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MED12, TERT promoter and *RBM15* mutations in primary and recurrent phyllodes tumours

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Background: *MED12* and *TERT* promoter mutations have been shown to be the most common somatic mutations in phyllodes tumours (PTs). The aims of this study were to determine the frequency of these mutations in recurrent PTs, assess whether *TERT* promoter mutations could be helpful in distinguishing fibroadenomas (FAs) from PTs and identify novel mutations that may be driving malignant progression.

Methods: *MED12* and the *TERT* promoter were Sanger sequenced in 75 primary PTs, 21 recurrences, 19 single FAs and 2 cases of multiple FAs with benign PTs. Whole-exome sequencing was performed on one borderline PT.

Results: Recurrent PTs and multiple FAs showed temporal discordance in *MED12* but not *TERT*. Recurrent samples did acquire *TERT* mutations, with recurrent benign PTs more likely to have mutations in both genes. *TERT* mutations were not helpful in differentiating between benign PTs and FAs in cases of multiple FAs/PTs. Exome sequencing revealed a nonsense mutation in *RBM15* and Sanger sequencing revealed another three *RBM15* mutations in malignant/borderline PTs.

Conclusions: This study has shown that *MED12* mutations can be heterogeneous in both synchronous and recurrent PTs unlike *TERT* mutations. We have also shown that *RBM15* mutations may be important in the pathogenesis of borderline/malignant PTs.

Phyllodes tumours (PTs) are rare fibroepithelial neoplasms of the breast composed of both stromal and epithelial elements similar to fibroadenomas (FAs) but with a more cellular stromal element. Histologically, PTs are classified as benign, borderline or malignant on the basis of stromal cellularity, nuclear atypia, mitotic activity, stromal overgrowth and type of border (infiltrating or pushing) (Lakhani *et al*, 2012). Borderline PTs have some but not all of the features of malignant PTs. Unlike FAs, PTs can recur locally and metastasise as sarcoma. Stromal atypia, mitoses, overgrowth and surgical margin status have been shown to be independent predictors of local recurrence (Tan *et al*, 2012). Most recurrent benign and borderline tumours are histologically similar to the primary neoplasms but can be more cellular, and malignant transformation has been described (Jones *et al*, 2008).

Recent sequencing studies have shown that *MED12* exon 2 mutations occur in approximately 70% of PTs and FAs (Cani *et al*, 2015; Nagasawa *et al*, 2015; Yoshida *et al*, 2015a; Lien *et al*, 2016a). Some studies suggest that the frequency is similar across both FAs and all grades of PTs (Cani *et al*, 2015; Yoshida *et al*, 2015a; Nagasawa *et al*, 2015; Lien *et al*, 2016a) and others that they are less frequent in malignant PTs (Pfarr *et al*, 2015; Piscuoglio *et al*, 2015) and FAs (Ng *et al*, 2015). This variation may be due to the

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type of FAs included in the series as two studies have shown *MED12* mutations are more common in intracanalicular than pericanalicular FAs (Yoshida *et al*, 2015a, b; Mishima *et al*, 2015). In both PTs and FAs, mutations are confined to the stromal component (Yoshida *et al*, 2015a, b; Mishima *et al*, 2015). PTs with *MED12* mutations tend to have lower recurrence rates than PTs without *MED12* mutations (Ng *et al*, 2015; Yoon *et al*, 2016). *MED12* exon 2 mutation can also help distinguish PTs from other spindle neoplasms of the breast, such as sarcomas (Lien *et al*, 2016b).

The finding in some studies that *MED12* mutations are less frequent in malignant PTs suggests that these tumours may be driven by other genetic events. Exome and targeted sequencing studies of malignant PTs have shown that they harbour recurrent mutations in the *TERT* promoter, *TP53*, *RB1*, *EGFR*, *PIK3CA*, *FGFR1*, *SETD2* and *KMT2D* (Tan *et al*, 2015; Gatalica *et al*, 2016; Liu *et al*, 2016), with *TERT* promoter mutations being the most frequent, occurring in ~70% of malignant/borderline PTs. *TERT* promoter mutations are also found in benign PTs (~50%) but are not as frequent as in malignant/borderline PTs (Yoshida *et al*, 2015b; Piscuoglio *et al*, 2016). They are rare in FAs (0–7%), suggesting that these mutations drive the progression of PTs. It has also been suggested that *TERT* mutations may be useful in distinguishing between benign PTs and cellular FAs (Tan *et al*, 2015), particularly in rare cases of multiple recurrent FAs where benign PTs have occasionally been described (Courtillot *et al*, 2010). *TERT* mutations can co-exist with *MED12* mutations and similar to *MED12* mutations are restricted to the stromal component (Yoshida *et al*, 2015b; Piscuoglio *et al*, 2016).

The aims of this study were to:

1. assess *MED12* and *TERT* promoter mutations in a series of recurrent PTs;
2. assess whether TERT mutations could be helpful in distinguishing FAs from PTs in patients with synchronous/metachronous multiple FAs and PTs; and
3. identify novel mutations in other genes that may be driving malignant progression of PTs.

MATERIALS AND METHODS

Sample collection. Formalin-fixed, paraffin-embedded tissue (FFPE) was obtained from 75 primary PTs and any ipsilateral recurrences together with two cases of multiple FAs that had developed benign PTs from 15 centres across the United Kingdom with ethical approval (MREC No. 03/12/083) and informed consent. Eleven cases had paired germline DNA extracted from blood samples. H&E-stained slides of each case were reviewed by a single histopathologist (AH) to confirm the diagnosis and DNA was extracted as previously described (Jones *et al*, 2008). Nineteen FAs (FFPE) and one fresh-frozen borderline PT were provided by the KHP Cancer Biobank (NHS REC ref. 12-EE-0493).

Sanger sequencing. The promoter region of *TERT* was amplified by PCR as previously described (Yoshida *et al*, 2015b) using the following primers: 5′-CAGCGCTGCCTGAAACTC-3′ and 5′-GTCCTGCCCCTTCACCTT-3′. *MED12* primers, targeting exon 2, were designed with the online tool Primer3: 5′-TGTTCTACACGGAACCCTCCTC-3′ and 5′-CTGGGCAAATGCCAATGAGAT-3′. Primers for the entire *RBM15* gene followed the same design and are listed in Supplementary Table 1. Sanger sequencing was performed in a 3730xl DNA Analyser (ThermoFisher, Waltham, MA, USA) according to the manufacturer's protocol. The analysis of the electropherograms was performed in the openly available 4peaks software.

Exome sequencing. A library was prepared from tumour and paired constitutional DNA using the SureSelect Human All Exon 50 Mb kit (Agilent, Santa Clara, CA, USA) and sequenced on Illumina HiSeq 2000 (San Diego, CA, USA) to a mean depth of $>100\times$. Subsequent analysis was performed using our in-house pipeline; in brief, sequencing reads were aligned to the reference human genome hg19 using NovoAlign (http://www.novocraft.com/products/novoalign/), Samtools (http://www.samtools.sourceforge.net/) was used to create a pileup file and VarScan2 (http://www.varscan.sourceforge.net/) was used to call somatic mutations and indels that were annotated using ANNOVAR (http://annovar.openbioinformatics.org/en/latest/) and cross referenced with dbSNP and 1000 Genomes. Somatic mutations were called if there was a minimum of $30\times$ coverage and the mutation was present in at least 10% of reads.

RESULTS

Seventy-five primary PTs (27 malignant, 22 borderline, 26 benign) were studied, of which 21 had recurred at least once (Table 1, Supplementary Table 2). Of the 21 recurrent cases, nine were benign (one of which recurred as a borderline PT), five were borderline (three of which recurred as malignant PTs) and seven were malignant. Nineteen FAs were also analysed.

Frequency of MED12 mutations. *MED12* mutations occurred in 22, 27, 54 and 21% of malignant, borderline and benign PTs and FAs, respectively (Table 2). *MED12* mutations were more common in benign PTs compared with malignant/borderline ($P=0.02$, Fisher's Exact Test).

MED12 mutations in recurrent PTs. Although *MED12* mutations appeared less common in PTs that recurred (19%) compared with those that did not (41%); this difference was not statistically significant ($P=0.1$, Fisher's Exact Test; Table 2).

In the four primary tumours with *MED12* mutations that did recur, two had the same *MED12* mutation in the paired recurrent tumour (one had two *MED12* mutations and both were seen in the recurrent sample), but in the other two cases (one malignant, one benign), there was no evidence of the original *MED12* mutation in the recurrence.

There was also evidence of recurrent tumours acquiring *MED12* mutations. Four cases (three benign, one borderline) with no evidence of a *MED12* mutation in the primary PT did have *MED12* mutations in the recurrent tumours (Table 1). Similarly, in three cases that developed a second recurrence, none had a *MED12* mutation in the primary, two had a *MED12* mutation in the first recurrence, and of these, one had the same *MED12* mutation in the second recurrence and the other a different *MED12* mutation.

Multiple MED12 mutations in the same lesion. We also found evidence of multiple *MED12* mutations in the same lesions (Supplementary Table 3). Five primary PTs (one of which recurred and both mutations were found in the recurrence) and another two recurrences had multiple (2–4) *MED12* mutations. Of these two recurrences, one had no evidence of *MED12* mutations in the primary and the other had a single *MED12* mutations in the primary lesion and then acquired another three mutations in the recurrence. All cases with multiple *MED12* mutations were benign.

Frequency of TERT promoter mutations. *TERT* promoter mutations occurred in 48, 55, 31 and 0% of malignant, borderline, and benign PTs and FAs, respectively (Table 2). There was no significant difference in the frequency of *TERT* mutations between the different subtypes of PT, but as expected a clear difference between PTs and FAs ($P=0.0001$, Fisher's Exact Test).

Table 1. Detailed table of recurrences and their *MED12* and *TERT* promoter mutations

Sample ID	Gene	Primary subtype	Primary mutation	Recurrence 1 subtype	Recurrence 1 mutation	Recurrence 2 subtype	Recurrence 2 mutation
1	MED12	M	No	M	No		
	TERT	M	c.-124C>T	M	c.-124C>T		
2	MED12	M	No	M	No		
	TERT	M	No	M	No		
3	MED12	M	No	M	No	M	No
	TERT	M	No	M	c.-124C>T	M	c.-124C>T
4	MED12	M	c.131G>A	M	No		
	TERT	M	No	M	No		
5	MED12	M	No	M	No		
	TERT	M	No	M	No		
6	MED12	M	No	M	No		
	TERT	M	No	M	No		
7	MED12	M	No	M	No		
	TERT	M	No	M	No		
8	MED12	Bo	No	M	No		
	TERT	Bo	c.-124C>T	M	c.-124C>T		
9	MED12	Bo	No	M	c.100-13T>C		
	TERT	Bo	No	M	No		
10	MED12	Bo	No	M	No		
	TERT	Bo	No	M	c.-124C>T		
11	MED12	Bo	No	Bo	No		
	TERT	Bo	No	Bo	No		
12	MED12	Bo	No	Bo	No		
	TERT	Bo	No	Bo	No		
13	MED12	B	No	B	c.138_164del27	B	Fail
	TERT	B	c.-124C>T	B	c.-124C>T	B	Fail
14	MED12	B	No	B	No		
	TERT	B	No	B	No		
15	MED12	B	c.123_152del30 and c.119A>T	B	c.123_152del30 and c.119A>T		
	TERT	B	No	B	c.-124C>T		
16	MED12	B	No	Bo	No		
	TERT	B	No	Bo	No		
17	MED12	B	c.131G>C	B	c.128A>C, 129A>G, c.131G>C, c.133_152del30		
	TERT	B	c.-124C>T	B	c.-124C>T		
18	MED12	B	No	B	c.120_146del27	B	c.113_151del39
	TERT	B	c.-124C>T	B	c.-124C>T	B	c.-124C>T
19	MED12	B	No	B	c.122_148_del27	B	c.122_148_del27
	TERT	B	c.-124C>T	B	c.-124C>T	B	c.-124C>T
20	MED12	B	c.124_156del33	B	No		
	TERT	B	No	B	No		
21	MED12	B	No	B	c.148G>A and c.133_147del15		
	TERT	B	No	B	c.-124C>T		

Abbreviations: B = benign; Bo = borderline; M = malignant.

TERT promoter mutations in recurrent PTs. Similar to *MED12*, there was no evidence that *TERT* promoter mutations were more or less common in cases that recurred (28% recurrent *vs* 50% non-recurrent). However unlike *MED12* all seven of the primary PTs with a *TERT* promoter mutation that recurred showed evidence of the mutation in the paired recurrence. Of those, three (benign) had a second recurrence, which also had evidence of the mutation.

There was also evidence of recurrent tumours acquiring *TERT* promoter mutations with four (one malignant, one borderline and two benign) acquiring a *TERT* promoter mutation in the first recurrence and again this was also found in subsequent recurrences (Table 1).

Table 2. Frequency of MED12 and TERT promoter mutations in recurrent and non-recurrent phyllodes tumours and fibroadenomas

	Malignant PTs			Borderline PTs			Benign PTs			Fibroadenomas
Mutation	All cases ($n=27$)	Cases that did not recur ($n=20$)	Cases that did recur ($n=7$)	All ($n=22$)	Cases that did not recur ($n=17$)	Cases that did recur ($n=5$)	All ($n=26$)	Cases that did not recur ($n=17$)	Cases that did recur ($n=9$)	($n=19$)
MED12 exon 2 mutations	6 (22%)	5	1	6 (27%)	6	0	14 (54%)	11	3	4 (21%)
TERT promoter mutations	13 (48%)	12	1	12 (55%)	11	1	8 (31%)	4	4	0

Abbreviation: PT = phyllodes tumour.

Table 3. Somatic mutations identified by whole exome sequencing of a single borderline phyllodes tumour

Chrom	Gene	Site of mutation	Annotation	% of reads with mutation
chr1	*RBM15*	Exonic	*RBM15*:NM_001201545:exon1:c.A583T:p.K195X: stopgain	64.20%
chr1	VASH2	Splicing	VASH2:NM_024749:exon4:c.366-1G>C	61.54%
chr11	ROBO3	Splicing	ROBO3:NM_022370:exon12:c.1785-4G>T	44.44%
chr11	TRIM49C	Exonic	TRIM49C:NM_001195234:exon3:c.T185C:p.I62T	41.63%
chr3	CAND2	Exonic	CAND2:NM_001162499:exon10:c.A2030G:p.D677G	40.23%
chr15	PLA2G4F	Exonic	PLA2G4F:NM_213600:exon1:c.27delG: frameshift deletion	37.88%
chr2	NEB	Exonic	NEB:NM_001164507:exon37:c.G4182T:p.K1394N	35.97%
chr10	ENKUR	Exonic	ENKUR:NM_145010:exon2:c.T198A:p.H66Q,	35.26%
chr1	YIPF1	Exonic	YIPF1:NM_018982:exon4:c.T89C:p.I30T,	34.45%
chr5	PCDHA11	Exonic	PCDHA11:NM_031861:exon1:c.C1446A:p.D482E	32.03%
chrX	FAM58A	Exonic	Unknown	21.86%
chr12	LIMA1	Splicing	LIMA1:NM_016357:exon9:c.973-3G>-	20.83%
chr1	CACNA1S	Splicing	CACNA1S:NM_000069:exon5:c.399-6T>C	17.43%
chr15	ZNF280D	Splicing	ZNF280D:NM_017661:exon11:c.781-8C>-	16.28%
chr14	RALGAPA1	Splicing	RALGAPA1:NM_014990:exon11:c.1012-8G>-,	15.58%
chr19	SSC5D	Exonic	SSC5D:NM_001144950:exon14:c.T4361C:p.L1454P	15.38%
chr11	BRSK2	Exonic	BRSK2:NM_001256630:exon1:c.A17C:p.H6P,	15.15%
chr20	BCAS4	Exonic	BCAS4:NM_198799:exon2:c.T191G:p.V64G	15%

Analysis of two cases of multiple FAs and PTs. In order to assess whether *TERT* promoter mutations could be useful in distinguishing between benign PTs and FAs in patients with multiple FAs, we analysed cases from two young women who developed bilateral multiple recurrent FAs and subsequently developed benign PTs (Supplementary Figure 1). The first, aged 22 years, developed multiple bilateral FAs over a period of 3 years and was diagnosed with benign PTs on the fourth recurrence. Nine lesions (7 FAs and 2 PTs) were examined from the 4-year period and none showed any evidence of *TERT* mutations. At the fourth recurrence, *MED12* mutations were identified in one cellular FA (c.130G>A) and one benign PT (c.130G>A and c.136_150del15). The second case occurred in a 20-year-old with bilateral multiple FAs that started to increase in size and so were excised and found to be a cellular FA and two benign PTs. Sequencing of each lesion showed no *TERT* mutations and a different *MED12* mutation in each of the lesions (c.107T>G, c.131G>A and c.100-8T>A).

Novel drivers of malignant and borderline PTs. In an attempt to identify other drivers of malignant and borderline PTs, we performed whole-exome sequencing of a single borderline case who had DNA available from fresh-frozen tumour and blood. Eighteen somatic mutations were identified but no *MED12* mutation (Table 3). The mutations were ranked according to the variant allele frequency within the tumour, on the assumption that those with a frequency of ~50% were less likely to be subclonal and more likely to be driver mutations. Five mutations with a variant frequency of 40–64% were identified and verified by Sanger sequencing in DNA extracted from FFPE material from the same tumour. Only one of these genes, *RBM15*, had previously been identified as harbouring a mutation in a PT (Tan *et al*, 2015; borderline PT with a frameshift *RBM15* mutation, c.598_601delGTAA). We therefore chose to Sanger sequence this gene in another 27 malignant, 17 borderline (including one recurrent sample) and 16 benign (including five recurrent samples) and found another three different mutations, two in malignant PTs and one in a borderline PT (Table 4, Figure 1, Supplementary Figure 2).

DISCUSSION

MED12 and *TERT* promoter mutations have previously been shown to be the most common mutations in PTs. This study, similar to others (Table 5A), has shown that *MED12* mutations are more common in benign PTs than malignant and borderline PTs

Table 4. *RBM15* mutations

Sample ID	Histopath subtype	Mutation DNA	Mutation AA	DANN[a] score	CADD[b] score
22	Borderline	c.583A>T	p.195K>[a]	0.999	26.3
23	Malignant	c.1924C>T	p.642R>[a]	0.995	36
24	Borderline	c.715delG	p.239V>fs[a]1	NA (del)	NA (del)
25	Malignant	c.2344C>T	p.782P>S	0.886	11.13

Abbreviations: NA = not applicable.
[a]DANN (https://doi.org/10.1093/bioinformatics/btu703) uses a 0–1, scale. Score ⩾0.96 identifies 92% of the true positive pathogenic variations, with 18.1% false positive benign variations.
[b]CADD (cadd.gs.washington.edu/). A scaled CADD score of 20 means that a variant is among the top 1% of deleterious variants in the human genome. Scaled CADD score of 30 means that the variant is in the top 0.1%.

and *TERT* promoter mutations are rare in FAs. By analysing paired primaries and recurrences, we have been able to assess whether these mutations are also present in paired recurrences or whether there is evidence of temporal heterogeneity. We have demonstrated that *MED12* is frequently heterogeneous between lesions from the same patient, in contrast to *TERT* promoter mutation that are consistently found in paired recurrences, including those with multiple recurrences. Lae *et al* (2016) also demonstrated temporal heterogeneity within *MED12* (they did not assess *TERT*). In their study, the heterogeneous recurrences had different *MED12* mutations from the primary case, this occurred in one of our recurrent cases (a second benign recurrence) but the remainder (one benign, one malignant) lacked a *MED12* mutation in the paired recurrent sample, suggesting that they were either new primaries or had arisen from a subclone within the primary PT that did not contain the *MED12* mutation. Unlike Lae *et al* (2016), we also have evidence of four cases of wild-type *MED12* primaries acquiring *MED12* mutations in the second event (three benign, one borderline) and of benign PTs harbouring multiple (2–4) *MED12* mutations in the same lesion. As Sanger sequencing will only detect clonal mutations, we cannot exclude the possibility that it was a subclonal mutation in the primary tumour which we cannot detect. These findings suggest that *MED12* are not only just early events in fibroepithelial tumours but also provide some growth advantage in established benign PTs.

A number of recurrences also acquired *TERT* promoter mutations, (4 cases – 2 benign and 2 malignant). In the series of PTs described by Yoshida *et al* 2015b; *TERT* and *MED12* mutations were frequently found together (Table 5B). We did not see this in the primary cases, 41% of PTs with *TERT* promoter mutations harboured *MED12* mutations, which is similar to (Piscuoglio *et al*, 2016) (52%) and (Liu *et al*, 2016) (50%). However, in the recurrent samples *TERT* and *MED12* mutations frequently co-occured ($P = 0.05$, Fisher's Exact test), particularly in benign cases where acquisition of *MED12* and *TERT* promoter mutations resulted in six out of nine benign recurrences having both a *TERT* and *MED12* mutation compared with one out of nine of their paired primary samples. In contrast, none of the malignant/borderline recurrences had mutations in both genes. This suggests that although they can co-exist *TERT* promoter mutations are not dependent on *MED12* mutations. As postulated by (Piscuoglio *et al*, 2016) *TERT* promoter mutations may allow the stroma of PTs to undergo more cycles of cell division and thus increase the chance of them acquiring a driver mutation.

The lack of *TERT* promoter mutations in FAs has led some authors to suggest that this mutation may be useful for distinguishing between FAs and benign PTs in rare cases of multiple FAs and benign PTs. In two such cases, we found no evidence of *TERT* promoter mutations in either the FAs or benign PTs. The analysis of these multiple tumour cases once again demonstrated heterogeneity of *MED12*. In the first case, no *MED12* mutation was present in the initial FAs and only appeared at the time of the fourth recurrence when a cellular FA and benign PT were diagnosed on histology – the presence of the same mutation suggested a common clonal origin, although the PT had also acquired a second *MED12* mutation. In contrast, in the second case (a cellular FA and two benign PTs), all three lesions had different *MED12* mutations, suggesting that they arose independently (Supplementary Figure 1).

The mechanism through which *MED12* mutations confer a growth advantage to FAs and benign PTs is not clear. *MED12* mutations are also frequent in uterine leiomyomas but less so in uterine leiomyosarcomas (Ravegnini *et al*, 2013). A possible explanation for this is that *MED12* mutations drive benign proliferation of smooth muscle and stroma in the uterus and breast, respectively, resulting in leiomyomas and FAs/benign PTs. In rare cases, these benign lesions progress to leiomyosarcoma and malignant PTs, but the majority of these malignant tumours arise *de novo* and therefore do not have *MED12* mutations.

The exome sequencing of the single borderline PT with fresh-frozen tissue did not reveal any *MED12* mutation and no *TERT* promoter mutation was found on Sanger Sequencing, but it did identify mutations in 18 genes, none of which had been previously identified as driver mutations in any type of cancer. The most frequent variant in this PT was a nonsense mutation in *RBM15*, an RNA-binding protein on 1p13.3 involved in regulating splicing of GATA1 and RUNX transcription factors in megakaryocyte differentiation and translocated in infant acute megakaryocytic leukaemia (Tran *et al*, 2016). Interestingly, previously published array CGH data of this tumour (Jones *et al*, 2008) showed loss in this region (1:105666811-117575143, GRCh37/hg19), Supplementary Figure 3.

A frameshift mutation (c.598_601delGTAA) in *RBM15* was also detected by exome sequencing by (Tan *et al*, 2015) in a borderline PT but was not remarked upon as a possible driver gene. All other PT sequencing studies have used targeted sequencing panels that have not included *RBM15*. Sanger sequencing of our tumour series identified a further three *RBM15* mutations in malignant/borderline PTs, two of which were nonsense or frameshift changes and one of which was a missense mutation, not predicted to be deleterious but located at the start of a highly conserved C-terminal SPOC (Spen paralog and ortholog C-terminal) domain. One hundred and sixty-six mutations in *RBM15* have been catalogued in COSMIC (http://cancer.sanger.ac.uk/cosmic), of which only 10 are nonsense and occur in a variety of solid tumours, including bladder, head and neck, colorectal, stomach, prostate and pancreas. As well as being involved in the development of megakaryocytic leukaemia, *RBM15* is an important factor in X chromosome silencing (Moindrot *et al*, 2015) and has been shown to be expressed in mammary tissue (https://www.gtexportal.org/home/). It is therefore not unreasonable to suggest that a truncated or absent RBM15 protein may confer a growth advantage in PTs.

Of the five *RBM15* mutations that have now been described in PTs (four in this study, one by Tan *et al*, 2015), three occurred in malignant/borderline PTs that did not harbour *MED12* or *TERT* promoter mutations. Previous literature also suggests that *PIK3CA* mutations are more common in PTs that do not harbour *MED12* mutations (Tan *et al*, 2015; Piscuoglio *et al*, 2016). In

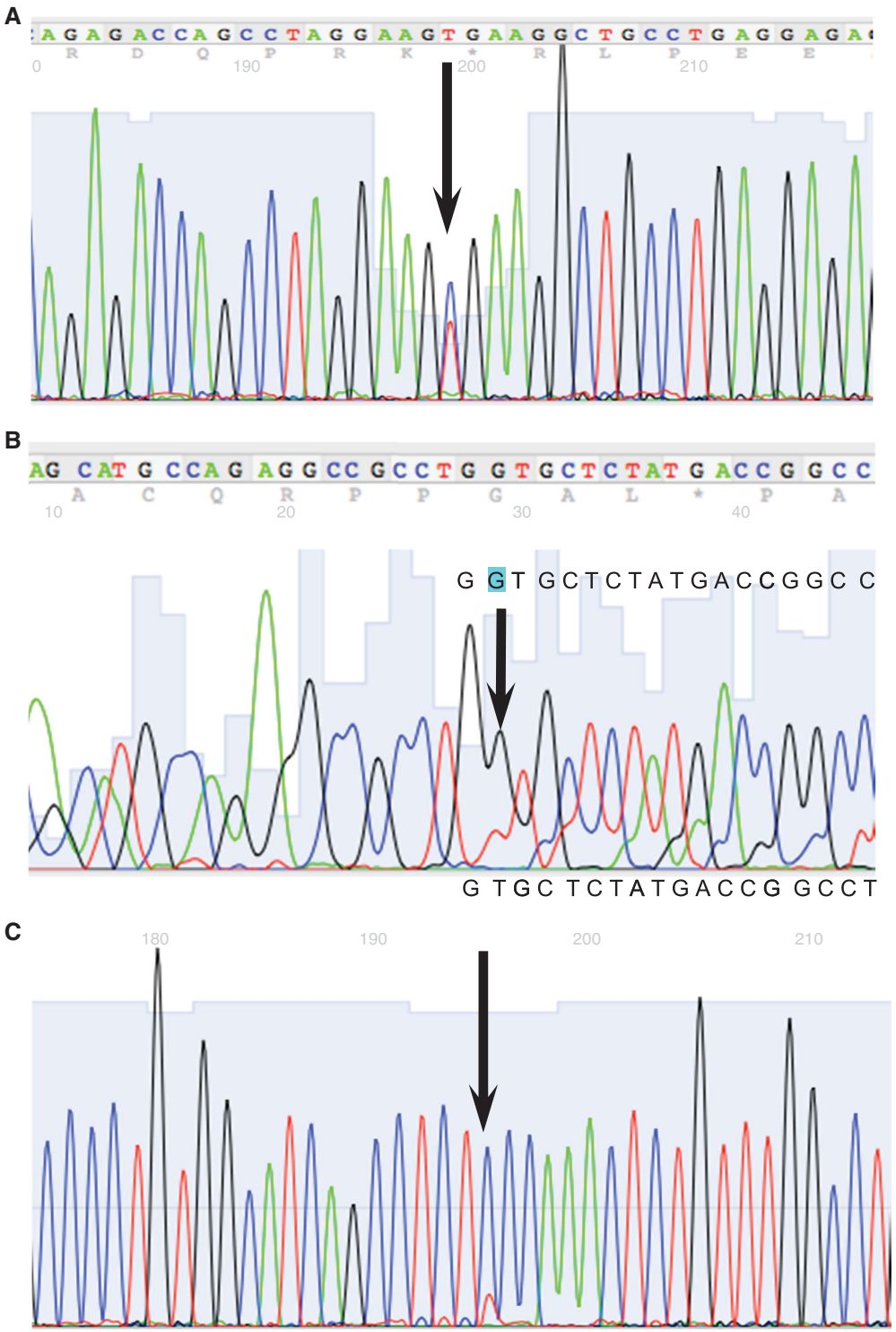

Figure 1. *RBM15* mutations identified by Sanger sequencing. (**A**) Sample 23 (c.1924 C>T, p.R642*). (**B**) Sample 24 (c.715delG, p.V239fs*1) – correct sequence shown above, shifted sequence shown below (reverse sequence shown in Supplementary Figure 2). (**C**) Sample 25 (c.2344C>T, p.P782S).

contrast, *RARA* mutations were more frequent in samples with *MED12* and *TERT* promoter mutations (Tan *et al*, 2015; Piscuoglio *et al*, 2016) and thus may provide a selective advantage when *TERT*/*MED12* are mutated.

In conclusion, we have shown that, although *MED12* mutations are common in both benign PTs and FAs, suggesting that they are sometimes early events in fibroepithelial lesions of the breast, they can be discordant in recurrent PTs, particularly in benign cases where they can be lost or acquired with some cases carrying multiple mutations in *MED12*. There was less evidence of temporal heterogeneity in *TERT* promoter mutations, but recurrent samples did acquire *TERT* promoter mutations, supporting previous data from our laboratory that recurrent samples often acquired new genetic changes (Jones *et al*, 2008).

Through exome sequencing of a single malignant PT, we have shown that *RBM15* may be a novel driver mutation in malignant/

Table 5A. Summary of previous published data on *MED12* mutations in FAs and PTs

Publication	Number of PTs in the study	Frequency in FAs	Frequency in benign PTs	Frequency in Bo PTs	Frequency in malignant PTs	Frequency in PTs that recurred *vs* none recurrent cases	Evidence of temporal heterogeneity in recurrent PTs	Evidence of acquisition of mutations in recurrent PTs	Evidence of heterogeneity in synchronous PTs/FAs
Lae *et al* (2016)	83 primary 14 recurrences	70%	58.3%	63.3%	27.6%	50% *vs* 49%	Yes 3/6 recurrences had different *MED12* mutations compared with primary tumour	No	NA
Cani *et al* (2015)	15	NA	80%	80%	40%	NA	NA	NA	NA
Lien *et al* (2016a)	49	47.1%	72.7%	70.6%	70%	NA	NA	NA	NA
Liu *et al* (2016)	30	NA	NA	NA	30%	NA	NA	NA	NA
Mishima *et al* (2015)	24 primary 2 recurrences	47%	80%	67%	0%	50% *vs* 82%	No	No	Yes: 6 cases
Nagasawa *et al* (2015)	11	67%	NA	NA	NA	NA	NA	NA	NA
Ng *et al* (2015)	112 primary 10 recurrences	59%	65.1%	65.6%	42.8%	Higher recurrence likelihood in those without *MED12* mutations	NA	NA	NA
Pfarr *et al* (2015)	16	62%	73%	NA	20%	NA	NA	NA	NA
Piscuoglio *et al* (2015)	47	65%	88%	78%	8%	NA	NA	NA	Yes: 4 cases
Piscuoglio *et al* (2016)	76	NA	80%	64%	23%	NA	NA	NA	NA
Tan *et al* (2015)	79	86%	82%	63%	60%	NA	NA	NA	No: 1 case
Yoon *et al* (2016)	176 number of recurrences not stated		71.4%	51%	26.9%	*MED12* mutations associated with a non-significant improvement in DFS	NA	NA	NA
Yoshida M *et al* (2015)	46	62%	83%	80%	77%	NA	NA	NA	NA
Current study	75 primary 21 recurrences	21%	54%	27%	22%	19 vs 41%	Yes	Yes	Yes

Abbreviations: DFS = disease-free survival; FA = fibroadenoma; NA = not assessed; PT = phyllodes tumour.

Table 5B. Summary of previous published data on *TERT* promoter mutations in FAs and PTs

Publication	Number of PTs in the study	Frequency in FAs	Frequency in benign PTs	Frequency in Bo PTs	Frequency in malignant PTs	Frequency in PTs that recurred *vs* none recurrent cases	Evidence of acquisition of mutations in recurrent PTs	Evidence of heterogeneity in synchronous PTs/FAs
Liu *et al* (2016)	30	NA	0%	33%	60%	NA	NA	NA
Piscuoglio *et al* (2016)	76	NA	18%	57%	68%	NA	NA	NA
Yoshida *et al* (2015a,b)	46	7%	50%	87%	62%	NA	NA	NA
Current study	75 primary 21 recurrences	0%	31%	55%	48%	28 vs 50%	Yes	No

Abbreviations: FA = fibroadenoma; NA = not assessed; PT = phyllodes tumour.

borderline PTs at a frequency of 7%, but this requires further validation in additional sample sets. In order to identify other drivers of malignant PTs, further analysis of these unusual tumours would be better carried out through exome or whole-genome sequencing rather than targeted sequencing in order to detect mutations in genes not currently known to be drivers of solid cancers.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Cani AK, Hovelson DH, McDaniel AS, Sadis S, Haller MJ, Yadati V, Amin AM, Bratley J, Bandla S, Williams PD, Rhodes K, Liu CJ, Quist MJ, Rhodes DR, Grasso CS, Kleer CG, Tomlins SA (2015) Next-gen sequencing exposes frequent *MED12* mutations and actionable therapeutic targets in phyllodes tumors. *Mol Cancer Res* **13**(4): 613–619.

Courtillot C, Chakhtoura Z, Bogorad R, Genestie C, Bernichtein S, Badachi Y, Janaud G, Akakpo JP, Bachelot A, Kuttenn F, Goffin V, Touraine P. Benign Breast Diseases Study Group (2010) Characterization of two constitutively active prolactin receptor variants in a cohort of 95 women with multiple breast fibroadenomas. *J Clin Endocrinol Metab* **95**(1): 271–279.

Gatalica Z, Vranic S, Ghazalpour A, Xiu J, Ocal IT, McGill J, Bender RP, Discianno E, Schlum A, Sanati S, Palazzo J, Reddy S, Pockaj B (2016) Multiplatform molecular profiling identifies potentially targetable biomarkers in malignant phyllodes tumors of the breast. *Oncotarget* **7**(2): 1707–1716.

Jones AM, Mitter R, Springall R, Graham T, Winter E, Gillett C, Hanby AM, Tomlinson IP, Sawyer EJ. Phyllodes Tumour Consortium (2008) A comprehensive genetic profile of phyllodes tumours of the breast detects important mutations, intra-tumoral genetic heterogeneity and new genetic changes on recurrence. *J Pathol* **214**(5): 533–544.

Laé M, Gardrat S, Rondeau S, Richardot C, Caly M, Chemlali W, Vacher S, Couturier J, Mariani O, Terrier P, Bièche I (2016) MED12 mutations in breast phyllodes tumors: evidence of temporal tumoral heterogeneity and identification of associated critical signaling pathways. *Oncotarget* **7**(51): 84428–84438.

Lakhani SR, Ellis IO, Schnitt SJ, Tan PH, van de Vijver MJ (2012) *World Health Organization Classification of Tumours of the Breast*. IARC: Lyon, France.

Lien HC, Huang CS, Yang YW, Jeng YM (2016a) Mutational analysis of *MED12* exon 2 in a spectrum of fibroepithelial tumours of the breast: implications for pathogenesis and histogenesis. *Histopathology* **68**(3): 433–441.

Lien HC, Huang CS, Yang YW, Jeng YM (2016b) *MED12* exon 2 mutation as a highly sensitive and specific marker in distinguishing phyllodes tumours from other spindle neoplasms of the breast. *APMIS* **124**(5): 356–364.

Liu SY, Joseph NM, Ravindranathan A, Stohr BA, Greenland NY, Vohra P, Hosfield E, Yeh I, Talevich E, Onodera C, Van Ziffle JA, Grenert JP, Bastian BC, Chen YY, Krings G (2016) Genomic profiling of malignant phyllodes tumors reveals aberrations in FGFR1 and PI-3 kinase/RAS signaling pathways and provides insights into intratumoral heterogeneity. *Mod Pathol* **29**(9): 1012–1027.

Mishima C, Kagara N, Tanei T, Naoi Y, Shimoda M, Shimomura A, Shimazu K, Kim SJ, Noguchi S (2015) Mutational analysis of *MED12* in fibroadenomas and phyllodes tumors of the breast by means of targeted next-generation sequencing. *Breast Cancer Res Treat* **152**(2): 305–312.

Moindrot B, Cerase A, Coker H, Masui O, Grijzenhout A, Pintacuda G, Schermelleh L, Nesterova TB, Brockdorff N. (2015) A pooled shRNA screen identifies Rbm15, Spen, and Wtap as factors required for Xist RNA-Mediated silencing. *Cell Rep* **12**(4): 562–572.

Nagasawa S, Maeda I, Fukuda T, Wu W, Hayami R, Kojima Y, Tsugawa K, Ohta T (2015) *MED12* exon 2 mutations in phyllodes tumors of the breast. *Cancer Med* **4**: 1117–1121.

Ng CC, Tan J, Ong CK, Lim WK, Rajasegaran V, Nasir ND, Lim JC, Thike AA, Salahuddin SA, Iqbal J, Busmanis I, Chong AP, Teh BT, Tan PH (2015) *MED12* is frequently mutated in breast phyllodes tumours: a study of 112 cases. *J Clin Pathol* **68**(9): 685–691.

Pfarr N, Kriegsmann M, Sinn P, Klauschen F, Endris V, Herpel E, Muckenhuber A, Jesinghaus M, Klosterhalfen B, Penzel R, Lennerz JK, Weichert W, Stenzinger A (2015) Distribution of *MED12* mutations in fibroadenomas and phyllodes tumors of the breast–implications for tumor biology and pathological diagnosis. *Genes Chromosomes Cancer* **54**(7): 444–452.

Piscuoglio S, Murray M, Fusco N, Marchiò C, Loo FL, Martelotto LG, Schultheis AM, Akram M, Weigelt B, Brogi E, Reis-Filho JS (2015) *MED12* somatic mutations in fibroadenomas and phyllodes tumours of the breast. *Histopathology* **67**(5): 719–729.

Piscuoglio S, Ng CK, Murray M, Burke KA, Edelweiss M, Geyer FC, Macedo GS, Inagaki A, Papanastasiou AD, Martelotto LG, Marchio C, Lim RS, Ioris RA, Nahar PK, Bruijn ID, Smyth L, Akram M, Ross D, Petrini JH, Norton L, Solit DB, Baselga J, Brogi E, Ladanyi M, Weigelt B, Reis-Filho JS (2016) Massively parallel sequencing of phyllodes tumours of the breast reveals actionable mutations, and *TERT* promoter hotspot mutations and *TERT* gene amplification as likely drivers of progression. *J Pathol* **238**(4): 508–518.

Ravegnini G, Mariño-Enriquez A, Slater J, Eilers G, Wang Y, Zhu M, Nucci MR, George S, Angelini S, Raut CP, Fletcher JA. *MED12* mutations in leiomyosarcoma and extrauterine leiomyoma. *Mod Pathol* (2013) **26**: 743–749.

Tan J, Ong CK, Lim WK, Ng CC, Thike AA *et al.* (2015) Genomic landscapes of breast fibroepithelial tumors. *Nat Genet* **47**(11): 1341–1345.

Tan PH, Thike AA, Tan WJ, Thu MM, Busmanis I, Li H, Chay WY, Tan MH, Phyllodes Tumour Network Singapore (2012) Predicting clinical behaviour of breast phyllodes tumours: a nomogram based on histological criteria and surgical margins. *J Clin Pathol* **65**(1): 69–76.

Tran NT, Su H, Khodadadi-Jamayran A, Lin S, Zhang L, Zhou D, Pawlik KM, Townes TM, Chen Y, Mulloy JC, Zhao X. (2016) The AS-*RBM15* lncRNA enhances *RBM15* protein translation during megakaryocyte differentiation. *EMBO Rep* **17**(6): 887–900.

Yoon N, Bae GE, Kang SY, Choi MS, Hwang HW, Kim SW, Lee JE, Nam SJ, Gong G, Lee HJ, Bae YK, Lee A, Cho EY (2016) Frequency of *MED12* mutations in phyllodes tumors: Inverse correlation with histologic grade. *Genes Chromosomes Cancer* **55**(6): 495–504.

Yoshida M, Sekine S, Ogawa R, Yoshida H, Maeshima A, Kanai Y, Kinoshita T, Ochiai A (2015a) Frequent *MED12* mutations in phyllodes tumours of the breast. *Br J Cancer* **112**(10): 1703–1708.

Yoshida M, Ogawa R, Yoshida H, Maeshima A, Kanai Y, Kinoshita T, Hiraoka N, Sekine S (2015b) *TERT* promoter mutations are frequent and show association with *MED12* mutations in phyllodes tumors of the breast. *Br J Cancer* **113**(8): 1244–1248.

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