Recent insights into the molecular pathogenesis of mammary phyllodes tumours

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ABSTRACT

Phyllodes tumours (PTs) of the breast are true biphasic neoplasms within which interactions between the epithelium and stroma are critical for tumour development and progression. Despite numerous studies reporting the results of ancillary marker investigations in PTs, the current histological grading systems remain unreliable at predicting clinical outcome even when supplemented by these markers. As a consequence, there has been much interest in the prospect of using molecular/genetic techniques to develop a more robust "grading" system. This review focuses on recent cytogenetic and molecular studies investigating the pathogenesis of PTs and those correlating molecular findings with clinicopathological features of the tumours. Recent data highlight that intratumoural genetic heterogeneity is common in PTs and may account for the reported lack of correlation between histological grading and clinical behaviour. The entire spectrum of molecular aberrations in PTs are yet to be fully defined, however recent array-based studies using comparative genomic hybridisation have reported that copy number changes increase with the progression from benign PT to malignancy. Tumour recurrence and progression is likely to reflect the presence of under-recognised subclones. $p^{16INK4a}$ *(CDKN2A)* inactivation also appears to be important in PT pathogenesis. Further additional studies will be required to identify and validate new prognostic markers and therapeutic targets in order to improve the diagnosis, classification, prediction of outcome and management of patients with this rare neoplasm. Data generated from modern sequencing technologies are likely to provide new insights into the disease and assist in this endeavour.

INTRODUCTION

Mammary phyllodes tumours (PTs) are rare fibroepithelial neoplasms of the breast. The term 'fibroepithelial' refers to their histological composition; they are composed of epithelial and stromal elements, like the common fibroepithelial lesion, fibroadenoma (FA). It is this combination of epithelial and stromal components, integral to the tumour that makes PTs an ideal model within which to study epithelial-stromal interactions.

The diagnosis and classification of PTs often presents challenges to pathologists, particularly in their distinction from FAs. The latter are one of the most frequent causes of a breast lump and approximately 50 times more common than PTs.[1] FAs and PTs differ in their biological behaviour; the latter has a much more frequent propensity for local recurrence and the potential to metastasise and hence there are important differences in how they are managed. However, both tumours are part of a morphological spectrum and can be very difficult to differentiate clinically, radiologically, on fine needle biopsy cytology, on core biopsy and even on excision histology. Cellular FAs and juvenile FAs can be particularly difficult to differentiate from low grade PTs. Although PTs historically occur in an older age group and can present with a larger, growing mass, there is significant overlap with the age at presentation, size and rate of growth of FAs. With the modern practice of population-based breast screening and improvements in public education, there is a tendency for PT to present at an early clinical stage when they are small in size. Furthermore, in Asian/Latina white women, PTs also tend to present at a young age. These factors compound the difficulty in diagnosis.[2]

THEORIES FOR THE ORIGIN OF PT

Various hypotheses have been proposed for the origin of PTs. Genome wide loss of heterozygosity (LOH) was studied in PTs and FAs by Wang *et al*.[3] They found one PT with malignant epithelium and stroma that appeared to share a LOH genotype suggestive of a common progenitor origin for both components.[3]

A case of metastatic malignant PT to the lung has been reported which had epithelial ducts in addition to liposarcomatous stroma, similar to the primary tumour in the breast.[4] The ductular structures had morphological features (double cell layer with outer smooth muscle actin positive clear cells indicative of myoepithelial cells) and immunoprofile (positive for hormone receptors and gross cystic duct fluid protein (GCDFP)-15) consistent with mammary origin from the PT. This metastatic spread of benign-appearing epithelial components and malignant stroma could support a common progenitor origin.

However, Sawyer *et al*[5] showed allelic imbalance by comparative genomic hybridisation (CGH) of chromosome 1q and 3p in the epithelium and stroma of PTs, sometimes independent of one another. In prostatic PTs, McCarthy *et al*[6] found evidence of clonal origin for the epithelium and stroma with significantly different patterns of allelic loss suggesting different clonal origins.

GRADING OF PTS

PTs have a spectrum of morphologies and are divided into benign/low grade tumours to

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borderline/intermediate grade tumours and malignant/high grade tumours (figure 1), in an attempt to predict their clinical behaviour. However, the reliability of prediction of clinical outcomes based on morphological features (grade), even with clinical and radiological correlation, is poor. Furthermore, the difficulty in predicting outcome is reflected in the use of a multitude of grading systems which further complicates interpretation of the literature.

All of the grading systems advocate assessment of the same histological parameters (namely stromal overgrowth, stromal cellularity, stromal atypia, stromal mitotic rate, margin characteristics); however the cut-offs used for each parameter in the various grading systems differ. Most authors use a three-tiered grading system: 'benign, borderline and malignant' PT. However, some prefer to refer to the three tiers as 'low grade, intermediate grade or high grade/malignant PT' to acknowledge the metastatic potential of even so-called 'benign' tumours (again reflecting the unreliability of the grading systems). Several authors advocate a two-tiered classification (low grade and high grade PT).[7]

The 2012 WHO classification of breast tumours recommends a three-tiered grading system presented in table 1. However it uses subjective cut-offs that limit its usefulness in clinical practice.[8] Recognition that histological parameters may be of different importance and weightage has led to a proposed predictive nomogram that can assist in calculating an individual's risk of recurrence based on criteria of stromal atypia, mitotic rate, stromal overgrowth and surgical margins.[9] The utility of this nomogram however, awaits validation by other series of PTs. The heterogeneity between and within individual PTs is marked and this may also cause some difficulty in applying the grading criteria. Most systems advocate grading on the worst area for any particular feature. Furthermore, it is recognised that tumour regions that appear homogenous on light microscopy may in fact be heterogeneous on a molecular level. These unrecognised subclones (discussed in detail below) may account for the unreliability of the histological grading schema.

An improved understanding of the biology of PTs may lead to the identification of better prognostic indicators and the development of a more robust grading system. The desired end result is to tailor management more effectively. PTs are often misdiagnosed or mismanaged with dominant themes of underdiagnosis and undertreatment. There is a need to more reliably identify the patients who may benefit from more aggressive/adjuvant treatment and monitoring. The PTs that will recur and/or metastasise are not well delineated with the currently available diagnostic tools. On the other hand, it is also likely that the more frequent PTs with true 'benign' behaviour are being overtreated.

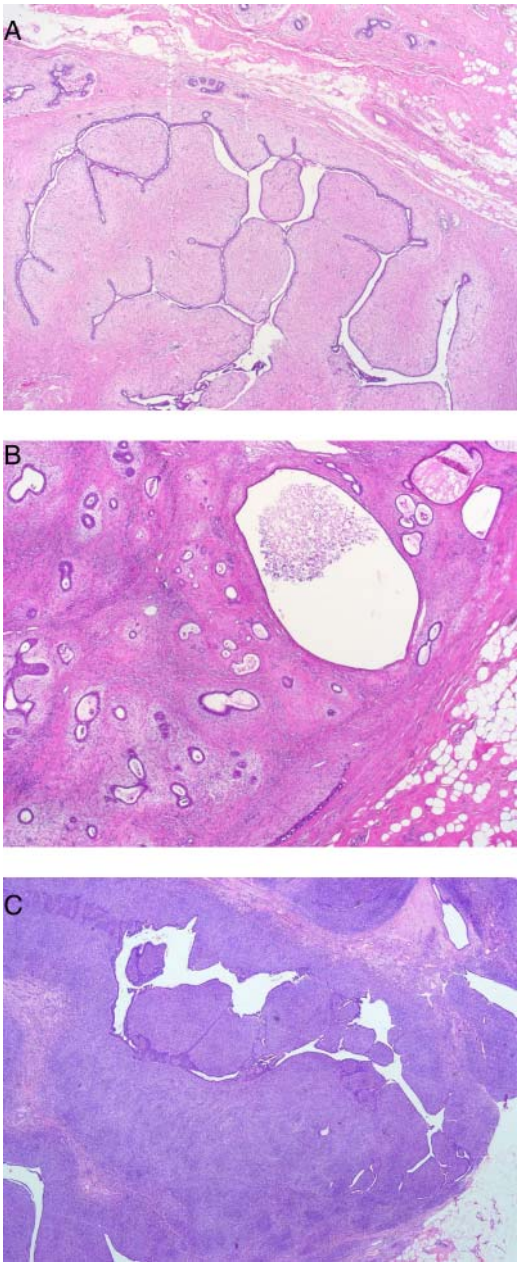

Figure 1 Histology of phyllodes tumours (PTs). (A) Benign PT: Classical leaf-like architecture with mild stromal cellularity. (B) Borderline PT: Increased stromal cellularity. (C) Malignant PT: Markedly cellular and atypical stroma with stromal overgrowth.

Table 1 The 2012 WHO three-tiered grading system for phyllodes tumours (PTs)[8]

	Grade of PT		
Criterion	**Benign**	**Borderline**	**Malignant**
Tumour border	Well-defined	Well-defined, may be focally permeative	Permeative
Stromal cellularity	Cellular, usually mild, maybe non-uniform or diffuse	Cellular, usually moderate, maybe non-uniform or diffuse	Cellular, usually marked and diffuse
Stromal atypia	Mild or none	Mild or moderate	Marked
Mitotic activity	Usually few (<5 per 10 hpf)	Usually frequent (5–9 per 10 hpf)	Usually abundant (>10 per hpf)
Stromal overgrowth	Absent	Absent or very focal	Often present
Malignant heterologous elements	Absent	Absent	May be present
Distribution relative to all breast tumours	Uncommon	Rare	Rare
Relative proportion of all PTs	60–75%	15–20%	10–20%

ANCILLARY TESTING

As studies of clinical and histological features had not yet yielded reliable prognostic factors, a variety of ancillary tests and biomarkers have been studied in PTs. Most of the studies used immunohistochemical assays, although in situ hybridisation techniques, flow cytometry and AgNOR counts have also been studied in PTs. These studies are summarised in table 2. Few have shown any correlation with outcome measures but some show stratification by grade.

There has been much recent interest in the prospect of genetic 'grading' with an increased focus on cytogenetic and molecular studies to advance knowledge of PT pathogenesis, upon which this review will focus (table 3).

CYTOGENETIC AND MOLECULAR GENETIC PATHOLOGY OF PTs

Clonality

PTs were previously believed to be stromal neoplasms with entrapped or benign 'innocent' epithelial elements. The original hypothesis was that FAs were polyclonal stromal and epithelial proliferations, and PTs were monoclonal stromal and polyclonal epithelial proliferations.[7] In 1993, Noguchi *et al*[10] showed that

Table 2 Studies of ancillary tests and markers in phyllodes tumours

Author	No. of cases	Test	Findings summarised
Layfield[54]	6 giant FAs, 10 PTs	Flow cytometry	No correlation between DNA ploidy histology, recurrence or metastasis
Samarat-unga[55]	28 PTs	AgNOR counts Flow cytometry	AgNOR counts and flow results ∝ survival status
Keelan[56]	60 PTs	Flow cytometry	Flow results did not correlate with outcome
Niezabit-owski[37]	118 PTs	Flow cytometry, p53*, Ki67*	S phase fraction and p53 ∝ DFS Ki67 ∝ OS
Kleer[24]	20 PTs	p53*, Ki67*	Ki67 and p53 ∝ grade but not recurrence
Yonemori[38]	41PT	p53*, Ki67*, EGFR* HER2*, CD117*	Ki67 and p53 ∝ DFS and OS EGFR no correlation HER 2 and CD117 no staining
Shpitz[57]	23 PTs	p53*, Ki67*, HERr2*	P53 ∝ grade but not recurrence
Tse[58]	186 PTs	p53*, MVD*	p53 ∝ MVD MVD ∝ cellularity, malignancy, margin status
Tse[59]	143 PTs	p53*	P53 ∝ grade, mitoses
Gatalica [43]	25 PTs	p53*	P53 ∝ grade
Millar[60]	20 FAs, 15 PTs	p53*	Strong stromal p53 in malignant PTs only
Erhan[39]	21 PTs	p53* and Ki67*	Ki67 and p53 stained most malignant PTs
Esposito[32]	30 PTs	p53*, Ki67*, CD117*, p16*, p21*, ED1*	CD117 and negative ED1 ∝ malignancy Ki67, p53, p16 and p21 differentially expressed though the grades
Dacic[34]	23 PTs	p53*, Ki67*, MVD*, bFGF*, UK*	Ki67 and p53 ∝ grade Correlation between epithelial and stromal expression of bFGF and UK which increased with grade.
Ridgway[35]	7 cellular FA, 15 PTs core biopsy	Ki67*	Ki67 may help differentiated cellular FA and PT on core biopsy
Kang[36]	20 FA, 20 juvenile FA, 92 PTs	Ki67* APC7*	Ki67 and APC7 ∝ grade
Noronha[33]	33 Pts	Ki67*, CD117*, CD34*	CD117 and CD34 differentially expressed with grade
Tan[45]	335 PTs	p53*, CD117*	CD117 and p53 ∝ grade CD117 ∝ recurrence
Korcheva[51]	7 FA, 31 PTs	P53*, CD117*, PH3*, mdm2*, cdk4* PCR for 30 cancer-related genes	p53 and PH3 ∝ grade S8R substitution in *FBX4* in 3 PTs
Tse[46]	179 PTs	CD117*	CD117 ∝ grade
Sawyer[47]	30 PTS	CD117* c-myc ISH and IHC	CD117 and c-myc ∝ grade
Bose[50]	17 Pts	CD117* *kit* mutation analysis	No activating mutations
Djordejevic[53]	12 FA, 9 cellular FA, 47 PTs	CD117* Toludine blue	CD117 staining is due to mast cells
Logullo[52]	14 FA, 12 juvenile FA, 70 PTs	CD117*	CD117 associated with benign lesions
Carvalho[48]	19 PTs	CD117*, PDGFRA* Direct sequencing of *c-kit* and *PDGFRA* gene	CD-117 increased in malignant PTs. No activating mutations
Chen[49]	19 PTs	CD117*, actin*, CD34* *c-kit* partial sequencing	CD34 expressed in benign PTs CD117 and actin expressed in malignant PTs Point mutations in c-kit in 2 malignant PTs
Tse[61]	143 PTs	ER*, PR*, AR* in epithelium	ER and PR inversely ∝ grade ER inversely ∝ mitoses
Rao[62]	13 Fas, 5 PTs	ER and PR biochemical assays	PR in stroma of all PTs and 11 Fas ER in epithelium of 1 PT only

Continued

Table 2 Continued

Author	No. of cases	Test	Findings summarised
Lien[63]	400 samples;44 PTs	GR*	GR++ in malignant PTs and MCa
Sapino[64]	33 Fas, 40PTs	ER-α*, ER-β*, SMA*, calponin* RT-PCR on stroma for ER-β mRNA	Only ER-β detected in stroma of PTs and Fas ER-β+ cells double stain with SMA ER-β+ FA patients are younger
Kuijper[65]	47 PTs	Ki67*, cyclin A*, cyclin D1*, pRb*, p53*, p16*, bcl-2*, p21*	Stromal over expression of p16, p53, pRb, p21 and cyclin A ∝ grade (cyclin A best) p53 ∝ DFS >3 over expressed stromal markers ∝ DFS No over expression in epithelium
Karim[66]	65 PTs	p16*, pRb, cyclin D1, Ki67	Stromal p16, stromal and epi pRb, stromal and epi Ki67 ∝ grade Epi pRb >7 ∝ reduced DFS
Tse[67]	40 PTs	MVD* via CD31*	Significant difference in MVD between benign and borderline PTs (higher in latter)
Tse[68]	185 PTs	VEGF*, MVD*	Stromal VEGF and MVD ∝ grade Stromal VEGF ∝ MVD
Tse[69]	167 PTs	e-NOS*, i-NOS*	Stromal e-NOS and i-NOS differed significantly between benign and malignant groups of PTs Stromal i-NOS ∝ VEGF and MVD
Tse[70]	461 PTs	ED-1*	ED-1 ∝ atypical histology
Tse[69]	33 FAs, 181 PTs	CD10*	Stromal CD10 ∝ benign (FA benign PT) vs Malignant (PT)
Tsai[71]	22 PTs	CD10*, SMA*, vimentin*	CD10, SMA and vimentin ∝ WHO grading
Zamecnik[72]	20 FAs, 6PTs	CD10*	Stromal CD10 does not distinguish between FA and PT.
Al-Masri[73]	43 PTs	CD10*	CD10 ∝ grade distant metastases
Moore[74]	15 FAs, 26 PTs, other spindle lesions	Bcl-2*, CD34*	Combined bcl-2 and CD34 expressed in stroma of FA, PTs and PASH
Dunne[75]	26 PTs, 18 MCa, 8 fibromatosis	Multiple IHCs	CD34 and bcl-2 + stroma distinguish PTs
Kuijper[76]	30 FAs, 37 PTs	HIF-1α*, CAIX*, VEGF*, p53*	HIF-1α ∝ DFS VEGF ∝ grade
Koo[77]	232 PTs	10E4* (HS), p53*, Ki67* CD117*, bcl-2*	Stromal 10E4 ∝ grade, Ki67 and p53
Feakins[78]	11 FAs, 46 PTs, 6 normal breast	PDGF*, PDGFRβ8	Stromal PDGFRβ8 and epithelial PDGF with stromal PDGFRβ8 ∝ death from disease, atypia and stromal overgrowth
Sawyer[79]	119 PTs	β-catenin*, cyclin D1* Wnt2 and Wnt5a ISH Mutation screening for β-catenin and APC genes	Stromal nuclear β-catenin ∝ epithelial Wnt5a
Sawyer[80]	16 FAs, 23 PTs	ILGF-I and ILGF-II ISH Correlated with 122	ILGF-1 ∝ nuclear β-catenin in PTs
Karim[81]	65 PTs	β-catenin*, Wnt1*, Wnt5a*, SFRP4*, E-cadherin*	Epi and stromal β-catenin, epi Wnt1, epi E-cadherin ∝ grade Epi E-cadherin ∝ decreased DFS
Lacroix-Trixi[82]	52 MCa, 8 fibromatosis, 23 PTs	β-catenin*	94% benign lesions positive 57% malignant lesions positive
Tsang[83]	158 PTs	Stromal α-catenin*, β-catenin*, E-cadherin*	α-catenin ∝ β-catenin α-catenin ∝ recurrence
Tsang[84]	155 PTs	Epi E-cadherin	E-cadherin ∝ stromal cellularity, overgrowth and mitoses E-cadherin ∝ increased recurrence and decreased DFS
Kersting[29]	58 PTs	EGFR FISH Gene sequencing of intron 1 of egfr Multiple IHCs	EGFR over expression ∝ grade, EGFR over expression ∝ intron 1 amplification, EGFR over expression ∝ p53, Ki67, CD117, p16, cyclin A and cyclin E Intron 1 amplification ∝ p53, p16 and p21
Suo[28]	22 PTs	EGFR*, c-erb B3*, c-erbB4*, ER*, PR*, p53*, Ki67*, BM28*	EGFR, c-erb B3, c-erbB4, p53, Ki67, BM28 ∝ malignancy
Tse[30]	453 PTs	EGFR* and FISH	EGFR ∝ grade egfr amplification in only 8%
Kwon[85]	207 PTs	N-Cadherin*, Twist*, TGF-β*, HMGA2*, S100A4*, Ezrin*, SDF1*, CXCR4*	Stromal Twist, HMGA2, S100A4, CXCR4, TGF-β ∝ grade Twist ∝ decreased DFS and OS
Kim[86]	82 PTs	MMP-1*, -2*, -7*, -9*, -11*, -13*, -14* TIMP-1*, -2*, -3*	Stromal MMP-14 ∝ grade
Chong[87]	145 PTs	Keratin 15*, TCN1*, HOXB13*	Keratin 15, TCN1 and HOXB13 ∝ grade
Chia[88]	109 PTs	Keratin panel*	Focal staining in stromal cells

AR, androgen receptor; APC7, anaphase promoting complex 7; BFGF, basic fibroblast growth factor; BM28, proliferative marker; CAIX, carbonic anhydrase IX; cdk4, cyclin dependent kinase 4; DFS, disease free survival; EGFR, epidermal growth factor receptor; Epi, epithelial; ED1, endothelin-1; ER, oestrogen receptor; e-NOS, endothelial nitric oxide synthase; FA, fibroadenoma; FISH, fluorescence in-situ hybridisation; GR, glucocorticoid receptor; HOXB13, antibody to homoebox gene; HIF-1α, hypoxia-inducible factor 1 α; HS, heparan sulphate; ISH, in situ hybridisation; i-NOS, inducible nitric oxide synthase; ILGF, insulin-like growth factor; MVD, microvessel density; MCa, metaplastic carcinoma; MMP, matrix metalloprotease; OS, overall survival; PASH, pseudoangiomatous stromal hyperplasia; PH3, phosphohistone 3; PDGRFA, platelet derived growth receptor A; PR, progesterone receptor; PDGF, platelet derived growth factor; PDGFRβ, PDGF receptor β; SMA, smooth muscle actin; SFRP4, secreted frizzled related protein 4; TIMP, tissue inhibitors of metalloproteases; TCN1, transcobalamin I; UK, urokinase; VEGF, vascular endothelial growth factor; ∝, correlates with; *, immunohistochemical stain (IHC).

FAs were polyclonal and suggested that they should be considered as hyperplastic lesions rather than neoplasms, in contrast to the monoclonal and truly neoplastic PT. Monoclonality does not necessarily equate with neoplasia and vice versa and more recent studies have found that the distinction is not so simple. Clonal analysis, based on trinucleotide repeat polymorphism and methylation of the X-chromosome linked androgen receptor gene, was also studied by Noguchi's group in three patients who developed PTs at the same site of a previous FA resection.[11] They showed that the original FAs were monoclonal as were the recurrent PTs. Furthermore, it was the same androgen receptor gene allele that was inactivated in each of the patients' tumours.[11] They therefore suggested that, in a proportion of FA, somatic mutations can result in monoclonal proliferation (that on histology appears identical to the polyclonal proliferation) which has a propensity to progression to PT and recurrence.[11] Areas of apparent stromal overgrowth in FAs have also been shown to be monoclonal by PCR-based clonality assays.[12] Kuijper *et al*[12] proposed a tumour progression model in which FAs (polyclonal) could progress in stromal and/or epithelial directions from polyclonal hyperplasia to monoclonal expansion, to PT or carcinoma in situ, respectively.

There are several recent studies that assessed clonality in the epithelial and stromal components in PTs and the results suggest that both elements are neoplastic and interact. Whether the epithelium and stroma have the same clonal origin is still controversial. Dietrich *et al*[13] reported the karyotypes of five PTs in 1994 and found that the epithelium was polyclonal. However, a subsequent publication by the same group reported that clonal abnormalities were found in the epithelium and stroma.[14] Kuijper *et al*'s[12] PCR-based clonal assay of PTs and FAs found that while in most cases PT stroma was monoclonal and epithelium was polyclonal, there were two cases with monoclonal epithelium and three cases with polyclonal stroma.

Karyotyping

No recurrent chromosomal aberrations specific to PTs have been identified to date. However, karyotypic complexity has been suggested as a marker of malignancy in PTs. Dietrich *et al*[13] detected clonal chromosomal abnormalities in the mesenchymal component of all five tumours that they studied in 1994. Four of the PTs (one benign, two borderline and one malignant with low grade areas) showed simple structural abnormalities, however the fifth PT was purely malignant and had a complex hypodiploid clone with evidence of clonal evolution. A recurrent PT had the identical karyotype to its original primary. Dietrich *et al*'s[14] subsequent study of six PTs showed that all had clonal karyotypic abnormalities and the five benign PTs had simple chromosomal abnormalities but the one malignant PT was nearly triploid. However, in contrast, in 2002 Ladesich *et al*[15] demonstrated a complex karyotype in a benign/low grade PT.

In experimental cell systems, two cell lines that were derived from a histologically benign PT by xenograft and direct cell culture[16] grew as monolayers of spindle cells with short doubling times (1.5 days), and were aneuploid with loss of an X chromosome. The authors suggested that the ability of the tumour to form stable cell lines in culture and the aneuploidy cast doubt on whether the PT classified as benign based on their morphological features were truly benign tumours.

Loss of heterozygosity

Wang *et al* compared LOH in PTs and FAs and found that LOH was frequent but heterogeneous in PTs, and largely absent in FAs, supporting their distinction as separate entities.[3] High grade PTs were more genetically unstable with a higher fractional level of LOH compared with lower grade tumours. Paired primary and recurrent tumours showed evidence of the same clonal origin and progression (increased numbers of LOH loci in the recurrent tumours). There were some repeated LOH loci (3p, 17q, 4q, 7q and 7p) raising the possibility that these chromosomal regions may include tumour suppressor genes important for the pathogenesis of PT. Loss at 7p12 was seen in 50% of the PTs studied and more frequently in high grade tumours. Loss at 3p24 was more frequent in benign and intermediate grade PTs, and is also present in tissue adjacent to breast carcinoma.[3] This suggests that loss of 3p24 may be an early event, and loss of 7p12 a later event in the neoplastic pathway.

Comparative genomic hybridisation

Chromosome based CGH allows genome-wide screening of the chromosomes for copy number gains and losses in a single experiment. Initial CGH studies by Lu *et al*[17] found that most chromosomes involved in PTs showed a pattern of gains and losses similar to breast carcinoma, but the genomic amplifications common in breast carcinomas were not identified in PTs. Gain of 1q was statistically significantly associated with stromal overgrowth and recurrence, and was suggested as a potential marker of local aggression. Similarly, Jee *et al*[18] found gain of 1q in 14 of 22 PT cases, but this included benign and malignant PTs and was not associated with grade or outcome.

Lae *et al*[19] presented the results of CGH on 30 PTs in 2007 which were graded by the then WHO criteria. The most frequent imbalances were gain of 1q, loss of 13q, loss of 6q, gain of 5p and loss of 10p. Recurrent imbalances were found in 55%, 91% and 100% of benign, borderline and malignant PTs, respectively. The mean numbers of chromosomal changes were 1, 6 and 6 in benign, borderline and malignant PTs, respectively. They found that the tumours appeared to segregate into two groups by patterns of genomic imbalance, thus providing molecular evidence in support of a two-tiered grading system. Borderline and malignant PTs could not be distinguished on the basis of their CGH changes in this study but there were numerous recurrent chromosomal changes that were distinct from benign PTs. The former group frequently showed gain of 1q and 13q. Gain of 1q is one of the most common changes observed in human solid tumours.[19] The loss of 13q involved a small region at 13q14.2, where the *Rb1* gene localises suggesting it could be the target of deletions.[19]

Lv *et al*[20] similarly found that the results of their CGH studies of the stroma of 36 PTs supported a two-tier grading system. The chromosomal copy number findings of 12 borderline and 12 malignant PTs were very similar, but could be distinguished from the 12 benign PTs by an increased number of chromosomal gains in a non-random distribution. Gains were more frequent than losses in borderline and malignant PTs, but they were in balance in benign PTs. However, no single recurrent alteration characterising the borderline and/or malignant PTs could be found. The most frequent abnormal chromosomal region was in Chr 4q12 and the authors postulated that it may harbour genes important in the progression of PTs. Candidate genes at this region include stem cell factor receptor oncogene homologue, kinase insert domain receptor and α-foetoprotein. Gain of 1q did not correlate with grade or any histological parameters in contrast to the earlier findings by Lu *et al*,[17] which may be related to the small number of cases in the latter.

Array based CGH studies

Array/microarray-based CGH uses genomic clones or oligonucleotides as targets in contrast to the metaphase chromosomal targets of chromosome based CGH[21] allowing direct mapping of alterations to the genome sequence and facilitating identification of oncogenes and tumour suppressor genes.

In an array-based CGH study of a large cohort of 126 PTs (37 benign, 41 borderline and 48 malignant; grading method unspecified), Jones *et al*[22] found that copy number changes were uncommon in benign PTs but increased in frequency in borderline and malignant tumours. The changes were on a chromosomal scale (whole or arms of chromosomes). The main genetic changes in the malignant and borderline PTs included gain of 1q, loss of 13q, loss of 6, gain of 5p and loss of 10p, in addition to loss of 9p and gain of 7 and 8. In contrast to Lae *et al*,[23] cluster analysis of Jones *et al*'s data supported division of PTs into two groups with malignant PTs in one category, and benign and borderline PTs together. These apparently contrasting results may reflect differences in the criteria employed for classifying PT or the application/interpretation of the diagnostic criteria therein.

In the study above, Jones *et al* studied expression using Affymetrix U1133A GeneChips, and noted that the 9p21 interstitial deletion involving the $p16^{INK4A}$ locus (the cyclin-dependent kinase inhibitor 2A (*CDKN2A)* gene) was present frequently in malignant and borderline PTs with some causing homozygous loss. There was concomitant loss of $p16^{INK4A}$ expression that was associated with the 9p deletion. The *CDKN2A* gene was sequenced in 35 PTs and one mutation was found. Methylation specific PCR was performed on nine PTs and also showed evidence of hypermethylation of *CDKN2A* in most of the borderline and malignant PTs. The authors concluded that inactivation of $p16^{INK4A}$ is important in the progression to malignant PTs.

In the same study, Jones *et al* microdissected out multiple areas of stroma from the individual tumours with apparent histological homology and performed array-based CGH. They also found marked intratumoural genetic heterogeneity despite corresponding histological monotony. Recurrent and primary paired samples of PTs were also analysed and recurrences were found to have acquired new genetic changes when compared with their respective primary tumours. Some benign PTs acquired genetic changes typical of malignant PTs, but there was no associated histological alteration. The authors suggest that the poor correlation between the current histological grading and behaviour is due to intratumoural genetic heterogeneity and the inability to identify these unfavourable subclones on histology. Furthermore, they suggested that disease progression usually occurs as a consequence of recurrence of a previously unidentified unfavourable subclone, although true malignant transformation of a truly benign residual PT cannot be excluded.

Another group to use array-based CGH to study PTs was Kuijper *et al*[21] who published their findings of an analysis of 11 PT s (five benign, one borderline and five malignant) and three FAs in 2009. They found no copy number changes in the FAs. Ten of the 11 PTs showed copy number changes but these were not correlated with tumour grade. Recurrent losses were found at 1q, 4p, 10, 13q, 15q, 16, 17p and 19. Recurrent gains were found at 1q, 2p, 3q, 7p, 8q, 16q and 20. Although several of the chromosomal regions affected by recurrent copy number changes include the location of known oncogenes and tumour suppressor genes, specific mutations/alterations were not found on sequencing.

All of the benign PTs in Kuijper *et al*'s study showed copy number changes, and the authors suggested that genomic instability could be an early initiating event in PT pathogenesis and the term 'benign' PT was misleading as these tumours had genetic complexity that distinguished them from the truly benign FAs, which could account for their unpredictable clinical behaviour.

Loss of material in the regions of 3p12 and 3p21 has been found on karyotyping and CGH studies.[5 14 17] The former region includes the location of the *FHIT* gene, commonly deleted in breast carcinoma.[5] The latter region covers the location of the microsatellite instability gene *hMLH1*.[5] Kleer *et al*[24] performed LOH studies on eight PTs for loci on chromosome 3p and found no allelic losses at the *FHIT* and *hMLH1* loci, although the number of cases studied is limited with no significant statistical power.

Gene expression profiling studies

A recent mRNA expression profiling study[25] found that 162 genes were upregulated in the combined borderline-malignant group, and this included genes implicated in disease development, mitoses, cell signalling, cell cycle progression, cell adhesion and extracellular matrix receptor adhesion. In situ hybridisation and immunohistochemistry confirmed that stromal overexpression of four gene products (*PAX3, SIX1, HMGA2, TGFB1*) was significantly associated with the borderline-malignant phenotype. *PAX3* knockdown cell lines were found to have decreased cellular proliferation in the studies of malignant and borderline cell lines. *SIX1* and *HMGA2* knockdown cell lines had decreased cellular proliferation in the malignant cell line only. *TGFB1* knockdown cell lines had decreased cellular proliferation in the borderline cell line only. No activating mutations, amplifications or translocations were found in any of the four genes. In contrast to the above study by Kuijper *et al*, in which a less specific methodology was employed, the authors concluded that these genes were important in the progression to borderline-malignant phenotype, but the mechanisms of genetic alterations were not yet elucidated.

Ang *et al*[26] profiled 21 PTs (6 benign, 10 borderline and 5 malignant) using Affymetrix U133Plus GeneChips. Comparison between the three PT grades yielded a list of 29 genes that accurately classified the tumours into their appropriate histological grades. They noted that upregulation of *HOXB13* was seen in malignant PTs but not in borderline PTs and suggested that it may be involved in the progression to malignancy. They also performed array-based CGH and found that the mean number of genetic changes significantly increased with tumour grade (2.7 in benign, 4.2 in borderline and 9 in malignant), similar to Jones *et al*'s findings.[21 22 26] Again, 1q was the most common chromosomal change in the borderline-malignant group.

Lee *et al* performed array-based CGH on one PT and found similar chromosomal gains and losses as Jones *et al* and Kuijper *et al*,[21 22 27] namely gain of 1q and 5p, and loss of 10p and 13q. However, they also demonstrated loss of 1p36 and 17q11.2. The 1p36 deletion involved a region with 65 genes, including succinate dehydrogenase enzyme subunit B that is lost in various cancers and is associated with Carney's triad. The 17q11.2 deletion contains the *NF1* gene, the germline mutation of which causes neurofibromatosis type 1. Both of these hereditary cancer syndromes are associated with an increased risk of developing stromal tumours, namely gastrointestinal stromal tumours. The authors suggest these genes as targets for further analysis.

Immunohistochemical studies

Several immunohistochemical studies have shown that stromal epidermal growth factor receptor (EGFR) expression increases significantly with increasing tumour grade.[28–30] Kersting *et al*[29] suggested that *EGFR* amplifications were related to PT progression in their gene dosage study assessing *EGFR* gene intron 1 and *EGFR* whole gene amplifications. The occurrence of intron 1 amplifications correlated significantly with PT grade but *EGFR* whole gene amplifications did not. The same group then performed global gene expression analysis by array based CGH on 10 PTs and found that 213 genes were upregulated and 17 were downregulated.[31] Amplifications within the regulatory sequences of *EGFR were* associated with expression of eps15 and caveolin-1. The latter is involved in storage of EGFR and mediating specific EGFR signals in mesenchymal cells; it may be linked to β-catenin and insulin-like growth factor receptor with phosphorylated Akt.

Tse *et al*'s[30] study of EGFR immunohistochemistry in 453 PTs is the largest published to date. They analysed cases positive on immunohistochemistry for EGFR by fluorescence in situ hybridisation for gene amplifications, which were found in only 8% of tumours. The authors concluded that EGFR overexpression was probably involved in the pathogenesis of PTs but mechanisms other than amplification were more likely to be the cause.

Ki67 is now a well-established and robust immunohistochemical marker that has been used for assessment of tumour cell proliferation activity. Many studies of mammary PTs have employed Ki67 immunostaining as an adjunct with other markers of proliferation or tumour aggressiveness. Not surprisingly, Ki67 index has been shown to increase with increasing tumour grades in PTs and correlates with EGFR overexpression.[28 29 32–36] Ki67 index also appears to be a useful prognostic indicator for patients with malignant PTs in which Ki67 expression may influence disease-free and/or overall patient survival.[37–39]

Methylation studies

Methylation of the promoters of cancer-related genes is a critical epigenetic abnormality in neoplasia, where tumour suppressor genes are silenced by CpG island hypermethylation. However, there are very few studies of hypermethylation in PTs. Huang *et al*[40] studied 26 FAs and 86 PTs to evaluate if methylation markers could differentiate between the two tumour types. They undertook methylation-sensitive high resolution melting to screen for promoter DNA methylation changes in 11 genes. Five gene promoters showed methylation in PTs (*RASSF1A, TWIST1, APC, WIFI1* and *MGMT*; the former two being significantly hypermethylated in some PTs). FAs showed background methylation levels in *RASSF1A* and *MGMT* only, suggesting a non-neoplastic origin.

Kim *et al*[41] studied hypermethylation via multiplex-nested PCR in 87 PTs and found a trend of increasing methylation with increasing grade, with a significant increase when borderline and malignant PTs were compared with benign PTs.

Jones *et al*'s[22] original study published in 2008 included methylation specific PCR of *CDKN2A* and showed methylation of the locus in two of three malignant PTs and all of five borderline PTs, confirming the importance of methylation in the pathogenesis of PT.

Mutation analysis

P53

PTs are common tumours in patients with Li-Fraumeni syndrome (caused by a germline mutation in *TP53).*[42] *TP53* has been found to be frequently overexpressed in PTs, especially malignant PTs.[7 43] Gatalica *et al*[43] sequenced *TP53* in one PT from a patient without known germline mutations and found a missense mutation on exon 7 in the microdissected malignant component. However, Wooley *et al*[44] showed, by *TP53* gene sequencing, that a metastatic malignant PT can have an intact wild type *TP53* gene sequence. Jones *et al*[22] screened 24 PTs for *TP53* mutations as 17p deletions were one of the most common array-based CGH change in their study of 126 PTs, but none were found, suggesting an alternate target for the deletion. They also screened for three potential tumour suppressor genes (*RBBP4*, *FABP3* and *HDAC1*) from the vicinity of 1p deletion but no mutations were found.

KIT

KIT is a proto-oncogene that encodes CD117, a type III membrane bound tyrosine kinase receptor.[45–47] c-kit mutations and activation are present in many human tumours, including gastrointestinal stromal tumours.[46] There has been renewed interest in the role of *KIT* in various tumours since the development of a number of clinical efficacious targeted therapeutic agents specific against tumours with activating mutations in *KIT.* There have been several studies investigating CD117 immunohistochemical expression in PTs which have all shown that stromal CD117 increases significantly with increasing tumour grade and is predictive of recurrence.[32 33 45 46 48 49] However, on sequencing, only rare point mutations in *KIT* are found in PTs and no activating mutations have been described to date.[47–50] These results suggest that targeted therapies for *KIT* mutations may not be effective therapies in PTs. Furthermore, some recent immunohistochemical studies have found no correlation between CD117 expression and PT grade, and that the reported staining may be due to background mast cells.[51–53] One of these studies also performed mutational analysis via a Sequenom MassARRAY system that screened for mutations in a panel of 30 cancer related genes in 31 PTs.[51] While no mutations in *KIT* were found, an S8R substitution in *FBX4* (an E3 ubiquitin ligase) was identified in three PTs (one benign and two borderline).

CONCLUSIONS

The epithelium and stroma components are critical in the development and progression of PTs and the former is no longer thought of as an 'innocent' bystander. Furthermore, epithelial-stromal interactions are important in this biphasic neoplasm. Intratumoural genetic heterogeneity has been demonstrated and may account for the lack of correlation between histological grading and clinical behaviour. Tumour recurrence and progression is likely to reflect the presence of under-recognised subclones, however true genetic progression may also occur. Large array-based CGH studies have found that copy number changes are uncommon in benign PTs but increase in number with the progression to malignancy (however this is controversial and

Table 3 Studies supporting various genetic grading schemas

Benign vs Borderline +Malignant (2 tier)	Benign+Borderline vs Malignant (2 tier)	Benign vs Borderline vs Malignant (3 tier)
Lae *et al*[23]	Jones *et al*[22]	Ang *et al*[26]
Lv *et al*[20]		
Kim *et al*[41]		

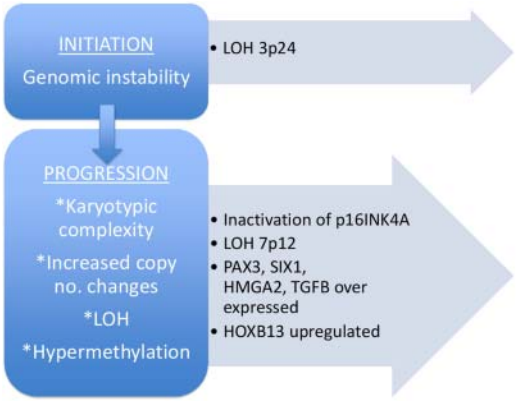

Figure 2 Molecular mechanisms of initiation and progression of phyllodes tumours.

some smaller studies suggest that genomic instability is an early event and important in tumour initiation). Changes tend to be large scale with whole chromosome or chromosome arms involved. It has been challenging to identify specific genes involved by mutation analysis, however $p^{16INK4a}$ inactivation appears to be important. Other mechanisms such as LOH and hypermethylation are also likely to be involved (figure 2). There are genetic differences between PTs and FAs and use of the term 'benign' PTs is being questioned in view of the differences seen in the genetics of a 'benign' PT when compared with a truly 'benign' FA. Some studies question the neoplastic nature of FA and suggest that they are in fact hyperplastic lesions. From the practical perspective of routine morphological diagnosis without the benefit of molecular genetic distinction, there has been recommendation of a conservative approach to benign fibroepithelial neoplasms with overlapping features of FA and benign PT, pending greater clarity in their biological differences.[8]

Take home messages

- There is marked intratumoural genetic heterogeneity in phyllodes tumours (PTs) that is not recognised clinically or histopathologically which contributes to the unpredictability of tumour behaviour. Under-recognised subclones may account for tumour recurrence and progression.
- Comparative genomic hybridisation studies have found that copy number changes in PTs tend to be large scale involving whole chromosomes or chromosome arms. There are some common gains and losses, however, mutation analysis of candidate genes at these sites has been largely unsuccessful in identifying the specific genes driving disease progression.
- Alternate mechanisms of gene inactivation including loss of heterozygosity and hypermethylation are important in PTs.

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Recent insights into the molecular pathogenesis of mammary phyllodes tumours

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