Next generation sequencing of the nidus of early (adenosquamous proliferation rich) radial sclerosing lesions of the breast reveals evidence for a neoplastic precursor lesion

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Abstract

We sought to determine if adenosquamous proliferation of early cellular radial sclerosing lesions of the breast harbours hot spot mutations and to help clarify its relationship to low-grade adenosquamous carcinoma as a potential form of early neoplasia. Four low-grade adenosquamous carcinomas, early radial sclerosing lesions from 13 individuals, and 4 benign proliferative breast lesions were microdissected and assessed with a 50-gene Hot-spot cancer panel. Early radial sclerosing lesions were selectively microdissected concentrating on their adenosquamous proliferation (nidus). Hot spot mutations in *PIK3CA* were detected in ten (77% of) radial sclerosing lesions, in one low-grade adenosquamous carcinoma, and in usual ductal hyperplasia and apocrine adenosis. Over three quarters of individuals with cellular (adenosquamous proliferation rich) early radial sclerosing lesions tested harboured somatic mutations in *PIK3CA* suggesting that adenosquamous proliferation is a clonal lesion. Its relationship to low-grade adenosquamous carcinoma remains unclear in view of the small sample size and unmatched radial sclerosing lesions and low-grade adenosquamous carcinomas.

Introduction

There is growing evidence that breast lesions characterized by adenosquamous proliferation (ASP), whether in the form of a radial sclerosing lesion (incorporating radial scar and complex sclerosing lesion; RSL), a sclerosing papilloma or similar lesions, are phenotypically similar to low-grade adenosquamous carcinoma (LGASC) [1]; their distinction is often subjective and is poorly defined in the literature. They may indeed form a continuous morphological spectrum with benign sclerosing lesions at one end and LGASC at the other, the latter typically being more extensive, and with prominent desmoplastic stroma [1,2]. RSLs have been shown to harbour areas that are clonal and neoplastic and clonal differences are seen within different areas of the same RSL [3]. Macrodissection studies have shown PI3K/AKT/MTOR pathway mutations in over 63% of

RSL, greater than twofold more frequent than in breast carcinoma [4]. The PI3K/AKT/MTOR pathway has been termed a pro-survival pathway because its activation is associated with cell survival, proliferation, migration and angiogenesis and as such it has a key role in normal human development. PI3K and PTEN have phosphorylating and dephosphorylating effects respectively on phosphatidylinositol. Phosphorylation by PI3K increases the activity of downstream kinases AKT and MTOR. PTEN thus has tumour suppressor activity. Inactivating germline mutations of *PTEN* are associated with hamartoma-tumour syndromes. Its somatic loss is associated with various sporadic human cancers [5]. Perhaps unsurprisingly, germline and mosaic (genetic or somatic) mutations in the PI3K/AKT/MTOR pathway are associated with overgrowth syndromes while somatic mutations are implicated in sporadic malignancies [6].

Phosphatidylinositol-3-kinase (*PIK3CA*) mutations are common in proliferative breast lesions [7] and indeed the majority component of RSL, particularly in late lesions, represents proliferative breast disease (PBD) such as usual ductal hyperplasia [8,9]. A similar lesion, which may form a spectrum with RSL, is infiltrative epitheliosis, an infrequently diagnosed breast lesion that comprises ASP in association with florid usual ductal hyperplasia in a non-stellate configuration [10]. Like RSL, infiltrative epitheliosis has been shown to harbour *PIK3CA* mutations, so is also thought to represent a clonal proliferation [11]. The neoplastic risk of RSL, the best documented of these lesions, for common cancers may merely reside in the fact that they occur in the context of PBD, which has an inherent neoplastic risk that is essentially the same as that stated for RSL [12]. It may in fact be the case that ASP arises in PBD due to this neoplastic risk, and that its subsequent growth and associated desmoplasia distorts the tissue within which it arises. The final histological diagnosis essentially depends upon whether this lesion resides centrally within a focus of PBD giving a stellate configuration (RSL), complicates a papilloma (sclerosing papilloma), arises intermixed with PBD, and/or papillomatosis at the nipple (syringomatous tumour), in a subareolar location (subareolar sclerosing duct hyperplasia) or in breast parenchyma (infiltrating epitheliosis). Although PBD itself is a risk factor for common cancers, ASP may be a direct precursor of low-grade metaplastic carcinoma, particularly LGASC [1]. Our aim was to determine whether microdissection specifically targeting the nidus and particularly the ASP of early RSL would reveal genetic mutations, isolated from that of background PBD. Four LGASCs, 13 early or cellular RSLs and four cases of PBD without ASP were assessed with a 50-gene hot-spot cancer panel using next generation (massive parallel) sequencing (NGS).

Materials and methods

Case selection

Archived cases from a 10-year period were identified utilizing a SNOMED search to identify examples of LGASC as defined by Rosen [13] and sufficiently cellular early RSL to enable adequate DNA yield, using the accepted definition of Anderson and Battersby [8,9]. Four examples of LGASC were identified and of sufficient cellularity such that over 90% of the lesion comprised tumour cells within prominent desmoplastic stroma, with several examples

showing perineural invasion (Figure 1). Over a hundred cases with RSL were examined from which 13 with cellular early RSL were selected, some of which were multiple and/or bilateral. ASP in several instances formed a discrete-tumoural mass confined within the RSL (Figure 2), but in most instances they ramified between benign ducts, which often showed usual ductal hyperplasia, which made it difficult to obtain samples of ASP exclusive of background breast tissue while obtaining a sufficient yield of assessable DNA. While over 90% of the tissue targeted for microdissection was from the nidus of the RSLs, the ratio of ASP to nidus density ranged from 10% to greater than 90% because these are often paucicellular lesions. Four separate cases of benign proliferative disease (controls) without a sclerosing lesion were selected because the examples of RSL had insufficient uninvolved tissue to achieve a pure DNA yield. It is acknowledged this is less ideal than selecting control tissue from same subjects. However, gene mutations are well documented in proliferative breast tissue [7]. Positive and negative controls (eg, for known *PIK3CA* mutations) were unavailable. The cases were anonymized prior to assessment. The study received institutional approval from the source laboratory. The cases were assessed with a cancer hot-spot panel to detect single nucleotide variants (SNVs) and short insertions and deletions (indels). (Table 1)

Microdissection and DNA extraction

Serial sections (8 μm) of formalin fixed paraffin embedded (FFPE) tissue from each case were similarly aligned on Superfrost™ Ultra Plus Adhesion Slides (Thermo Scientific). The first and sixth serial sections were stained with haematoxylin and eosin (H&E) (Figure 3). Using the first H&E section regions enriched with RSLs were imaged and selected using a P.A.L.M. DuoFlexCombi System (Zeiss). Reference points throughout sections one to six were used to map the RSL-rich regions-of-interest onto each of these slides in turn. The laser on the P.A.L.M. DuoFlexCombi System was used to etch around the ASP foci on the unstained slides and the second H&E stained slide was used to confirm the regions of interest retained the enriched RSL morphology throughout the serial sections. In each case, the unstained cellular nidus, rich in ASP, were then precisely microdissected using a sterile needle under a stereo-microscope. Genomic DNA was then purified using the GeneRead DNA FFPE Kit (Qiagen). Briefly, following deparafinization, proteinase K digestion was performed at 56°C for 1 hour.
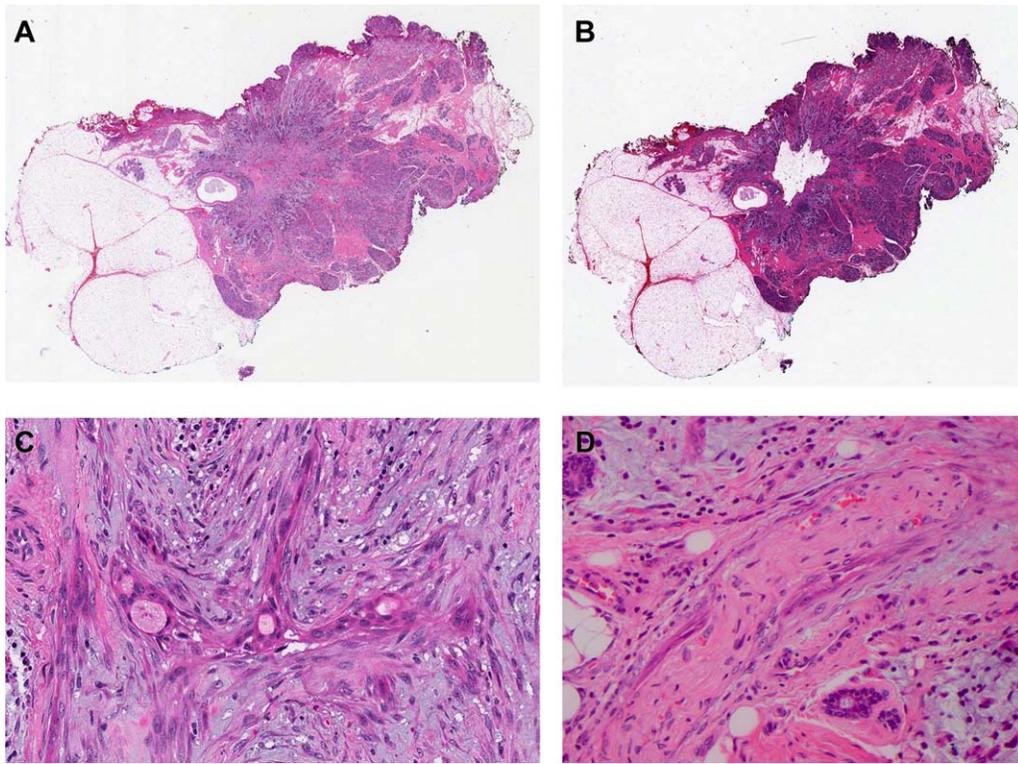
Figure 1. Sample A1: Low-grade adenosquamous carcinoma. Displayed somatic mutation in *PIK3CA*: H1047L (H&E). A. Whole slide scan – pre-dissection. B. Whole slide scan – post-dissection. C. Adenosquamous proliferation of LGASC (scan 28×).D. Perineural invasion (scan 40×).
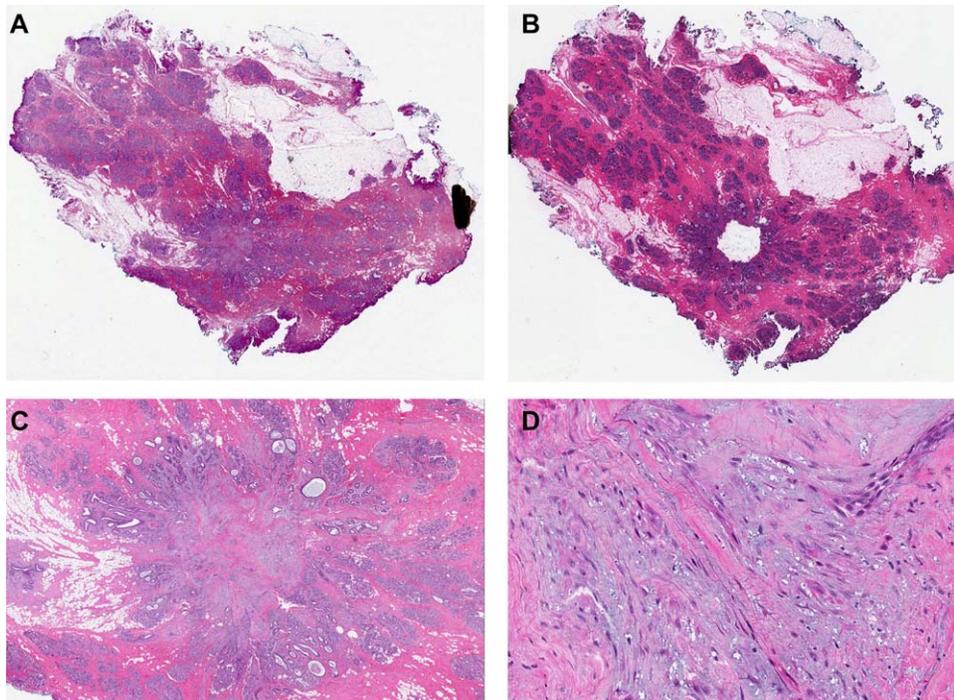

Figure 2. Sample B3: Early radial sclerosing lesion (RSL) with dense tumour-like nidus. Displayed somatic mutation in PIK3CA: H1047R (H&E). A. Whole slide scan – pre-dissection. B. Whole slide scan – post-dissection. C. Adenosquamous proliferation of early RSL (scan 1.7×). D. Adenosquamous proliferation of early RSL (scan 29×).

Table 1. Case and control samples selected for cancer hot-spot sequencing

Sample code	Diagnosis	Age (yr)	Laterality	Size (mm)	ASP nidus density (%)	Estimated sample purity (%)	DNA yield (ng/μL)
A1	LGASC	45	Right	25	>90	>90	31.2
A2	LGASC arising in IE?	48	Right	20	>90	>90	42.8
A3	LGASC	78	Right	17	>90	>90	18.5
A4	LGASC	65	Right	13.5	>90	>90	17.2
B1	RSL x 2 (incorporated intraduct papilloma in one)	41	Left	<10	40	>90	30.2
B2	RSL	45	Left	5	10	>90	33.4
B3	RSL	45	Right	Each <10	>90	>90	11
B4	RSL x 4	46	Left	2–5	80	>90	25
B5	RSL	44	Right	12	30	>90	15.8
B6	RSL	48	Left	10	30	>90	30.8
B7	RSL	49	Left	10	40	>90	28
B8	RSL (*BRCA* mutation, previous left carcinoma)	38	Bilateral: Right Left	<10 <10	10 50	>90	23.2
B9	RSL x 5	45	Left	Each <10	10	>90	32
B10	RSL (adenomyoepithelial hyperplasia present)	38	Left	12	30	>90	19.5
B11	RSL	40	Bilateral: Right Left	≤12 8	50	>90	23.2
B12	RSL	47	Left	7	35	>90	37
B13	RSL	24	Right	<10	40	>90	38.2
C1	UDH	56	N/A	N/A	>50	>90	12.8
C2	UDH	49	N/A	N/A	>50	>90	37.2
C3	UDH, CCC	32	N/A	N/A	>50	>90	22
C4	AA	53	N/A	N/A	>50	>90	26.4

Sample codes: A = LGASC, B = RSL, C = PBD controls.
AA: apocrine adenosis. CCC: columnar cell change. IE: Infiltrating epitheliosis. LGASC: low-grade adenosquamous carcinoma. PBD: proliferative breast disease. RSL: radial sclerosing lesion. UDH: usual ductal hyperplasia. N/A: not available.

Formaldehyde modification of nucleic acids was reversed by incubation with buffer FTB and Uracil-N-glycosylase added to reverse cytosine deamination. Following RNase digestion, DNA was washed on the spin column, eluted and quantified using a Qubit® 3.0 Fluorometer. DNA were then stored at −20°C until sequence analysis.

Hot-spot panel with next generation (massive parallel) sequencing

We targeted a set of ~2800 variants in known cancer genes present in the COSMIC database using an Ion Ampliseq Cancer hotspot v2 assay (Thermofisher Scientific) [14]. The data were collected using TorrentSuite 4.2 and analyzed using Ion Reporter 4.6. Data were filtered to remove intronic, synonymous and common variants (based on 1000 genomes frequencies >1%). Overall, there was insufficient extracted DNA to allow confirmation of results by an orthogonal method (Sanger sequencing). However,

1) Areas of ASP identified on slide 1 were selected using PALM imaging software
2) ASP-rich ROIs were then mapped onto images of slides 2–6
3) Slide 6 was used to confirm ASP region was retained throughout serial sections
4) ROIs were etched onto the unstained slides using the PALM laser
5) Etched regions were microdissected and DNA extracted for analysis
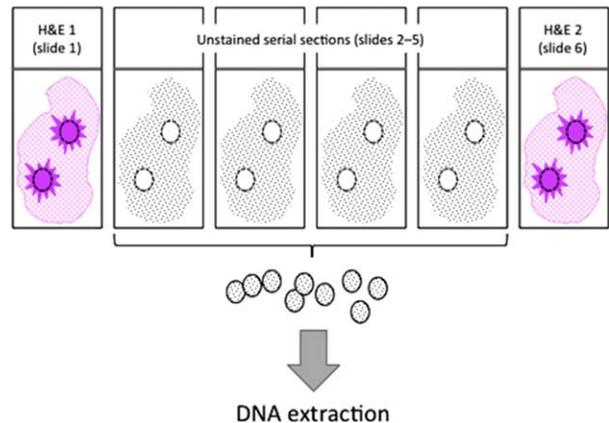

Figure 3. Schematic diagram of tissue selection for microdissection on PALM laser. ASP: adenosquamous proliferation. ROI: region of interest.

Table 2. Results of cancer hot-spot sequencing

Sample code	Gene	Locus	Type	Ref	Genotype	Variant Allele Frequency	Exon	Protein
A1	PIK3CA	chr3:178952085	SNV	A	A/T	0.15	21	p.His1047Leu
A2	JAK3	chr19:17945696	SNV	C	C/T	0.54	16	p.Val722Ile
A3	–	–	–	–	–	–	–	–
A4	PDGFRA	chr4:55141026	SNV	G	G/A	0.07	7	p.Arg234Gln
	PTEN	chr10:89717676	SNV	C	C/T	0.07	12	p.Arg558Cys
	STK11	chr19:1220394	SNV	G	G/A	0.06	4	p.Gly163Ser
B1	PIK3CA	chr3:178952085	SNV	A	A/G	0.06	21	p.His1047Arg
	RET	chr10:43609955	SNV	C	C/T	0.06	11	p.Thr636Met
B2	PIK3CA	chr3:178952085	SNV	A	A/G	0.08	21	p.His1047Arg
B3	ATM	chr11:108138003	SNV	T	T/C	0.60	17	p.Phe858Leu
	JAK3	chr19:17945696	SNV	C	C/T	0.47	16	p.Val722Ile
	PIK3CA	chr3:178952085	SNV	A	A/G	0.15	21	p.His1047Arg
B4	PIK3CA	chr3:178952085	SNV	A	A/G	0.08	21	p.His1047Arg
B5	PIK3CA	chr3:178952085	SNV	A	A/G	0.26	21	p.His1047Arg
B6	ATM	chr11:108155132	SNV	G	G/A	0.48	26	p.Ala1309Thr
	PIK3CA	chr3:178952085	SNV	A	A/G	0.28	21	p.His1047Arg
B7	–	–	–	–	–	–	–	–
B8	SMAD4	chr18:4860475	SNV	A	A/G	0.52	12	p.Ile525Val
	PIK3CA	chr3:178952085	SNV	A	A/G	0.27	21	p.His1047Arg
B9	–	–	–	–	–	–	–	–
B10	PIK3CA	chr3:178936091	SNV	G	G/A	0.19	10	p.Glu545Lys
B11	PIK3CA	chr3:178936091	SNV	G	G/A	0.16	10	p.Glu545Lys
B12	PIK3CA	chr3:178952085	SNV	A	A/G	0.26	21	p.His1047Arg
	SMARCB1	chr22:24134064	SNV	C	C/A	0.06	2	p.Thr72Lys
B13	–	–	–	–	–	–	–	–
C1	PIK3CA	chr3:178952085	SNV	A	A/T	0.16	21	p.His1047Leu
C2	AKT1	chr14:105246551	SNV	C	C/T	0.08	3	p.Glu17Lys
C3	SMARCB1	chr22:24134064	SNV	C	C/A	0.06	2	p.Thr72Lys
C4	PIK3CA	chr3:178952085	SNV	A	A/T	0.17	21	p.His1047Leu

Sample codes: A1–A4 = LGASC, B1–B13 = RSL, C1–C4 = PBD controls.
Genotype codes: A: adenine. C: cytosine. G: guanine. T: thymine. SNV: single nucleotide variant.

there is well-documented high concordance between NGS of whole-exome and for cancer hot-spot mutations, and Sanger sequencing. The latter is of use when there is poor gene coverage or there are novel variants detected by NGS [15,16].

Results

Cases and controls were all female. The age at diagnosis of LGASC ranged from 45 to 78 (mean 59) years whereas for the 13 with early RSL it was 24–49 (mean 42) years. The age of the individuals with PBD ranged from 32 to 56 (mean 47) years. The 21 samples (4 LGASC, 13 RSL and 4 PBD) were assessed with a cancer hotspot-sequencing panel, yielding between 11 and 81 variants per sample (average 21/sample). In most there were less than 30

variants, but one sample had nearly 81 variants, which were likely FFPE artefacts present at extremely low allelic frequency (61/81 variants with allele fraction < 0.05, and 62/81 C–T or A–G variants). Overall there were 448 candidate variants in 21 samples. The average depth of sequencing for variants was 988X (range 54–2000X), with 22 being below 500X. After filtering to remove intronic and synonymous coding region variants, as well as variants found in the thousand genomes project [17] at $>1\%$ (common inherited variants) there were 123 variants (average 5.8/sample). When working with DNA from FFPE tissue, there is a significant low level of artefactual mutation resulting in variants with an allele frequency of 1–10%. Unfortunately, there are also likely real variants present at low level. Hence the traditional approach is to exclude variants below 5% when looking for likely pathogenic variants, especially in tissues that have been selected to

contain likely high mutation loads. When we excluded variants that were only present at a very low frequency in each sample (<5% allele frequency), 26 SNV remained (Table 2). Of these, 13 were in *PIK3CA,* of which 11 were variants affecting codon 1047, while the remaining 2 affected codon 545. Ten of the thirteen (77%) B samples (RSL) harboured mutations in *PIK3CA* of which eight (61.5%) had A/G (His1047Arg) mutations and two (15.5%) had G/A (Glu545Lys) mutations. In contrast, the A (LGASC) and C (PBD) samples had A/T (His1047Leu) mutations in one and two individuals respectively. The variants were generally present at 6–28% allelic frequency in each sample. There were a number of variants at high allelic frequencies (eg, JAK3 p.Val722Ile) which were deemed likely to be rare inherited heterozygous variants. Other variants of note were a *PTEN* variant present at low level in sample A4 (Arg558Cys) and an *AKT* variant present at low level in sample C2 (AKT1 Glu17Lys). The hot-spot variants detected were not novel and included known tumour drivers. Hence, the chance of them being false positives was regarded to be zero. As stated above, Sanger confirmations are usually employed when a variant is novel.

Discussion

RSL is an apparently self-limited benign sclerosing lesion, categorized into early and late forms [8,9]. The phenotypic similarity of ASP in benign sclerosing lesions to the characteristic neoplastic ducts of LGASC suggests that ASP represents a form of early neoplasia [1,2]. Clonality has been demonstrated within RSL [3,4] and more recently in infiltrating epitheliosis [11], lesions that contain ASP. We sought to determine if selective microdissection of the ASP-rich nidus of early RSLs, attempting to avoid sampling background benign PBD, would reveal evidence of specific mutations and show evidence of clonality. *PIK3CA* mutations were present in 13 samples. Ten (77%) of the RSLs harboured mutations in *PIK3CA* of which eight (61.5%) had H1047R mutations and two (15.5%) had E545K mutations, while one LGASC and two PBD (usual ductal hyperplasia and apocrine adenosis) samples had H1047L mutations. One LGASC showed a *PTEN* (Arg558Cys) mutation. Our results support the notion that ASP in the setting of RSL is a clonal proliferation, but which appears to involute more often, creating a paucicellular elastotic 'late' RSL [1]. However, because ASP infrequently formed a pure mass, there was a variable

content of normal breast tissue and PBD, which is difficult to avoid because the ASP arises within PBD. Our results suggest that the presence of *PIK3CA* mutations in RSL may not be only attributable to the common association of usual ductal hyperplasia, which has recently been suggested to be an early clonal neoplastic proliferation [23], and that ASP may be a clonal lesion in its own right. Its relationship to LGASC remains unclear in view of the small sample size and the fact that our RSL and LGASC samples were unmatched because of the nature of the lesions in the cases selected.

The PI3Ka protein is a lipid kinase involved in cellular processes vital for cancer progression, such as cell growth, proliferation, motility, survival and metabolism. Dysregulation of PI3Ka signaling is therefore one of the most frequent oncogenic events. Gain of function somatic mutations within the gene encoding PIK3CA on chromosome 3q26.3 are frequently observed in a variety of human tumors, including in up to one third of breast carcinomas. One of the commonest and most frequently studied mutations is an amino acid substitution at position 1047 from a histidine (H) to an arginine (R) H1047R (His1047Arg) [18], which results in a gain of protein function. In mouse models, over expression of PIK3-CA^{H1047R} has been shown to induce mammary cells to de-differentiate into a multipotent stem cell state and to then differentiate into either luminal, basal or dual cell lineages. This induced cell plasticity results in multi-lineage tumours [19,20]. Other common gain of function mutations in PIK3CA are found at codons 542 and 545.

Studies of early neoplasia in breast have shown that single nucleotide variations (mutations) are common, that aneuploidy may also be present and that these changes may be detected in a patient's early neoplasia and associated cancer indicating a common ancestral clone. However, although activating *PIK3CA* mutations are commonly detected in early neoplasia and are common in breast cancer, they do not necessarily correlate with progression to invasive carcinoma or play a prominent role in promoting the progression to cancer at an early stage [21]. So, while *PIK3CA* mutations may provide a growth advantage in early neoplasia they may not be present in any ensuing carcinoma, which might explain why they were more common in RSL than in LGASC in this series. *PIK3CA* mutations are present in up to 50% of benign proliferative breast lesions (usual ductal hyperplasia and columnar cell change) [7], in 22% and 42% of intraduct papillomata with and without hyperplasia [22], and in nearly 64% of macrodissected radial scars [4]. It has recently been

proposed that due to evidence of i) activating mutations in the PI3K/AKT/MTOR pathway, ii) loss of heterozygosity and iii) its mass forming proliferative nature, usual ductal hyperplasia is best regarded as an early neoplastic intraductal proliferation [23]. Indeed, *PIK3CA* mutations are common in infiltrating epitheliosis, a lesion which contains mass forming usual ductal hyperplasia [11]. Papillomata without cytological atypia have an up to two fold increased risk of subsequent breast cancer, similar to that seen with usual ductal hyperplasia/proliferative breast disease. RSL have a similar risk of subsequent carcinoma but this risk may be attributable to coexistent PBD including usual ductal hyperplasia because RSLs arise in that setting [12]. The frequency of *PIK3CA* mutations in macrodissected RSL could therefore potentially reflect a high content of PBD. Infiltrating epitheliosisis also a lesion characterized by a high content of usual ductal hyperplasia (epitheliosis) along with scleroelastic stromal changes containing tubules, strands and cords of spindle cells with variable squamous differentiation (adenosquamous proliferation/ASP), which resembles a miniature LGASC [10]. In a recent study of infiltrating epitheliosis, all eight cases showed mutations in components of the PI3K pathway with seven cases harbouring *PIK3CA* mutations representing six hotspot H1047R mutations and one E542K mutation, and the remaining case harbouring a *PIK3R1* (L380del) small deletion. In one case the same *PIK3CA* (H1047R) and *SF3B1* (K700E) mutations were identified in infiltrating epitheliosis, ductal carcinoma *in situ* (DCIS) and LGASC, suggesting that the lesions were clonally related and that the infiltrating epitheliosis and DCIS may have represented a 'substrate for the development of the LGASC'. Unfortunately, none of our LGASC cases had an adjacent RSL to enable such comparison. The authors also suggested that infiltrating epitheliosis may form a spectrum with RSL [11].

In summary, our data support the notion that RSL may represent a form of early neoplasia. The detection of recurrent *PIK3CA* mutations in these cases implies clonality. The mutation drives cellular proliferation and provides a survival advantage. The factors that determine whether ASP involutes or persists (in the setting of a RSL), and possibly even progresses to other forms of neoplasia, remain unknown and would indeed be an interesting new line of research.

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Author contributions

MW and TO conceived the study. MW selected the cases for assessment. TO performed the microdissection, macrodissection and DNA extraction. RA performed the genetic testing and analyses. MW wrote the first draft of the manuscript, which was subsequently expanded and edited by all authors.

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