interpreted the data. F. Pareja, M. S. Toss and E. A. Rakha wrote the first manuscript, which was reviewed by all co-authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used for Sanger sequencing analysis of the *HRAS* Q61 hotspot locus.

Table S2. Sequencing platform, *HRAS* mutational status and clinicopathological characteristics of the 26 breast adenomyoepitheliomas included in this study.