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Stromal *MED12* exon 2 mutations in complex fibroadenomas of the breast

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ABSTRACT

Aims Here we explore the presence of mediator complex subunit 12 (*MED12*) exon 2 and telomerase reverse transcriptase (*TERT*) promoter hotspot mutations in complex fibroadenomas (CFAs) of the breast.

Methods The stromal components from 18 CFAs were subjected to Sanger sequencing of *MED12* exon 2 and the *TERT* promoter hotspot loci. The epithelial and stromal components of two *MED12* mutated CFAs were subjected to laser capture microdissection, and Sanger sequencing of *MED12* exon 2, *TERT* promoter and *PIK3CA* exons 9 and 20, separately.

Results *MED12* exon 2 mutations were identified in the stroma of 17% of CFAs. The analyses of epithelial and stromal components, microdissected separately, revealed that *MED12* mutations were restricted to the stroma. No *TERT* promoter or *PIK3CA* mutations in exons 9 and 20 were detected in analysed CFAs.

Conclusions Like conventional fibroadenomas, *MED12* exon 2 mutations appear to be restricted to the stromal component of CFAs, supporting the notion that CFAs are stromal neoplasms.

INTRODUCTION

Fibroadenomas (FAs) of the breast are a group of benign biphasic neoplasms consisting of a proliferation of both epithelial and stromal components.¹ After the seminal publication by Lim *et al*,² describing mediator complex subunit 12 (*MED12*) exon 2 mutations in approximately 60% of FAs, our group and others have not only confirmed the presence of these mutations in FAs, but also demonstrated that they are restricted to the stromal component of these lesions.^{2–5} *MED12* exon 2 mutations have also been detected in other fibroepithelial neoplasms, including phyllodes tumours (PTs), where these mutations are present in 88% of benign, in 78% of borderline and in 8% of malignant PTs.^{5–8} In addition, our group initially described the presence of telomerase reverse transcriptase (*TERT*) promoter hotspot mutations in PTs,⁷ which have been confirmed to increase in frequency from benign to malignant PTs and to be vanishingly rare in conventional FAs.^{9–11}

FAs comprise histologic variants, including juvenile fibroadenoma, cellular FAs and complex fibroadenomas (CFAs), which display different histologic features and variations in their clinicopathologic characteristics. A subset of FAs, characterised by

abundant myxoid matrix and hypocellular stromal component, was previously categorised as myxoid fibroadenomas (MFAs) as per fourth edition WHO classification.¹² These lesions were reported to lack *MED12* exon 2 mutations in the stromal component, and to harbour recurrent mutations in *TP53*, *PIK3R1*, *ERCC5* and *PIK3CA* in the epithelial rather than the stromal component.¹³ Importantly, in one case of MFA included in Lozada *et al*,¹³ a mutation affecting *PRKARIA* was detected in the stromal component, and the case was subsequently reclassified as a myxoma. Per fifth edition WHO classification of breast neoplasms,¹ CFA is a variant of fibroadenoma that contains one or more of the following histological findings: cysts >3 mm, sclerosing adenosis, epithelial calcifications and papillary apocrine metaplasia. As compared with conventional FAs, CFAs have more abundant epithelial components sharing histological features of proliferative fibrocystic disease of breast and less characteristic neoplastic stroma. An increased relative risk (RR) of subsequent invasive breast carcinoma was reported for patients with CFAs (RR 3.10; 95% CI 1.9 to 5.1) in comparison to patients with conventional FAs (RR 2.17; 95% CI 1.5 to 3.2).¹⁴ Other studies, however, have indicated that CFAs may not result in increased breast cancer risk beyond the presence of other established risk factors such as proliferative disease or atypical epithelial hyperplasia of breast. The clinical behaviour of CFAs, however, remains contentious, and the current recommendation is to manage patients with CFA with a conservative approach, similarly to the approach employed for the management of women with conventional FAs.^{15 16}

The genetic underpinning of CFAs, whether the abundant epithelial component contributes to the tumorigenesis or whether they are genetically related to conventional FAs or PTs is unclear. Here we conducted an exploratory analysis to determine whether *MED12* exon 2 and *TERT* promoter hotspot mutations would be present in CFAs. Furthermore, given our previous findings that *PIK3CA* hotspot mutations were present in PTs without FA-like areas and in a subset of FAs, classified as MFAs in the prior (fourth edition) WHO classification,^{8 12 13} in addition to the *MED12* exon 2 and *TERT* promoter hotspot mutations, we also assessed the presence of mutations in exons 9 and 20 of *PIK3CA* in separate epithelial and stromal components of two CFAs subjected to laser capture microdissection (LCM).

MATERIALS AND METHODS

Subjects and samples

Following institutional review board approval, slides and formalin-fixed paraffin-embedded (FFPE) tissue blocks of 24 CFAs from 23 adult patients were retrieved from the archives of the Department of Pathology of Memorial Sloan Kettering Cancer Center (New York, USA). All cases were reviewed by nine breast pathologists (APMS, FP, ME, TMD, MPM, HYW, EB, JSR-F and HZ) and were reclassified according to the WHO criteria fifth edition (2019).¹ A diagnosis of CFA was rendered when at least one or more complex features were present (ie, cysts >3 mm, sclerosing adenosis, epithelial calcifications and papillary apocrine metaplasia). Eighteen CFAs (from 17 anonymised patients) were included in this study on a consensus diagnosis being rendered. All cases are unique to this study, and none has been previously reported.

Tumour microdissection and sequencing

Eight micrometre thick FFPE representative histological sections of 18 CFAs were microdissected under a stereomicroscope (Olympus SZ61) to ensure a stromal cell content >80%. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Sanger sequencing was used for the assessment of mutations affecting *MED12* exon 2 and *TERT* promoter hotspots in the stromal components of 18 CFAs. Sanger sequencing and PCR amplification methods were performed as previously described¹⁷ and are detailed in online supplemental methods and table S1.

To define whether the mutations affecting the exon 2 of *MED12* would be restricted to the stroma or would also be present in the epithelial component in the *MED12* mutated cases, representative samples from these tumours were subjected to LCM to obtain the epithelial and stromal components separately (online supplemental methods). Due to the limited material available in one of the cases (CFA7), LCM was performed in two (CFA18 and CFA22) CFAs harbouring *MED12* exon 2 mutations. DNA samples from the epithelial and stromal components were subjected to Sanger sequencing of *MED12* exon 2, *TERT* promoter hotspots and *PIK3CA* exons 9 and 20. Sequence electropherograms of the forward and reverse strands were analysed using Mutation Surveyor (SoftGenetics) and the mutations identified were manually curated. All analyses were performed in duplicate.

RESULTS

Our study included 18 CFAs from patients with a median age of 48 years at diagnosis (range 33–82 years, (online supplemental table S2). All CFAs harboured at least one complex feature, such as sclerosing adenosis or fibrocystic changes (figure 1A and online supplemental table S2). Fibrocystic changes including usual ductal hyperplasia without atypia, apocrine metaplasia, columnar cell changes, cyst formation and stromal fibrosis were identified in all CFAs, and sclerosing adenosis were present in the adjacent breast tissue in 13 (72%) cases. Based on the data retrieved from the pathology reports, concurrent ductal carcinoma *in situ* (67%, 12/18), invasive ductal carcinoma (22%, 4/18), invasive lobular carcinomas (6%, 1/18), lobular carcinoma *in situ* (11%, 2/18) or atypical ductal hyperplasia (6%, 1/18) were identified in the breast tissue away from CFAs included in this study.

MED12 exon 2 mutations were identified in the stromal component of 17% (3/18) of CFAs (figure 1B and online supplemental table S2). Two CFAs harboured the hotspot p.G44S

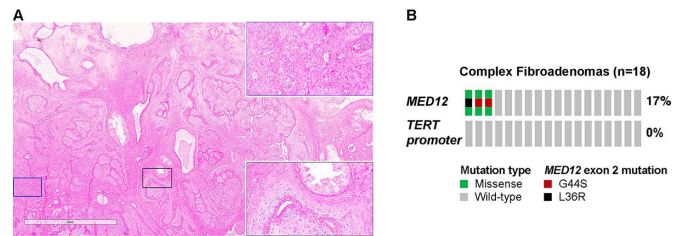


Figure 1 Histologic features and frequency of *MED12* exon 2 and *TERT* promoter mutations in complex FAs of the breast. (A) Representative H&E micrograph of a complex FA displaying apocrine metaplasia (bottom right) and sclerosing adenosis (top right), scale bar, 2 mm. (B) Frequency of *MED12* exon 2 and *TERT* promoter hotspot mutations in the stromal component of 18 CFAs included in this study. CFAs, complex fibroadenomas; FAs, fibroadenomas; *MED12*, mediator complex subunit 12; *TERT*, telomerase reverse transcriptase.

(c.130G>A) and one case harboured the pathogenic p.L36R (c.107T>G) *MED12* mutations (figure 2A–C and online supplemental table S3). No differences in the histologic and clinical features of the CFAs, according to the presence or absence of *MED12* mutations were found online supplemental figure S1 and table S2. No *TERT* promoter hotspot mutations were detected in the CFAs analysed (figure 1B, online supplemental table S2).

In CFA18 and CFA22, where the epithelial and stromal components were successfully separated, Sanger sequencing analysis revealed that the *MED12* mutations (p.G44S) were exclusively restricted to their respective stromal components. No *MED12* mutations were found in the epithelial component of the CFAs (figure 2D and online supplemental table S4). No *TERT* promoter hotspot mutations or *PIK3CA* mutations in exons 9 or 20 were found in either the epithelial or stromal component of the CFAs subjected to LCM.

DISCUSSION

CFAs are a variant of FAs¹² and comprise 22.7% to 40.4% of diagnosed FAs.^{14 18} Often clinically overlooked, CFAs tend to occur in patients older than those with conventional FAs^{15 18} with an average size of half of other FAs.¹⁵ Histologically, the presence of epithelial alterations overlapping with fibrocystic changes inside the lesion is characteristic of this FA subtype. Tubular adenoma of breast, which is a rare epithelial proliferation arising from the terminal duct lobular unit, is occasionally in the differential diagnosis due to the presence of prominent sclerosing adenosis inside the CFA.¹⁹ Although some studies suggest that CFAs carry a slightly increased risk of subsequent development of breast cancers comparing to general population,¹⁴ others did not confirm this risk.^{15 16}

The exploratory, hypothesis generating analysis performed here revealed that, akin to conventional FAs, a subset of CFAs harbour pathogenic mutations in *MED12* exon 2, although at a lower frequency (17% CFAs vs up to 65% conventional FAs).^{5 17} These findings support the notion that while CFAs and conventional FAs are morphologically and, to a certain extent, clinically separate entities, they share similar genetic alterations in *MED12* in a subset of cases, in agreement with what was reported by Lien *et al.*²⁰ Due to the rich nature of epithelial component and its integral growth mixed with stromal components, it is technically challenging to obtain adequate amount of DNA from the separate components in CFAs by LCM. We successfully assessed mutations in *MED12* exon 2 and *TERT* promoter loci in the separately laser microdissected epithelial and stromal components of

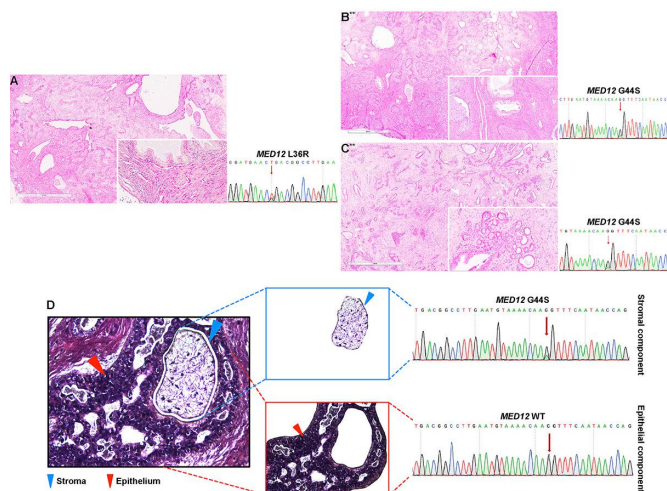


Figure 2 Complex FAs have *MED12* exon 2 mutations restricted to the stromal component. Representative H&E micrographs of CFAs and Sanger sequencing electropherograms of (A) *MED12* exon 2 L36R mutation (CFA7), (B–C) *MED12* exon 2 G44S mutations (CFA18 and CFA22, respectively) identified, scale bar, 2 mm, (D) CFA18 subjected to LCM and independent sequencing of the epithelial and stromal components (left, blue arrow—stroma, red arrow—epithelium); representative sequence electropherograms of exon 2 of *MED12* in the stroma (right top) and the epithelial component (right bottom). ** *MED12* mutated CFAs subjected to laser capture microdissection. CFA, complex fibroadenomas; FAs, fibroadenomas; LCM, laser capture microdissection; *MED12*, mediator complex subunit 12; *TERT*, telomerase reverse transcriptase.

two CFAs. These cases were also subjected to Sanger analysis of *PIK3CA* exons 9 and 20. Given that *MED12* mutations are restricted to the stromal component, CFAs, akin to conventional FAs, likely constitute stromal rather than fibroepithelial neoplasms. No *PIK3CA* mutations in exon 9 and 20 were identified in these cases, consistent with previous observations derived from the analysis of fibroepithelial lesions harbouring *MED12* mutations.¹⁰

Our study has limitations. Due to limited archived FFPE tissue availability, we restricted our analysis to specific genetic alterations. Our analysis was restricted to the mutations affecting *MED12* exon 2, *TERT* promoter hotspot mutations, and in two *MED12* exon 2 mutated cases, also *PIK3CA* exons 9 and 20. Although our findings support the notion that *MED12* exon 2 mutations do not constitute the main driver of CFAs, we cannot rule out that there are other genes which may play a role in the biology of CFAs. Moreover, due to the size of our cohort and rarity of recurrent events, our study may have a limited power to assess the significance of the presence of *MED12* exon 2 mutations in the stromal component of CFAs. All CFAs included in this study were retrieved from the pathology archives of a cancer centre, which might have contributed to the high frequency of co-occurrent invasive and/or *in situ* carcinomas at the time of the diagnosis of CFA. Given the scope of this study, we could not determine the prognostic significance of *MED12* exon 2 mutations and its association with the presence of co-occurrent invasive and/or *in situ* carcinomas identified in our cohort. Further studies to assess the impact of *MED12* exon 2 mutations in CFAs in the risk of development of breast cancer are warranted. Despite these limitations, here we demonstrated that CFAs harbour *MED12* exon 2 mutations in the stromal component,

akin to other histologic variants of FAs. At variance with conventional FAs, however, the frequency of *MED12* exon 2 mutations in CFAs appears to be lower than that of conventional FAs. Further studies are warranted to define the genetic underpinning of *MED12* wild-type CFAs.

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Contributors FP, EB, JSR-F and HZ conceived the study. EmdS and HZ coordinated the retrieval of samples. APMS, MPM, FP, HYW, TMD, ME, EB, JSR-F and HZ performed the pathology review. EmdS, APMS, CS and ADCP performed experiments and analysis. EmdS, FB, BW, JSR-F and HZ analysed and interpreted the data. EmdS, FB, JSR-F and HZ wrote the first manuscript, which was reviewed by all coauthors.

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Competing interests JSR-F reports receiving personal/consultancy fees from Goldman Sachs and REPARE Therapeutics, membership of the scientific advisory boards of VolitionRx, REPARE Therapeutics and Paige. AI, membership of the Board of Directors of Grupo Oncoclínicas, 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. HZ reports consultancy fee from Roche/Genentech, outside the scope of the submitted work. FB is currently an employee and equity holder of Seattle Genetics, Inc but the present work is outside the scope of his role.

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SUPPLEMENTARY MATERIALS

Stromal *MED12* Exon 2 Mutations in Complex Fibroadenomas of the Breast

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Supplementary Figure S1

Supplementary Methods

Supplementary Tables S1-S4

Supplementary Figure 1. Histologic features of complex fibroadenomas of the breast.

Representative hematoxylin and eosin micrographs of complex fibroadenomas lacking *MED12* exon 2 mutations included in the study. Scale bar, 2mm. Corresponding sample IDs are indicated on each image (top left) and insert (bottom right) depicting histological features identified in the complex fibroadenomas.

Supplementary Methods

Supplementary Table S1: Primers used for Sanger sequencing.

Supplementary Table S2. Clinicopathological characteristics, *MED12* exon 2 and *TERT* promoter status of the complex fibroadenomas of the breast included in this study.

Supplementary Table S3. *MED12* exon 2 mutations identified in the complex fibroadenomas of the breast and their predicted pathogenicity.

Supplementary Table S4. *MED12* exon 2, *TERT* promoter and *PIK3CA* exon 9 and exon 20 status in the complex fibroadenomas of the breast subjected to laser capture microdissection of the epithelial and stromal components.

Supplementary Methods

Laser capture microdissection

Eight- μ m-thick FFPE sections of cases CFA18 and CFA22 were cut using a microtome cryostat (Leica) and mounted onto Arcturus PEN Membrane glass slides (Life Technologies). Slides were dehydrated and stained with hematoxylin. Laser capture microdissection was performed using an LMD 6500 instrument (Leica Microsystem). The stromal and epithelial components of a given case were outlined using the associated software and then captured separately using an infrared capture laser. DNA from the microdissected components was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer' instructions and subjected to mutation screening described below.

PCR amplification and Sanger sequencing

Putative somatic mutations in selected genes of interest were investigated by Sanger sequencing. PCR amplification of 10ng of genomic DNA was performed using the AmpliTaq 360 Master Mix Kit (Life Technologies) as previously described.^{1,2} PCR products were purified (ExoSAP-IT, Thermo Fisher Scientific) and subjected to Sanger sequencing encompassing *MED12* exon 2, *TERT* promoter hotspot locus and *PIK3CA* exons 9 and 20 (**Supplementary Table S1**). All analyses were performed in duplicate.

Supplementary References

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Supplementary Table S1: Primers used for Sanger sequencing.

Gene	Forward	Reverse
<i>MED12</i> exon 2	TGTTCTACACGGAACCCTCCTC	CTGGGCAAATGCCAATGAGAT
<i>TERT</i> promoter	CCAGGGCTTCCCACGTGC	ACTGGGGACCCGGGCACC
<i>PIK3CA</i> exon 9	CTGTGAATCCAGAGGGGAAA	GCACTTACCTGTGACTCCATAGAA
<i>PIK3CA</i> exon 20	CTCAATGATGCTTGGCTCTG	TGGAATCCAGAGTGAGCTTTC

Supplementary Table S2. Clinicopathological characteristics, *MED12* exon 2 mutations and *TERT* promoter status of the complex fibroadenomas of the breast included in this study.

Sample ID	Age (years)	Menopausal status	Sclerosing adenosis	Fibrocystic changes	Size (cm)	<i>MED12</i> mutation	<i>TERT</i> status
CFA 1	54	Post	Y	Y	NA		WT
CFA 2	67	Post		Y	NA		WT
CFA 4	62	Post		Y	1		WT
CFA 5	39	Pre	Y	Y	NA		WT
CFA 6	41	Pre		Y	1.5		WT
CFA 7	36	Pre		Y	NA	c.107T>G	WT
CFA 8	63	Post	Y	Y	2.1		WT
CFA 9	47	Pre	Y	Y	NA		WT
CFA 10	52	Pre	Y	Y	NA		WT
CFA 11	63	Post	Y	Y	NA		WT
CFA 13	33	Pre	Y	Y	NA		WT
CFA 16	33	Pre	Y	Y	NA		WT
CFA 17	82	Post	Y	Y	NA		WT
CFA 18	41	Pre	Y	Y	NA	c.130G>A	WT
CFA 19	45	Pre		Y	0.9		WT
CFA 21	37	Pre	Y	Y	1.8		WT
CFA 22	52	Peri	Y	Y	1.6	c.130G>A	WT
CFA 24	48	Pre	Y	Y	NA		WT

NA, not available; WT, wild type

Supplementary Table S3. *MED12* exon 2 non-synonymous somatic mutations identified in the complex fibroadenomas of the breast and their predicted pathogenicity.

Sample ID	<i>MED12</i> mutation	<i>MED12</i> Amino Acid Change	Start Position	Mutation Taster	Provean	Polyphen-2	CADD	ClinVar Interpretation	COSMIC Mutation ID
CFA 1									
CFA 2									
CFA 4									
CFA 5									
CFA 6									
CFA 7	c.107T>G	p.L36R	70339230	disease causing	deleterious	probably damaging	deleterious	Not provided	COSM131590
CFA 8									
CFA 9									
CFA 10									
CFA 11									
CFA 13									
CFA 16									
CFA 17									
CFA 18	c.130G>A	p.G44S	70339253	disease causing	deleterious	probably damaging	deleterious	other	COSM131594
CFA 19									
CFA 21									
CFA 22	c.130G>A	p.G44S	70339253	disease causing	deleterious	probably damaging	deleterious	other	COSM131594
CFA 24									

Supplementary Table S4. *MED12* exon 2, *TERT* promoter and *PIK3CA* exon 9 and exon 20 status in the complex fibroadenomas of the breast subjected to laser capture microdissection of the epithelial and stromal components.

Sample ID	Component	<i>MED12</i> exon 2	<i>TERT</i> promoter	<i>PIK3CA</i> exon 9	<i>PIK3CA</i> exon 20
CFA18	Stroma	p.G44S	WT	WT	WT
	Epithelium	WT	WT	WT	WT
CFA22	Stroma	p.G44S	WT	WT	WT
	Epithelium	WT	WT	WT	WT

WT, wild type

Supplementary Figure S1

