

Keywords: *MED12*; phyllodes tumour; fibroadenoma; breast

Frequent *MED12* mutations in phyllodes tumours of the breast

M Yoshida¹, S Sekine^{*1,2}, R Ogawa², H Yoshida¹, A Maeshima¹, Y Kanai², T Kinoshita³ and A Ochiai¹

¹Pathology and Clinical Laboratories, National Cancer Center Hospital, Tokyo 104-0045, Japan; ²Division of Molecular Pathology, National Cancer Center Research Institute, Tokyo 104-0045, Japan and ³Breast Surgery Division, National Cancer Center Hospital, Tokyo 104-0045, Japan

Background: Phyllodes tumours are rare fibroepithelial tumours of the breast, that include benign, borderline, and malignant lesions. Although the molecular basis of phyllodes tumours largely remains unknown, a recent exome study identified *MED12* mutations as a sole recurrent genetic alteration in fibroadenoma, a common benign fibroepithelial tumour that shares some histological features with the phyllodes tumour.

Methods: Forty-six phyllodes tumours and 58 fibroadenomas of the breast were analysed for *MED12* mutations by using Sanger sequencing.

Results: *MED12* mutations were identified in 37 out of the 46 phyllodes tumours (80%). The prevalence of *MED12* mutations was similar among benign (15/18, 83%), borderline (12/15, 80%), and malignant tumours (10/13, 77%). *MED12* mutations were also identified in 36 of the 58 fibroadenomas (62%). The mutations were frequent among intracanalicular-type (24/32, 75%) and complex-type lesions (4/6, 67%), but were significantly less common among the pericanalicular-type lesions (8/20, 40%). A microdissection-based analysis showed that *MED12* mutations were confined to the stromal components in both phyllodes tumours and fibroadenomas.

Conclusions: *MED12* mutations were frequent among the phyllodes tumours of the breast, regardless of the tumour grade. Phyllodes tumours and fibroadenomas share, at least in part, a common genetic background.

Fibroepithelial tumours of the breast are a heterogeneous group of tumours consisting of variable proportions of epithelial and stromal components (Tavassoli and Eusebi, 2009; Tan *et al*, 2012; Yang *et al*, 2014). Fibroadenoma and phyllodes tumour are the major histological entities of this group of tumours. Fibroadenomas are common benign neoplasms that predominantly affect young adults. Studies on autopsy cases have reported a prevalence of fibroadenoma of 15–23% (Frantz *et al*, 1951; Bhathal *et al*, 1985; Bartow *et al*, 1987; Nielsen *et al*, 1987). Phyllodes tumours are rare, accounting for less than 1% of all breast tumours and include benign, borderline, and malignant lesions (Reinfuss *et al*, 1996; Tavassoli and Eusebi, 2009), some phyllodes tumours can lead to local recurrences and distant metastasis (Chaney *et al*, 2000).

Phyllodes tumours are histologically characterised by a pronounced intracanalicular growth and increased cellularity (Tavassoli and Eusebi, 2009; Tan *et al*, 2012; Yang *et al*, 2014). Additionally, they may exhibit nuclear atypia and increased mitotic counts, and they may have an obvious sarcomatous component. However, the histological discrimination of phyllodes tumours from fibroadenomas sometimes can be a diagnostic challenge. In particular, a significant overlap exists in the histological features of intracanalicular-type fibroadenomas and benign phyllodes tumours, and no clear-cut morphological criteria exist for distinguishing these two lesions (Giri, 2009; Lawton *et al*, 2014). Furthermore, some studies have suggested the potential progression of fibroadenomas to phyllodes tumours (Noguchi *et al*, 1995; Hodges *et al*, 2009; Abe *et al*, 2011).

*Correspondence: Dr S Sekine; E-mail: ssekine@ncc.go.jp

Received 21 November 2014; revised 18 February 2015; accepted 7 March 2015; published online 2 April 2015

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The molecular basis for the tumourigenesis of fibroepithelial breast tumours has been poorly understood, but a recent study by using massively parallel sequencing identified *MED12* mutation as the sole and highly recurrent genetic alteration of fibroadenomas (Lim *et al*, 2014). Based on its somatic nature and high prevalence, the *MED12* mutation is expected to be a critical driver mutation in the development of fibroadenoma. Interestingly, uterine leiomyomas have also shown to frequently harbour *MED12* mutations of a similar spectrum, implying a common role of *MED12* mutations in the development of these two oestrogen-dependent neoplasms (Makinen *et al*, 2011; McGuire *et al*, 2012; Matsubara *et al*, 2013; Kampjarvi *et al*, 2014). A recent study showed that uterine leiomyoma-associated *MED12* mutations disrupted the MED12-CyclinC interface and resulted in loss of mediator-associated CDK activity, suggesting a role in aberrant CDK8/19 activity (Turunen *et al*, 2014).

We postulated that phyllodes tumours might share a common genetic background with fibroadenomas based on the partial morphological resemblance of these tumours and the results of previous reports suggesting a histogenetic relationship between fibroadenomas and phyllodes tumours. In the present study, we conducted a mutation analysis of *MED12* in a series of fibroadenomas and phyllodes tumours.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan. All the tissue samples were obtained at the National Cancer Center Hospital, Tokyo, Japan. The resected specimens were routinely fixed in 10% formalin and embedded in paraffin. This study examined 104 fibroepithelial tumours of the breast, including 46 phyllodes tumours and 58 fibroadenomas. The phyllodes tumours were subclassified according to the WHO classification as benign, borderline, or malignant lesions, based on semi-quantitative assessments of stromal cellularity, cellular pleomorphism, mitotic activity, tumour margin/border appearance, and stromal distribution/overgrowth (Figure 1A–C) (Tan *et al*, 2012). Fibroadenomas were primarily classified into intracanalicular- and pericanalicular-types depending on the predominant growth patterns (Figure 1D and E). In addition, fibroadenomas showing cyst formation, sclerosing adenosis, and/or apocrine metaplasia were classified as complex type (Figure 1F) (Dupont *et al*, 1994).

Sections of tumour specimens were deparaffinised, stained briefly with hematoxylin and subjected to DNA extraction. The stromal components of phyllodes tumours and fibroadenomas were dissected by using sterilised toothpicks under a microscope. Non-tumourous areas were separately dissected when a sufficient amount of tissue was available. The dissected samples were incubated in 50 μ l DNA extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetra-acetic acid, 0.5% v/v Tween 20, 200 μ g ml⁻¹ proteinase K) at 50 °C overnight. Proteinase K was inactivated by heating at 100 °C for 10 min. Then, the samples were subjected to polymerase chain reaction (PCR) by using a pair of primers encompassing the frequently mutated region of *MED12* (5'-ACTCTCCCACCCCTTCCCC-3' and 5'-GGCAGGATTGAA GCTGACGTTTC-3'; Matsubara *et al*, 2013). The PCR products were purified by using the Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA). The isolated PCR products were then bidirectionally sequenced by using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Mutations were confirmed by using at least two independent analyses.

A microdissection-based analysis was performed in selected cases with a *MED12* mutation. The epithelial and stromal components were microdissected separately by using a laser microdissection system (MMI CellCut system; Molecular Machines and Industries,

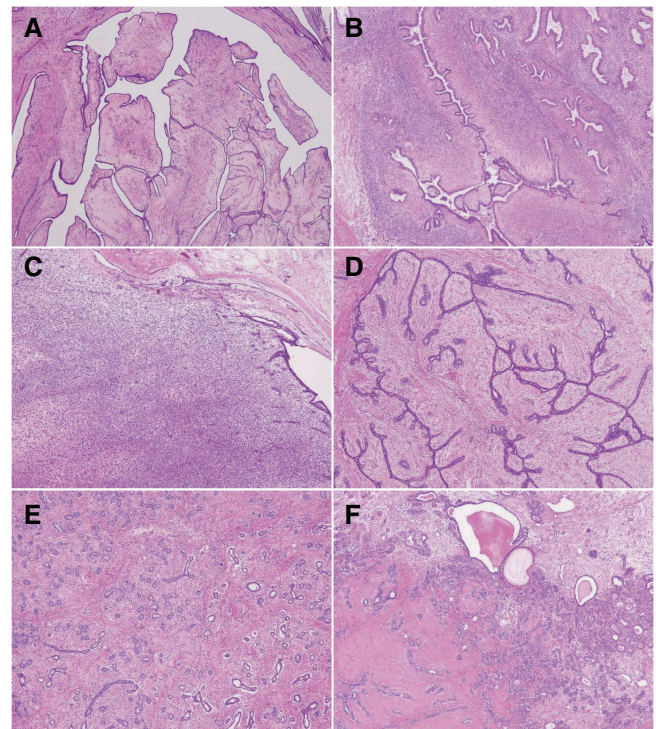


Figure 1. Representative histology of phyllodes tumour (A–C) and fibroadenoma (D–F). (A) Benign phyllodes tumour showing a classical leaf-like structure. (B) Borderline phyllodes tumour with stromal expansion resulting in duct compression. (C) Malignant phyllodes tumour showing prominent stromal overgrowth and a high cellularity. (D) Intracanalicular-type fibroadenoma. The ducts are compressed and exhibit a slit-like structure. (E) Pericanalicular-type fibroadenoma. The pericanalicular proliferation of the stroma and tubular glands are seen. (F) Complex fibroadenoma. Cyst formation and an area of stromal hyalinisation are noted.

Glattbrugg, Switzerland). The isolated samples were then examined for the presence of *MED12* mutations as described above.

The Fisher exact test was used to analyse categorical data. The Mann–Whitney U test or the Kruskal–Wallis test were used for the analysis of continuous variables. $P < 0.05$ was considered statistically significant.

RESULTS

The phyllodes tumours were histologically subclassified into 18 benign, 15 borderline, and 13 malignant lesions (Table 1). Patients with malignant or borderline tumours were significantly older than those with benign lesions ($P = 0.045$). Malignant tumours were larger than benign and borderline tumours ($P = 0.034$). The fibroadenomas consisted of 32 intracanalicular-type, 20 pericanalicular-type, and 6 complex-type lesions. The pericanalicular-type fibroadenomas were significantly larger than the intracanalicular-type lesions ($P = 0.020$).

MED12 mutations were identified in 37 (80%) phyllodes tumours and 36 (62%) fibroadenomas (Table 1). Even though *MED12* mutations tended to be more prevalent in the phyllodes tumours, the difference did not reach the statistical significance ($P = 0.053$). The prevalence of *MED12* mutations was similar among the phyllodes tumours of different tumour grades. Among the fibroadenomas, *MED12* mutations were more common among the intracanalicular-type lesions than among the pericanalicular-type lesions ($P = 0.019$). Non-tumour tissue samples were available for 26 phyllodes tumours and in 50 fibroadenomas, but none of

Table 1. MED12 mutations in phyllodes tumours and fibroadenomas of the breast

Histology	Number of cases	Age, year old	P-value	Tumour size, mm	P-value	MED12 mutation	P-value
Phyllodes tumour	46	48.5 (26–67)		46.5 (10–160)		37 (80%)	
Benign	18	41.5 (26–65)	0.045 ^a	33.5 (10–100)	0.034 ^a	15 (83%)	0.90 ^a
Borderline	15	55 (26–67)		48 (28–130)		12 (80%)	
Malignant	13	55 (36–64)		75 (17–160)		10 (77%)	
Fibroadenoma	58	39 (18–66)	9.0 × 10 ⁻⁶ ^b	15 (6–45)	6.5 × 10 ⁻¹¹ ^b	36 (62%)	0.053 ^b
Intracanalicular	32	39 (21–63)	0.78 ^c	14 (6–45)	0.020 ^c	24 (75%)	0.019 ^c
Pericanalicular	20	37.5 (18–66)		24 (6–45)		8 (40%)	
Complex	6	38.5 (32–46)		10 (9–16)		4 (67%)	

Age and tumour size are indicated as the median (range).
^aBenign vs borderline vs malignant grade.
^bPhyllodes tumour vs fibroadenoma.
^cIntracanalicular vs pericanalicular subtype.

these samples showed mutations, indicating the somatic nature of these mutations.

Most of the mutations identified were missense mutations, in-frame deletion/insertions within exon 2, or deletions involving a splice acceptor site (Table 2, Figure 2A). Two cases had an intronic nucleotide change 8 base pairs upstream of exon 2, which was predicted to result in the insertion of two amino acids. In both phyllodes tumours and fibroadenomas, missense mutations affecting codon 44 were the most frequent (Figure 3). No apparent differences in the mutation patterns were observed among these tumours.

To test whether MED12 mutations were also present in the epithelial component, we performed a microdissection-based analysis of five MED12-mutated phyllodes tumours and five MED12-mutated fibroadenomas. The epithelial and stromal components were separately microdissected and analysed for the presence of mutations. As a result, MED12 mutations were identified exclusively in the stromal components in all the lesions that were examined (Figure 2B). Thus, the MED12 mutation was absent in the epithelial components of fibroadenomas and phyllodes tumours.

Finally, we tested whether the presence of MED12 mutations was related to the abnormal expressions of p53, p16, or Rb. Immunohistochemistry showed p53 overexpression, p16 loss, and/or Rb loss in nine (69%) of the malignant phyllodes tumours and two (13%) of the borderline phyllodes tumours, but in none of the benign phyllodes tumours or fibroadenomas (Supplementary Table 1, Supplementary Figure 1). We tested the correlation between the MED12 mutation status and the abnormal expression of each tumour suppressor gene product, but did not find any significant correlations (Supplementary Table 2).

DISCUSSION

The present study identified MED12 mutations in the majority of phyllodes tumours of the breast. The mutation frequency was similar among benign, borderline, and malignant lesions. These observations suggest that MED12 mutations have a critical role in the tumorigenesis of most phyllodes tumours and are not involved in the progression from benign to malignant tumours. While this study was under review, Cani *et al* (2015) also reported the presence of MED12 mutations in phyllodes tumours. Their study identified MED12 mutations in four out of five benign, four out of five borderline, and two out of five malignant phyllodes tumours. Even though the number of analysed tumours was rather limited, these findings are consistent with ours in that MED12 mutations were found to be present in phyllodes tumours regardless of the tumour grades. MED12 mutations were also common in fibroadenomas, similar to the results of a previous study reporting a mutation rate of 62% (Lim *et al*, 2014). The mutation frequency was significantly higher in intracanalicular-type lesions than in pericanalicular-type

lesions. Since both phyllodes tumours and intracanalicular-type fibroadenomas are histologically characterised by an intracanalicular growth pattern (Tan *et al*, 2012), mutated MED12 may be involved in stromal cell proliferation and the formation of the characteristic morphology of these tumours.

Besides fibroadenomas, recurrent MED12 mutations have also been described in leiomyomas of the uterus, with reported frequencies of 52–82% (Makinen *et al*, 2011; McGuire *et al*, 2012; Matsubara *et al*, 2013; Kampjarvi *et al*, 2014). The mutation spectrum in uterine leiomyomas is similar to that observed in fibroepithelial tumours of the breast identified in the present study. The comparable mutation patterns among these tumours imply a common biological role of MED12 mutation in the tumorigenesis of these tumours.

We also analysed the expressions of p53, p16, and Rb, which are often altered in malignant phyllodes tumours (Tse *et al*, 2002; Tan *et al*, 2005; Lae *et al*, 2007; Jones *et al*, 2008; Cimino-Mathews *et al*, 2013). In agreement with previous studies, more than half of the malignant phyllodes tumours showed p53 overexpression, and the loss of p16 and/or the loss of Rb, indicating the inactivation of these tumour suppressors (Jones *et al*, 2008; Yachida *et al*, 2012; Tan *et al*, 2014). These abnormalities were rare or absent in other groups of tumours. On the other hand, no significant correlations were observed between the abnormal expressions of these proteins and the MED12 mutation status. The inactivation of these tumour suppressors is likely to have a role in the progression of phyllodes tumours regardless of the presence or absence of MED12 mutations.

By definition, phyllodes tumours and fibroadenomas are biphasic tumours consisting of epithelial and stromal components (Tavassoli and Eusebi, 2009; Tan *et al*, 2012; Yang *et al*, 2014). However, the exclusive presence of MED12 mutations in the stromal components of fibroadenomas and phyllodes tumours, in agreement with a previous study, suggests that these tumours are primarily a stromal neoplasms (Lim *et al*, 2014). Consistent with this hypothesis, a study using an X chromosome inactivation-based clonality analysis showed that the stromal component was monoclonal, whereas the epithelial component was polyclonal, in the majority of phyllodes tumours that were examined (Kuijper *et al*, 2002). Another study examining the EGFR copy number by using fluorescent *in situ* hybridisation detected amplification events only in the stromal components of phyllodes tumours (Kersting *et al*, 2006). This study also demonstrated the presence of a tumour driver gene alteration that was restricted to the stromal component, similar to the results of present study.

On the other hand, the consistent presence of epithelial components in phyllodes tumours and fibroadenomas implies their requirement in the growth of these tumours. Importantly, several previous studies have suggested critical roles of epithelium-derived factors in the growth of stromal cells in these tumours (Sawhney *et al*, 1992; Sawyer *et al*, 2002). The interdependence between the non-neoplastic epithelial component and the neoplastic stromal

Table 2. MED12 mutations in phyllodes tumours and fibroadenomas of the breast

Nucleotide	Amino acid	Mutated cases in phyllodes tumour (n = 46)	Mutated cases in fibroadenoma (n = 58)
c.122T>A	p.V41E	0	2
c.128A>C	p.Q43P	1	0
c.130G>A	p.G44S	1	2
c.130G>C	p.G44R	3	0
c.130G>T	p.G44C	1	2
c.131G>A	p.G44D	8	13
c.131G>C	p.G44A	2	1
c.131G>T	p.G44V	2	3
c.100-8T>A	p.E33_D34insPQ	2	0
c.100-2_141del44	Loss of splice acceptor	1	0
c.100-37_141del79insTTC	Loss of splice acceptor	1	0
c.100-6_157del64	Loss of splice acceptor	1	0
c.100-7_134del42	Loss of splice acceptor	1	0
c.100-11_129del41	Loss of splice acceptor	0	1
c.100-22_100-1del22	Loss of splice acceptor	0	1
c.101A>G, c.104_112del9	p.D34G, p.E35_T37	1	0
c.104_121dup18	p.E35_N40dup	1	0
c.104_121del18	p.E35_N40del	0	1
c.110_118dup9	p.T37_L39dup	0	1
c.111_155del45	p.A38_S52del	1	1
c.113_121del9	p.A38_N40del	0	1
c.117_134del18	p.L39_G44del	1	0
c.119_148del30	p.N40T, p.V41_A50del	0	1
c.120_125del6	p.N40_V41del	0	1
c.122_148del27	p.V41_P49del	1	1
c.122_157del36	p.V41_S52del	0	2
c.123_158del36	p.K42_G53del	1	0
c.123_164del42	p.K42_E55del	0	1
c.124_129dup6	p.K42_Q43dup	1	0
c.124_150del27, c.150T>C	p.K42_A50del, p.A50A	1	0
c.133_147del15	p.F45_P49del	0	1
c.129A>G, c.133_150del18	p.Q43Q, p.F45_A50del	1	0
c.138_164del27	p.N46_E55del	1	0
c.141_167del27	p.Q48_H56del	2	0
c.146_166del21	p.P49_E55del	1	0

component could conceivably produce the peculiar morphology of these fibroepithelial tumours. Interestingly, previous studies showed that allelic imbalance and monoclonality can also occur in the epithelium of phyllodes tumours (Sawyer *et al*, 2000; Kuijper *et al*, 2002). Considering the exclusive presence of MED12 mutations in the stromal component, clonal expansion may take place in the epithelial component independent of the stromal component.

The discrimination of phyllodes tumours and fibroadenomas is a clinically important issue because their management differs considerably. Fibroadenomas do not recur after complete resection, and conservative management, including a follow-up without excision, could be a valid option (Cant *et al*, 1995). On the other hand, phyllodes tumours, including benign lesions, may cause local recurrences and borderline and malignant lesions can develop

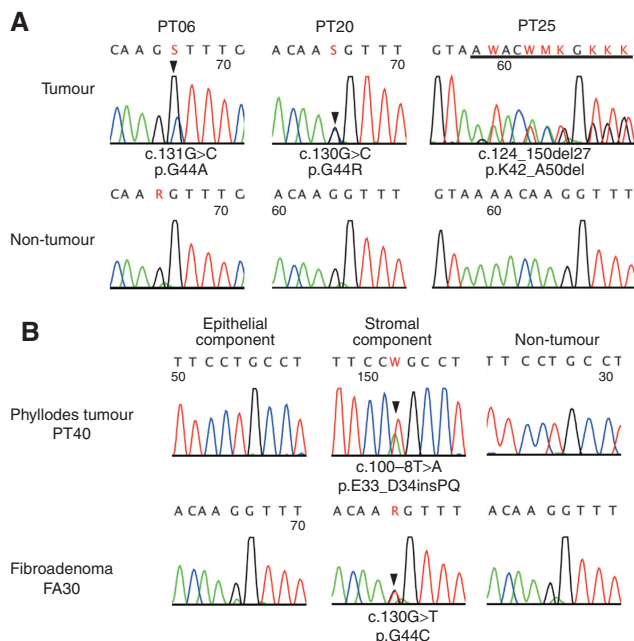


Figure 2. MED12 mutations in phyllodes tumours and fibroadenomas of the breast. (A) MED12 mutations in phyllodes tumours. **(B)** Microdissection-based analysis of MED12 mutations. Epithelial and stromal components of phyllodes tumours or fibroadenomas were separately microdissected and analysed for the presence of MED12 mutations. The arrowheads indicate missense mutations. The underline indicates deletions.

distant metastases (Chaney *et al*, 2000; Chen *et al*, 2005). However, the histological distinction of fibroadenomas and phyllodes tumours is based on several subjective findings, including the status of the tumour border, cellularity, and stromal atypia, and is not always straightforward (Lee *et al*, 2007; Tavassoli and Eusebi, 2009; Tan *et al*, 2012; Yang *et al*, 2014). A recent interobserver variability study highlighted a significant overlap in the morphology of cellular fibroadenomas and benign phyllodes tumours; a concordant diagnosis among 10 pathologists, who specialised in breast pathology, was made for only 2 out of 21 tumours (Lawton *et al*, 2014). The present study revealed that phyllodes tumours and fibroadenomas share a common genetic abnormality, in addition to their morphological features. This raises the possibility that fibroadenoma and phyllodes tumours represent a histomorphological continuum, rather than two distinct biological entities.

Some authors have suggested that phyllodes tumours arise from a terminal duct lobular unit *de novo* (Tan *et al*, 2012); on the other hand, several lines of evidence have suggested that phyllodes tumours might arise from fibroadenomas. A retrospective review of 36 malignant phyllodes tumours identified 11 cases that had transformed from fibroadenomas during a follow-up period (Abe *et al*, 2011). Also, some studies have reported that a subset of phyllodes tumours is associated with a previous history of resection for fibroadenomas (Lindquist *et al*, 1982; Chen *et al*, 2005). Furthermore, Noguchi *et al* (1995) analysed phyllodes tumours that developed following the resection of a fibroadenoma by using an X chromosome inactivation-based clonality analysis and suggested that phyllodes tumours could arise from preceding fibroadenomas. Although further evidence is required to reach a definite conclusion, the common presence of MED12 mutations in phyllodes tumours and fibroadenomas is consistent with the idea that fibroadenomas are potential precursors to phyllodes tumours.

The present study revealed the presence of MED12 mutations in the majority of phyllodes tumours, regardless of the tumour grade. Similar to

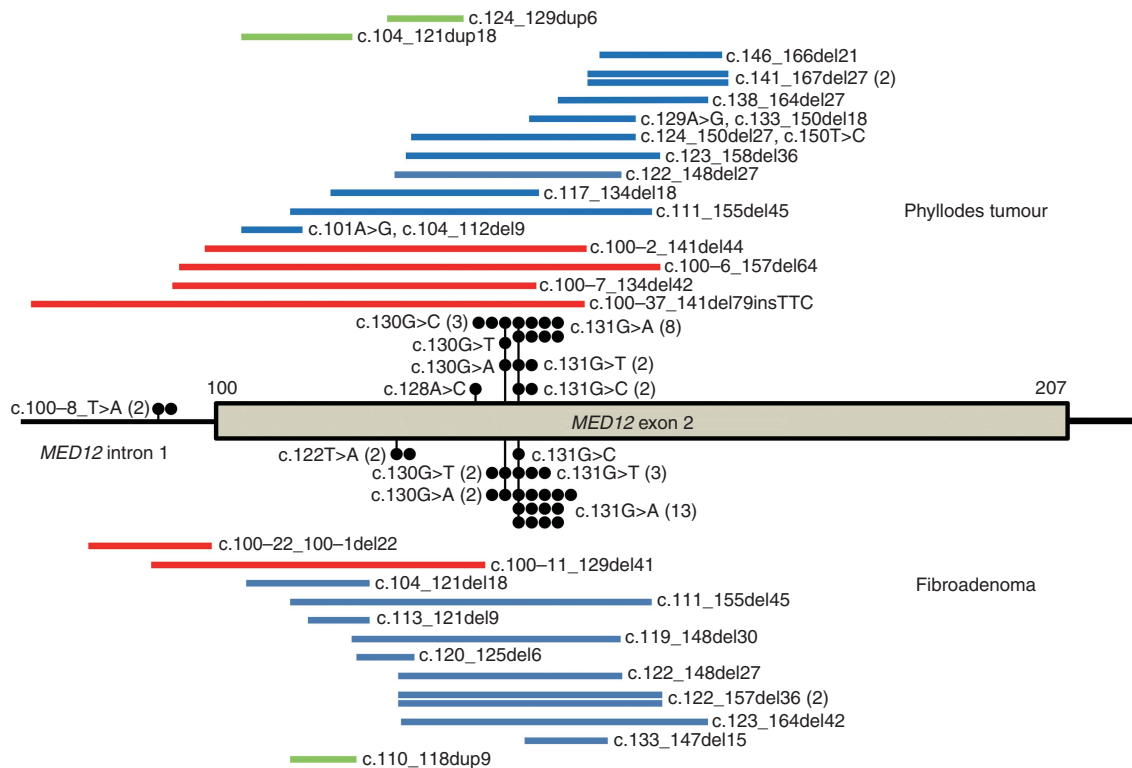


Figure 3. Distribution of MED12 mutations in phyllodes tumours and fibroadenomas of the breast. Closed circles, point mutations; red bars, deletions involving splice site acceptor; blue bars, in-frame deletions; green bars, duplications.

their involvement in fibroadenomas, MED12 mutations are thought to have a critical role in the tumourigenesis of phyllodes tumours. Furthermore, the common presence of MED12 mutations in these tumours may imply a histogenetic relationship between these two lesions.

ACKNOWLEDGEMENTS

We are grateful to Ms. Sachiko Miura, Ms. Toshiko Sakaguchi and Ms. Chizu Kina for the technical assistance. This work was supported in part by the National Cancer Center Research and Development Fund (26-A-4).

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Supplementary Information accompanies this paper on British Journal of Cancer website (<http://www.nature.com/bjc>)