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

Running Title: *MED12*, *TERT* promoter, *RBM15* mutations in phyllodes tumours

Diego A. Garcia-Dios[1], Dina Levi[1], Vandna Shah[1], Cheryl Gillett[1], Michael A. Simpson[2], Andrew Hanby[3], Ian Tomlinson[4], Elinor J. Sawyer[1]

1. Division of Cancer Studies, Guy's Hospital, King's College London, London SE1 9RT, UK.

2. Medical and Molecular Genetics, Guy's Hospital, King's College London, London, UK.

3. Leeds Institute of Cancer and Pathology, Cancer Genetics Building, St James's University Hospital, Beckett Street, Leeds LS9 7TF

4. Institute of Cancer and Genomic Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT

Corresponding author:

Name: Dr Elinor Sawyer

Innovation Hub,

Guy's Cancer Centre,

Guy's Hospital,

London SE1 9RT, UK

Telephone: +44 (0) 20 7188 7843

FAX: +44 (0) 20 7188 0919

e-mail: elinor.sawyer@kcl.ac.uk

Abstract

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 two 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.

Conclusion: 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.

Introduction

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 fibroadenomas, 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*, 2011). 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 phyllodes tumours and fibroadenomas (Cani *et al*, 2015; Yoshida *et al*, 2015a; Nagasawa *et al*, 2015; Lien *et al, 2016*). Some studies suggest that the frequency is similar across both FAs and all grades of PTs (Cani *et al*, 2015; Yoshida *et al*, 2015; Nagasawa *et al*, 2015; Lien *et al,* 2016a) and others that they are less frequent in malignant PTs (Piscuoglio *et al*, 2015; Pfarr *et al*, 2015) and FAs (Ng *et al*, 2015). This variation may be due to the 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*, 2015, Mishima *et al*, 2015). In both PTs and FAs, mutations are confined to the stromal component (Yoshida *et al*, 2015, 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 fibroadenomas (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 fibroadenomas (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 like *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;
3. identify novel mutations in other genes that may be driving malignant progression of PTs.

Material & 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 UK with ethical approval (MREC No. 03/12/083). 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 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 the Supplementary Table 1. Sanger sequencing was performed in a 3730xl DNA Analyser (ThermoFisher) 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 50Mb kit (Agilent) and sequenced on Illumina HiSeq 2000 to a mean depth of >100x. 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://www.annovar.openbioinformatics.org/)and cross referenced with dbSNP and 1000 Genomes. Somatic mutations were called if there was a minimum of 30x coverage and the mutation was present in at least 10% of reads.

Results

75 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, 9 were

benign (1 of which recurred as a borderline PT), 5 were borderline (3 of which recurred as malignant PTs) and 7 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 fibroadenomas respectively (Table 2). *MED12* mutations were more common in benign PTs compared to 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 to 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 (3 benign, 1 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 3 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).

TERT promoter mutations in recurrent PTs

Like *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 (1 malignant, 1 borderline and 2 benign) acquiring a *TERT* promoter mutation in the first recurrence and again this was also found in subsequent recurrences, Table 1.

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 developed multiple bilateral FAs aged 22 over a period of 3 years and was diagnosed with benign PTs on the fourth recurrence. Nine

lesions (7FAs and 2 PTs) were examined from the four-year period and none showed any evidence of *TERT* mutations. At the 4th 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 2 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 that had DNA available from fresh frozen tumour and blood. 18 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 sub-clonal 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*, 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, like others,Table 5a, has shown that *MED12* mutations are more common in benign PTs than malignant and borderline PTs 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 which 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 (1 benign, 1 malignant) lacked a *MED12* mutation in the paired recurrent sample suggesting that they were either new primaries or had arisen from a sub-clone within the primary PT that did not contain the *MED12* mutation. Unlike Lae *et al* we also have evidence of 4 cases of wild type *MED12* primaries acquiring *MED12* mutations in the second event (3 benign, 1 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 was a subclonal mutation in the primary tumour which we cannot detect. These findings suggest that *MED12* are not just early events in fibro-epithelial 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*, *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* (52%) and Liu *et al* (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 6/9 benign recurrences having both a *TERT* and *MED12* mutation compared to 1/9 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* *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 4th 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 2 benign PTs), all three lesions had different *MED12* mutations suggesting 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 on1p13.3 involved in regulating splicing of GATA1 and RUNX transcription factors in megakaryocyte differentiation and translocated in infant acute megakaryocytic leukemia (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* 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. 166 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 & neck, colorectal, stomach, prostate and pancreas. As well as being involved in the development of megakaryocytic leukemia, *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*), 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*, Piscuoglio *et al,* Liu *et al)*. In contrast *RARA* mutations were more frequent in samples with *MED12* and *TERT* promoter mutations (Tan *et al*, Piscuoglio *et al*) 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 they are sometimes early events in fibroepithelial lesions of the breast, they can be discordant in recurrent PTs, particularly 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 lab 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 / 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 done 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:

None

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Titles and legends to figures

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)

Supplementary Figure 1: MED12 mutations in two cases of multiple FAs and PTs

Supplementary Figure 2: Forward and reverse sequence of sample 24 (c.715delG, p.V239fs*1)

Supplementary Figure 3: Array CGH showing loss (arrow) between chr1:105666811(clone RP11-118G19) - 117575143 (clone RP11-27K13)

Nearest clone to RBM15 on array: RP11-284N8 (chr1:111095753-111288935)