Pathogenesis and Progression of Fibroepithelial Breast Tumors

Arno Kuijper

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Pathogenesis and Progression of Fibroepithelial Breast Tumors

Pathogenese en progressie van fibroepitheliale borsttumoren
(met een samenvatting in het Nederlands)

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Voor Danielle

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Chapter 1

General Introduction and Outline of Thesis

The group of fibroepithelial breast tumors consists of fibroadenoma, phyllodes tumor, sclerosing lobular hyperplasia and hamartoma. These tumors are called “fibroepithelial” because two components can be discerned: an epithelial and a mesenchymal (stromal, fibrous) component. These fibroepithelial tumors are described in detail in **chapter 2**, where extensive information is provided on such topics as microscopy, clinical behavior, molecular biology and genetics. This thesis will further focus on fibroadenoma and phyllodes tumor, the most frequent, clinically most relevant, and molecularly most interesting entities. By choosing these two representatives we investigate two extremes within the group of fibroepithelial tumors: a common benign tumor (fibroadenoma) and a rare tumor of unpredictable behavior (phyllodes tumor). Phyllodes tumors are graded as benign, borderline or malignant, with chances of recurrence and metastases rising with grade. In general, the aim of this thesis can be summarized as a study of progression in fibroepithelial tumors, i.e. epithelial (to carcinoma) and stromal (to phyllodes tumor) progression in fibroadenomas and progression in grade of phyllodes tumors.

Fibroadenoma is the most common fibroepithelial tumor. An autopsy study found an, mostly microscopical, incidence of 9% for fibroadenoma of the breast [1]. Of patients visiting a breast clinic, approximately 7% were diagnosed with fibroadenoma [2]. The age distribution ranges from early teens to over 70 years, with a mean age of about 30 years [3]. Although fibroadenoma in itself is a benign tumor, large epidemiological studies have related its presence to an increased risk for invasive breast cancer in later life [4-7]. Dupont et al found a relative risk (RR) of up to 4 depending on presence of so-called complex changes within the fibroadenoma, benign proliferative disease in the surrounding parenchyma and a positive family history for breast cancer [4]. To put this in perspective, a RR of 4 is nearly twice the risk of breast cancer for women with a first degree relative with breast cancer [8]. Further, a small number of fibroadenomas will give rise to breast carcinoma [9-12]. Malignancy arising from within a fibroadenoma may be difficult to detect since clinical and radiological signs may be masked until breach of the pseudocapsule [13]. Although it is probably not necessary to remove all fibroadenomas, no clear-cut directives exist for its clinical management. In **chapter 3** we therefore studied in detail the histologic features of a large group of fibroadenomas and its surrounding tissue and related these findings to clinical features in order to construct a proposal for clinical management. We paid special attention to signs of malignant progression in the epithelial or stromal compartments.

The relation between a positive family history and risk of breast cancer is well known. It is not known whether familial predisposition for breast cancer facilitates malignant transformation of fibroadenomas. In **chapter 4** we describe an

extraordinary case of a woman from a hereditary breast/ovarian cancer family with multiple fibroadenomas, three of which simultaneously gave rise to carcinoma *in situ*.

In contrast to fibroadenoma, phyllodes tumor is a rare lesion and of unpredictable behavior. A population-based study conducted in the USA revealed an annual age-adjusted incidence of 2.1 per 1 million women [14]. The mean age at diagnosis is about 45 years, approximately 15 years older than that of fibroadenoma patients [15]. Phyllodes tumors are graded as benign, borderline or malignant based on histologic features [16]. Recurrence occurs in 8 to 65% of cases, depending on grade of the primary tumor [17]. Further, metastases are encountered in up to 22% of malignant tumors [16]. The stromal component of phyllodes tumors is more prominent as compared to fibroadenomas and it is this component that metastasizes. The distinction between fibroadenoma and benign phyllodes tumor can be difficult to make. Further, morphological observations have suggested that fibroadenoma may progress to phyllodes tumor [15,18]. In our study as described in chapter 3 we identified several cases of fibroadenoma which seem to harbor an area of stromal progression. Previous studies using PCR-based clonality analysis suggested that the stromal component of fibroadenoma is polyclonal and that of phyllodes tumor is monoclonal [19]. In **chapter 5**, we carefully microdissected the areas of stromal progression in fibroadenomas and subsequently performed clonality analysis, in an attempt to test the hypothesis that fibroadenomas may progress to phyllodes tumors. Further, to construct a model in which the relation between both tumors is described, we analyzed “normal” fibroadenomas and phyllodes tumors as well.

With higher tumor grade, the microscopic appearance of phyllodes tumors becomes increasingly alarming, with increasing cellular atypia, increased number of mitoses and invading tumor margins. Not much is known on the molecular mechanisms driving progression of phyllodes tumors to higher grade. DNA is replicated and equally distributed over two daughter cells in the cell cycle which is divided into the interphase (G1-, S-, G2-phase) and the M(itosis) phase. Distortion of the cell cycle machinery regulating this process is a major phenomenon in carcinogenesis. Only a limited number of studies have addressed the role of cell cycle disturbances in phyllodes tumors [20-24]. These studies mainly focussed on p53, a tumor suppressor gene which seems to play an important role in phyllodes tumor development. No comprehensive studies on cell cycle proteins in phyllodes tumor exist though. In **chapter 6** we therefore studied a group of phyllodes tumors of different grades for expression of several important cell cycle regulators, such as p53, pRb and cyclin D1. Follow up data was gathered to determine the prognostic value of the different alterations. Although the stromal component of phyllodes tumors is dominant, several reports on phyllodes tumors describe that the epithelial component is more than merely passive and may even be neoplastic [25]. We

therefore carefully examined the epithelial component as well for altered expression of these cell cycle proteins.

As a tumor grows beyond a volume of several mm^3, it needs to develop a vascular system in order to maintain a steady supply of oxygen and nutrients. Numerous cytokines with either pro- or anti-angiogenic properties form a complex network of interaction in which neovascularization is determined by the balance between these factors. Expression levels of angiogenic factors, such as VEGF, and microvessel counts are of prognostic relevance in many types of invasive cancer. As compared to invasive breast cancer, fibroadenomas are capable of forming similar quantities of microvessels [26]. In phyllodes tumors, numbers of microvessels seem to increase with higher tumor grade [27]. Although there seems to be active neovascularization in fibroepithelial tumors, little is known on the proteins which stimulate this process. Recently, it was discovered that hypoxia inducible factor 1α (HIF-1α) is a pivotal factor in the adaptive response to changing metabolic demands in growing tumors [28]. HIF-1α activates several genes involved in angiogenesis, glycolysis, erythropoiesis and apoptosis. This protein has however not been studied in fibroepithelial breast tumors, which urged us to evaluate the expression and prognostic relevance of HIF-1α and its downstream targets VEGF and CAIX in fibroadenomas and phyllodes tumors of various grades. This work is described in **chapter 7**.

Classic cytogenetic studies using short-term culture and G-banding have identified clonal aberrations in several dozens of fibroadenomas but recurrent alterations have rarely emerged [29-34]. For phyllodes tumors similar results have been noted [35]. The disadvantages of short-term culture are evident, though. Comparative genomic hybridization (CGH) is a recently developed technique which allows whole genome screening for cytogenetic changes by mixing tumor and normal reference DNA and subsequent hybridization to normal metaphase chromosomes. CGH detected copy number changes scattered throughout the genome in fibroadenomas [36,37]. Further, using the same technique, recurrent aberrations have emerged in phyllodes tumors [38,39]. Gain at 1q was prominent in both studies. However, due to the limited resolution of chromosome CGH, the underlying genes suffering from copy number change remain unidentified. Recently, array based CGH has made its advent in cancer research [40]. This technique uses small DNA fragments (BAC, P1, cosmid or cDNA clones) as hybridization targets and this approach results in much improved resolution and sensitivity. Array CGH may therefore much better pinpoint cancer related genes from known genomic regions of altered DNA copy number [41]. In **chapter 8**, we therefore used array CGH to obtain genomic profiles of fibroadenomas and different grades of phyllodes tumors, hereby

hoping to narrow down gained or lost chromosomal loci to facilitate identification of genes responsible for tumorigenesis and progression.

With the introduction of targeted therapies such as geftinib and cetuximab in clinical practice, there has been a growing interest in the expression of epithelial growth factor receptor (EGFR) in human tumors. Since disseminated phyllodes tumor is notoriously difficult to treat, detailed knowledge about EGFR expression in phyllodes tumors may be relevant. Scattered reports however exist on EGFR expression in fibroepithelial tumors, often with conflicting results. Zelada et al found high stromal expression of EGFR in a small group of fibroadenomas [42]. Another report however described absent EGFR staining in stroma of fibroadenomas but frequent positive staining of the epithelial component [43]. Suo and Nesland found increasing expression of EGFR with higher grade in phyllodes tumors [44]. Since *EGFR* whole gene amplification only accounts for a small percentage of EGFR overexpressing breast cancers, it has been assumed for long that expression of EGFR is regulated mainly at the transcriptional level. Recently, a relation between the length of a CA sequence repeat in intron 1 of the *EGFR* gene and EGFR protein expression was found [45]. In **chapter 9** we studied EGFR expression by immunohistochemistry, *EGFR* whole gene amplification by fluorescence in situ hybridization (FISH) and amplification status of a short CA repeat within intron 1 of *EGFR* in phyllodes tumors and fibroadenomas. We further relate these findings to expression of several cell cycle markers which were assessed by immunohistochemistry on tissue microarrays. By this approach we hope to gain insight in the possible role of EGFR in the pathogenesis of fibroepithelial tumors.

Array-based gene expression analysis has been a major advance in cancer research [46]. DNA microarrays allow simultaneous analysis of the expression of tens of thousands of genes in a tissue in a single experiment. Gene expression profiling may even predict prognosis with higher accuracy than classical parameters [47]. Further, chances of treatment response may be assessed by DNA microarrays as well [48]. Phyllodes tumor and fibroadenoma are both biphasic breast tumors and share morphological similarities. Expression analysis has been successfully applied to identify discriminating genes in morphologically similar tumors, hereby revealing novel diagnostic markers [49]. Reasoning from our previously proposed model of fibroepithelial tumor genesis, in **chapter 10** we compare expression profiles between fibroadenoma and normal breast, between phyllodes tumor and normal breast, and between phyllodes tumor and fibroadenoma. Validation of some genes with altered expression will be performed on a tissue microarray composed of 58 phyllodes tumors and 167 fibroadenomas. A tissue microarray is composed of multiple cylindrical tissue cores and allows high-throughput analysis of protein expression with preservation of the histological context [50]. This approach will make it able to

determine which compartment, ie epithelium or stroma, is the source of the altered expression of a gene. The aim of this study is to detect genes involved in fibroepithelial tumor genesis. Since fibroadenoma and phyllodes tumor show different clinical behavior, molecular profiling may reveal prognostic genes in addition to markers which could prove to be a diagnostic aid.

Finally, **chapters 11** and **12** summarize and discuss all these studies.

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Chapter 2

Fibroepithelial Breast Lesions

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Contents

2. Fibroepithelial tumors

These tumors represent a heterogeneous group of lesions that contain both mesenchymal (stromal) and epithelial components. According to morphology and clinic they are classified into fibroadenomas, phyllodes tumors, hamartomas, sclerosing lobular hyperplasia and fibroadematoid hyperplasia.

2.1 Fibroadenoma

2.1.1 Synonyms

Some prefer adenofibroma as in other organs [1], but the consensus is to use only fibroadenoma for breast lesions.

2.1.2 Definition

Fibroadenoma is a well-demarcated benign fibroepithelial tumor with a relative balance between stromal and epithelial components. It contains elongated ducts surrounded by stroma. Fibroadenoma arises from the epithelium and stroma of the terminal duct-lobular unit.

2.1.3 Conceptual approach

Fibroadenoma is to be placed within the spectrum of fibroepithelial breast lesions as it is composed of both a stromal and an epithelial component, arising from the epithelium and stroma of the terminal duct-lobular unit. Thus the epithelial structures contain Ck5/14 positive progenitor cells with its glandular and myoepithelial progeny, whereas the stromal component shows vimentin positivity.

Fibroadenomas have been suggested to arise within sclerosing lobular hyperplasia which is present in the surrounding breast tissue of about 50% of fibroadenomas [2], and one can imagine that some fibroadenomas arise as localized foci of accelerated proliferation in a background of sclerosing lobular hyperplasia. On the other hand, fibroadenoma may even arise from the continuous expansion of only one lobule. However, this is probably rare, as only a single case of fibroadenoma with monoclonal stroma has been described [3]. Using the HUMARA assay, normal/hyperplastic epithelium and stroma microdissected from fibroadenomas was polyclonal in all cases [4]. As the lobular unit is the monoclonal ”patch” of the human breast meaning that all cells in one lobule derive from one progenitor cell [5], we conclude that most fibroadenomas probably derive from several lobules. Fibroadenomas may develop usual ductal hyperplasia (which is polyclonal) [4,6], CIS (either DCIS or LCIS) which is monoclonal [4,7] and even invasive carcinoma [8-10]. Likewise, the stromal component may expand polyclonally to form benign phyllodes tumor [4] or there may be clonal expansion of phylloid areas within fibroadenomas to phyllodes tumor [3,4,10,11]. Although, progression of a fibroadenoma to phyllodes

tumor most likely is a rare event [12], this makes clear that especially fibroadenomas and phyllodes tumors are not clearly separate entities, but form a morphological and molecular genetic spectrum.

Being a biphasic tumor, epithelial-stromal interactions are of special interest. Mitotic figures in the stroma of fibroadenomas are located preferentially in the proximity of the epithelium, suggesting the production of stromal stimuli by the epithelial cells [13]. Indeed, both acidic fibroblast growth factor (aFGF) and one of its receptors (FGFR4) have been detected in the epithelium of fibroadenomas, whereas the stroma was strongly positive for FGFR4 but only weakly positive for aFGF [14]. These findings are suggestive of the control of stromal proliferation by a paracrine loop where aFGF is mainly produced by the epithelium and FGFR4 in the stromal compartment itself. A comparable mechanism was found for the epidermal growth factor (EGF) and its receptor (EGFR) [15]. Furthermore, by assessing stromal expression of PDGF and PDGFR, Feakins et al found evidence for autocrine stimulation of stromal growth in fibroadenomas [16].

2.1.4 Clinical features

Clinical presentation

Fibroadenoma is the most common “benign” breast tumor. The age distribution ranges from the early teens to over 70 years, with a mean age of about 30 years [17]. Less than 5% of women with a fibroadenoma are post-menopausal. Clinically, it usually presents as a palpable well-demarcated firm mobile tumor, that shows a tendency to slightly enlarge at the end of the menstrual cycle and during pregnancy. However, with the advent of mammography screening more and more non-palpable Fibroadenomas are going to be detected. The left breast is slightly more often affected than the right, and the preferred site within the breast is the upper outer quadrant [17]. In about 15% of patients, multiple fibroadenomas occur syn- and metachronously in the same or opposite breast. Recurrences usually develop in the same quadrant as the first fibroadenoma after a mean interval of about 4 years in 36% of cases [17]. The familial syndrome in which myxoid fibroadenomas are associated with cutaneous and cardiac myxomas and endocrine over-activity is known as Carney's syndrome [18]. A few cases occurring in ectopic breast tissue in the axilla have been described [19].

About 90% of fibroadenomas are smaller than 4.0 cm [17]. Occasionally, they grow to huge sizes to involve most or all of the breast, especially during adolescence. They may develop as solitary or multiple tumors shortly after puberty [20,21,22] affecting one or both breasts.

There have not been many studies evaluating risk factors for development of fibroadenomas. Rarely racial [23] or familial predisposition may play a role [24,25]. Use of oral contraceptives [26,27], high Quetelet index and high number of full-term pregnancies [28] have been shown to reduce fibroadenoma risk, but exogenous

estrogen replacement therapy may increase risk [26] although not all studies agree on this [28].

Treatment and prognosis

Fibroadenomas can in occasional cases progress in both epithelial and stromal directions [4] to malignant tumors. However, as fibroadenomas have a tendency to be self-limiting or even regress (even giant fibroadenomas [1,29,30]), it is probably not necessary to remove them all [31-33]. Continuous growth, complaints, positive family history and age > 35 years are, however, indications for surgery [12]. Besides, many women prefer excision even if FNA indicates a benign lesion [31].

Most solitary fibroadenomass can well be treated by local excision. It is preferable to include some of the surrounding normal breast to allow assessment of proliferative changes adjacent to the fibroadenoma, and this helps to avoid re-excision when the tumor turns out to be a phyllodes tumor or contains carcinoma *in situ*. An exception to this are adolescent fibroadenomas that should be excised while preserving as much breast tissue as possible, since leaving a minimal amount of residual normal tissue may lead to near normal breast development [1].

Several epidemiological studies have shown that the risk of developing invasive breast cancer is increased in women with a history of fibroadenoma. The reported relative risks vary from 1.6 to 2.6 [34-38]. Features that further increase this risk to 3 are presence of cysts, sclerosing adenosis, calcifications, or apocrine metaplasia within the fibroadenoma ("complex fibroadenoma"), proliferative changes in the surrounding breast tissue, and a family history of breast carcinoma (relative risk 3.7) [36,37,39]. Interestingly, atypical (ductal or lobular) hyperplasia within fibroadenomas does not seem to indicate a further increased relative risk [40].

Fibroadenomas also occur in the male breast, although rarely. We described ourselves one case of fibroadenoma in a male-female transsexual [41].

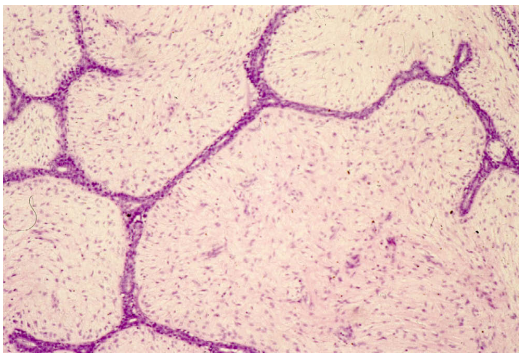


Figure 1. Fibroadenoma of intracanalicular type characterized by elongated two-layered ducts and homogenous low-cellular stroma with a pushing growth pattern into the epithelial strands forcing the ducts into slit-like elongated half-moon shaped structures (haematoxylin and eosin, original magnification x50).

2.1.5 Pathology

Macroscopy

Fibroadenoma usually presents with a smooth, bosselated contour. The cut surface shows a well demarcated, firm white to grey tumor surrounded by a fibrous pseudocapsule. Some tumors appear to be composed of several nodules divided by septae. Cysts of varying sizes may be present.

Microscopy

The typical fibroadenoma has well-defined borders and is composed of elongated ducts lined with two layers of epithelium surrounded by a more or less cellular fibrous stroma (Fig 1). Fibroadenoma can display a large variety of histological changes otherwise seen in the non-fibroadenomatous breast.

Several studies have characterized the stromal cells of fibroadenomas as fibroblasts [42-44]. The stroma of fibroadenomas may display an intracanalicular (60%), pericanalicular 20%) or mixed (20%) growth pattern [12]. In case of an intracanalicular growth pattern, the stroma pushes into the epithelial structures from one side, forcing the ducts into slit-like elongated half-moon or circular shaped structures. A pericanalicular growth results from stroma growing around the ducts allowing them to maintain their usual shape. Few, if any, mitotic figures are found in the stromal compartment.

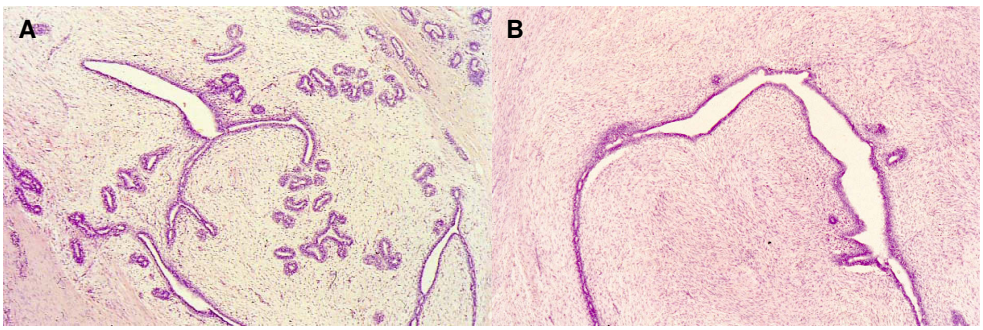
The stroma can be sparsely cellular, myxoid or hyalinised, or moderately cellular with a modest degree of pleomorphism. Myxoid fibroadenomas are especially associated with Carney's syndrome [18]. Sometimes stromal giant cells are found characterized by multiple hyperchromatic or vesicular nuclei, often arranged in a semicircular or florette pattern. They can get quite numerous, up to 10 per high-power field [45-48]. Because of their disturbing morphology, detection of giant cells can cause doubt about the benign nature of the lesion, especially when found in fine needle aspirates [49]. However, these cells do not influence the clinical course of lesion. Several rare forms of stromal differentiation are found in fibroadenomas. Although sometimes hard to detect, smooth muscle differentiation is present in a few percent of fibroadenomas [12,50,51]. Chondroid and osseous metaplasia is seen even more seldom [52]; the latter is found almost exclusively in fibroadenomas of post-menopausal women. As these are changes of the differentiation state of fibroblasts they are regarded as a form of mesenchymal metaplasia (Sm-actin and vimentin positive). They have to be distinguished from similar mesenchymal conversions of Ck5/14 positive progenitor cells, which occur in adenomyoepithelial tumors (see chapter 3). Pseudo-angiomatous stromal change is a rare finding (4% of fibroadenomas) [12]. Sometimes, phylloid lesions can be found in an otherwise "normal" fibroadenoma showing hypercellular stroma and increased numbers of mitotic figures (Fig 2) [10,12,53,54]. Recently, a clonality analysis of three

fibroadenomas recurring as phyllodes tumors provided evidence for a relation between both lesions [55].

The epithelial component of the tumor can display a broad range of changes. These include different types of benign proliferation and metaplastic changes described in previous chapters. When found in a fibroadenoma, the so-called complex lesions such as apocrine metaplasia, cysts, sclerosing adenosis or epithelial calcifications, are associated with an increased relative risk for breast cancer in later life [37]. A fibroadenoma should harbor at least one of these changes to be classified as complex. Dupont et al classified 23% of fibroadenomas as complex [37]. A recent study, however, could classify nearly twice as many fibroadenomas as complex [12], possibly attributable to more extensive sampling. The most frequently found feature is apocrine metaplasia (28%) followed by sclerosing adenosis (12%) [12]. Indeed, when Azzopardi found apocrine metaplasia in 14% of fibroadenomas and sclerosing adenosis in 6% he remarked that "more extensive sampling would reveal its presence even more" [9]. Calcifications and cysts are both found in a few percent of fibroadenomas. Furthermore foci of tubular or even secretory adenoma are exceptions [56].

Complex changes are not associated with epithelial proliferative disease in the adjacent tissue [12]. Therefore, the raised relative risk associated with these changes remains unexplained. Changes only rarely seen are papilloma, microglandular adenosis, pseudolactational changes and squamous metaplasia [12,57]. Infarction can be found in approximately 0.5% of fibroadenomas [24]. A sudden onset of pain is suggestive of infarction [58]. Fine needle aspiration biopsy is known to be able to induce such infarction [59].

Figure 2. In this otherwise inconspicious fibroadenoma of mixed type (A) an area of increased stromal cellularity containing 8 mitosis per 10 HPF was detected (B), to be interpreted as a phylloid area within fibroadenoma (haematoxylin and eosin, original magnification x50).



Using Page's criteria for diagnosing epithelial proliferative disease [60], usual ductal hyperplasia is frequently seen in breast fibroadenomas [12]. After excluding mild ductal hyperplasia, ductal hyperplasia of at least moderate grade can be found in approximately 30% of fibroadenomas. It can be found at all ages. Although finding hyperplasia in the otherwise normal breast is associated with a relative risk of 2-4, the meaning of ductal hyperplasia within a fibroadenoma is unknown, but it can be assumed to indicate a similar risk. It is however not correlated with proliferative disease in the tissue adjacent to the fibroadenoma [40]. Although once thought otherwise [61], hyperplastic changes within fibroadenoma are not associated with oral contraconceptive use [23,62]. A special problem in diagnosing hyperplasia in fibroadenomas is detachment and curling up of the epithelium into the duct leading to a widened duct filled with epithelial strands [10,12].

The study of Carter et al [40] showed that ADH or ALH is observed at a frequency of 0.81% within fibroadenomas. It does not seem to further increase the breast cancer risk.

Frequencies of CIS within fibroadenoma available in literature vary from 0.1% to 2.0% [12,63-65]. Ductal carcinoma in situ is found about as frequently as LCIS (Fig 3) [66].

CIS in a fibroadenoma is found in women two decades older than the mean age of all women with fibroadenomas [12,66]. It seems therefore that removal of fibroadenomas in women above the age of 35 or 40 years will reveal most fibroadenomas with CIS [12,64]. In 38 to 50% of fibroadenomas with CIS the proliferation can also be found in the adjacent tissue [12,64,67]. Therefore, if CIS is detected in a fibroadenoma the surrounding tissue should be explored as well. Invasive cancers arising within fibroadenoma are rare, are of ductal [68] or lobular [69] types, and should be treated as in the otherwise normal breast [64,67].

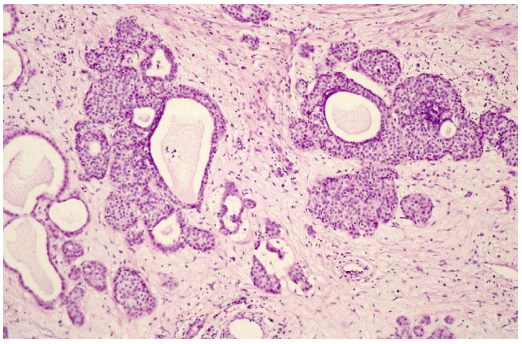


Figure 3. Fibroadenoma with an extensive lobular carcinoma in situ component (haematoxylin and eosin, original magnification x100).

Some consider "juvenile fibroadenomas" as a separate subtype, occurring mainly in teenage girls and characterized by rapid growth which may cause deformation of the breast. However, this is not exclusive for this age-group [29,30]. There are no features that histologically distinguish juvenile from usual fibroadenoma although the former more often shows cellular stroma and epithelial hyperplasia [1, 30]. There are therefore few arguments to consider "juvenile" as a useful term [29].

Cytology

Aspirates from fibroadenomas usually contain a mixture of epithelial and mesenchymal elements. In a cytological study Bottles et al. [70] found that abundant bipolar stromal cells, usually seen as bare nuclei, "antlerhorn clusters" (irregular flat stretches) and "honeycomb sheets" (fenestrated sheets of epithelium composed of uniform polygonal cells) are the most important features that favor a fibroadenoma instead of carcinoma. Still, when typical stroma is absent, a positive predictive value of 92% is reached by combining presence of ‘multilayered' fragments of epithelium in a background of bare nuclei [71]. Another paper found that typical stroma is present in 57%, antlerhorn clusters in 90% and honeycomb sheets in 81% of cytologically diagnosed fibroadenomas, somewhat reducing the clinical value of the features mentioned earlier [72]. It was determined that sensitivity and specificity of a cytologic diagnosis of fibroadenoma are respectively 87% and 94% [73].

Up to fifty percent of aspirates from fibroadenomas contain foam cells and apocrine cells [70], prominent nucleoli are seen in the epithelium of at least 80% and pleomorphic nuclei in 25% of fibroadenomas [70]. Failure to appreciate the cytologic variability that may be found in FNA specimens from fibroadenomas can therefore easily lead to a false suspicion of or diagnosis of carcinoma [74]. Special problems are presented by fibroadenomas harboring malignancy [75], and pregnant women where the cytological variability is more conspicuous, and epithelial cohesion is less, aspirates showing more loose atypical cells. Occasionally, the aspirate of a breast carcinoma may mimic the cytologic appearance of fibroadenoma [74,76,77].

2.1.6 Immunohistochemistry

The epithelial cell layers are usually Ck5/14- and Ck8/18/19-positive. Usually they express BRST2 and the luminal sides show MUC1 immunostaining. The myoepithelial layer is Ck5/14- and Sm-actin-positive, the stromal component shows the expected vimentin and focal Sm-actin expression, but does not show Ck5/14.

Most fibroadenomas express the progesterone receptor, whereas the estrogen receptor is expressed in a minority of tumors [77-79]. The estrogen receptor is mainly confined to the epithelial component, whereas the progesterone receptor is located in both the epithelial and stromal compartments [77,80,81]. It seems that the levels of the sex hormone receptors in fibroadenoma tissue vary during the menstrual cycle [82]. In addition, it was found that levels of estrogen and sulfatase enzyme are higher

in fibroadenoma than in normal breast tissue [83]. Also CD34 and bcl-2 staining is more abundant in the stroma of fibroadenoma than in the normal breast [84]. p53 staining in fibroadenomas is of wild type (weak in few cells) [84].

Some authors distinguish a so-called cellular variant of fibroadenoma. This variant from usual fibroadenoma by stromal cellularity of over 125 cells/high power field. Stromal cellularity was found to be correlated with bFGF and FGFR expression [86]. Based on expression patterns of several growth factors the authors placed this variant between phyllodes tumor and usual fibroadenoma. No clonality studies have been performed to obtain further evidence for this.

Immunohistochemical evaluation of the MIB1 index can discriminate between most fibroadenomas and phyllodes tumors. The distinction between fibroadenoma with high MIB1 index and benign phyllodes tumor remains problematic [54].

Recently, frequent overexpression of insulin-like growth factor II (IGF-II) was found in the stromal component of fibroadenomas. In addition, stromal overexpression of IGF-I was related to nuclear β-catenin accumulation. These results suggest a role for IGFs in the pathogenesis of fibroadenomas [87].

2.1.7 Core and vacuum assisted biopsy

The diagnosis fibroadenoma can in general well be made on a core biopsy. However, the differentiation between fibroadenoma and phyllodes tumor can at times be difficult [88].

2.1.8 Genetics

By cytogenetic studies of fibroadenomas 10 to 40% of tumors seem to display clonal chromosomal aberrations [89-96]. Most authors could not assign a specific abnormality to fibroadenomas, i.e. no preferential involvement of a chromosomal region or specific breakpoint has been recorded. A recent study, however, found that the majority (84%) of fibroadenomas is characterized by 6q alterations [97]. Since a similar high frequency of 6q alterations is found in premalignant lesions and carcinomas of the breast, the authors conclude that genes located on 6q are among the earliest events in pathogenesis of cancer. A shortcoming of most cytogenetics studies is that it does not become clear in which compartment the aberrant clone is located. By a combined immunohistochemistry/cytogenetic technique Fletcher et al could assign the clonal aberrations found in fibroadenomas to the mesenchymal compartment [89]. Dietrich, however, did find chromosomal abnormalities in cultures enriched for epithelial cells, which, however, were not found in cultures enriched for fibroblasts [96].

Clonality in fibroadenomas has been studied by a different approach as well. Taking advantage of polymorphic repeats in X-chromosome-linked genes and random inactivation of these genes by methylation, it was found that both the stromal and epithelial cells represent polyclonal cell populations [3,9,98,99]. This holds even

true for complex fibroadenomas [3]. Monoclonality, however, was observed in the stroma of a few simple and complex fibroadenomas. [55]. In our own study, microdissected stroma of fibroadenomas was polyclonal in all fibroadenomas, but phylloid areas in three fibroadenomas were found to be monoclonal [4].

The aberrant clones detected in cytogenetic studies are usually small and mostly balanced, which could explain why an early study by means of comparative genomic hybridization (CGH) was unable to detect any genomic imbalances in fibroadenomas [100]. However, a recent study did find several DNA copy number changes in fibroadenomas [101]. Gains of 5p14 (43%) and 5q34-qter (26%) were seen most frequently. A small study of 8 fibroadenomas found copy number alterations on many chromosomes, with gain of 8q and 5q as most frequent changes (3/8 cases both) [102]. As no microdissection was however done in these studies, it is unclear whether these cytogenetic alterations were present in stroma or epithelium.

Molecular studies found microsatellite instability (MIN) in 8% and loss of heterozygosity (LOH) in 10% of fibroadenomas, respectively [103]. Others however did not detect MIN, LOH or p53 gene mutations in fibroadenomas by Southern analysis [104] or by PCR-based techniques [105]. In addition, MIN, LOH or p53 mutations were not found in fibroadenomas which developed in the same breast as invasive breast cancer [106].

Low levels of telomerase activity were detected in 9/20 fibroadenomas in one study so far [107].

The reduced apoptosis to mitosis ratio in fibroadenomas compared to the normal breast [108] suggest that genes regulating proliferation and apoptosis may play a role in the development of these lesions.

2.1.9 Differential diagnosis

The differential diagnosis comprises hamartoma, tubular adenoma, sclerosing lobular hyperplasia, and especially phyllodes tumor.

Hamartomas show a normal lobular arrangement, and lack elongated ducts and cellular/edematous/myxoid stroma. Furthermore fibrocystic changes and epithelial hyperplasia are rare. Sclerosing lobular hyperplasia is less well demarcated than fibroadenoma, shows more vague and often enlarged lobular architecture, and the stromal component is more sclerotic. Tubular adenoma contains tubular/acinar structures with scant stroma tissue and is thus easily distinguished from fibroadenoma. Phyllodes tumor and fibroadenoma of intracanalicular type may be difficult to discriminate. Compared to fibroadenoma, phyllodes tumors show overgrowth of the stromal compartment with increased cellularity, especially in the periductal stromal areas. Mitotic activity is low in the stroma of fibroadenomas, and may be substantial in phyllodes tumors. The epithelial clefts are usually more elongated in phyllodes tumors, although phyllodes tumors of pericanalicular type do

occur. A particular problem is presented by fibroadenomas with focal areas with phylloidal features [10,12,53]. Marked atypia and sarcomatous differentiation are features of phyllodes tumors.

2.2 Phyllodes tumor

2.2.1 Synonyms

The term was introduced by Johannes Müller in 1838 for a tumor that was characterized by its leaf-like growth pattern (phyllos=leaf). The terms cystosarcoma phyllodes or cystosarcoma phylloides are still used by some [10] but the term phyllodes tumor is to be preferred. To designate lesions that are often benign as “sarcoma” is confusing for clinicians (especially “benign (cysto)sarcoma”), and these tumors are clearly not always cystic. A term that generically seems to be even better is “periductal stromal tumor”, as it better emphasizes the putative origin from periductal stroma, but this term is yet less popular and does not reflect the leaf-like growth pattern that is often present.

2.2.2 Definition

Phyllodes tumor applies to mixed epithelial-mesenchymal lesions with often a foliated structure, a double layered epithelial component and an overgrowth of the stromal component. The latter shows increased cellularity and proliferative activity, or even a sarcomatous appearance.

2.2.3 Conceptual approach

Phyllodes tumors are thought to derive from the perilobular-periductal stroma [10]. Within the spectrum of fibroepithelial breast tumors, phyllodes tumors are to be placed at the far end of stromal progression. Phyllodes tumors may derive from clonal expansion of the stromal part of (very rarely) hamartomas [109] or fibroadenomas [4]. In fact, one could easily imagine a phyllodes tumor (of intracanalicular type) to develop from expansion of the stroma of an intracanalicular fibroadenoma. Coexistent fibroadenomas are found in nearly 40% of phyllodes tumors [110], and phylloid areas with increased cellularity and mitotic activity [10,54] that are clonal in HUMARA analysis have been described [4]. Benign phyllodes tumors may have polyclonal stroma, indicating that they may not be truly neoplastic [4]. However, borderline and malignant phyllodes tumors have clonal stroma, which fits well with their neoplastic morphology and their clinical nature.

Although the stromal part of phyllodes tumors is the dominant component, the epithelial component may rarely be proliferative, showing usual ductal hyperplasia or even carcinoma in situ [111]. Expression of EGFR, c-erbB-3 and c-erbB-4 proteins have been detected in the neoplastic mesenchymal cells [112]. In the study of Feakins et al [113] neoplastic stromal cell positivity for PDGF-R was found in almost

50% of phyllodes tumors and for PDGF in 24%, associated with prominent nuclear was found in 15% of phyllodes tumors, and for epithelial PDGF and stromal PDGF-R in 43%, pointing to the importance of auto- and paracrine loops [113]. A similar phenomenon has been described for bFGF and FGFR [86]. Sawyer et al. found a positive relation between epithelial Wnt5a expression and stromal nuclear β catenin accumulation in benign phyllodes tumors. Their results suggested that in early stages of tumor development stromal proliferation is under epithelial control [114]. In later stages, initiated by unknown mutations, stromal growth becomes autonomous. These studies suggest that the role of the epithelial component in phyllodes tumor development might be more than mere passive.

The combined expression of CD34 and bcl-2 suggests that fibroadenomas, phyllodes tumors and pseudoangiomatous hyperplasia may arise from long-lived bcl-2-positive mesenchymal cells in the breast in a manner similar to that proposed for solitary fibrous tumors [84].

2.2.4 Clinical features

Clinical presentation

These tumors are rare. A population-based study conducted in the USA revealed an annual age-adjusted incidence of 2.1 per 1 million women [115]. Patients present at a wide age range [116-121]. The mean age at diagnosis is about 45 years, approximately 15 years older than that of fibroadenoma patients [10]. Most tumors occur in women 45 to 49 years old [115], but they can present during adolescence [118,122-128], also malignant ones [123-125,129] and rarely even before the age of ten [130]. Some rare cases of phyllodes tumors in ectopic breast tissue in the vulva [131,132] or the axilla [133] have been described.

Patients present with a well demarcated, firm, palpable tumor, clinically indistinguishable from fibroadenoma [116]. Size varies as does growth rate, rapid growth more often indicating a malignant phyllodes tumor, especially when occurring in a previously stable pre-existing tumor. Some cases have been described with multifocal phyllodes tumors in a single breast [110,134] or both breasts [110,117,129,135-138], and a few cases in men [48,139,140,142]. Large tumors may invade the skin or extend into the chest wall [69,129,139]. One case that presented with bloody nipple discharge was caused by spontaneous infarction of the tumor has been described [128].

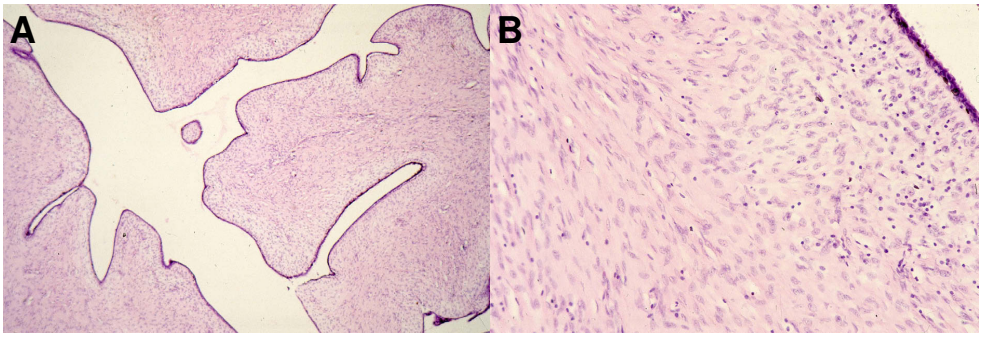
Risk factors including ethnicity; Asian and Latino patients present at younger age than non-Latina whites, and foreign-born Latino women had a three- to fourfold higher phyllodes tumor risk than Latino women born in the United States [115]. Besides, fibroadenoma is probably also a risk factor. Coexistent fibroadenomas are found in about 40% of phyllodes tumor cases [110], and may actually progress from fibroadenomas [4].

Treatment and prognosis

Grading of phyllodes tumors (see below) parallels the clinical behavior. Benign phyllodes tumors do not metastasize and have a low probability for local recurrence if completely excised [10] (Fig 4). In a series of 51 benign phyllodes tumors, 14 (27%) recurred locally, 6 of them within a year, and the others took 3-17 years. Borderline phyllodes tumors have a low probability (<5%) to metastasize, and are likely (>25%) to recur locally unless widely excised [10]. In a series of 22 borderline phyllodes tumors, 7 (32%) recurred locally, 4 within a year, the others taking up to 15 years. Several patients had multiple local recurrences [110]. About 25% of malignant phyllodes tumors develop metastases, and they are also prone to local recurrence [10,143].

Those few benign or borderline phyllodes tumors that metastasize almost always developed local recurrences with higher grade features prior to occurrence of systemic lesions [10].

Figure 4. Benign phyllodes tumor. A. Stromal compartment growing in classical leaf like pattern with flat epithelium and moderate stromal overgrowth pushing into the epithelium. B. Detail of the stromal component with moderate cellularity, mild atypia and absent mitotic figures. (haematoxylin and eosin, original magnifications x50 and x200, respectively).



Metastases usually occur at distant sites and only rarely (less than 1%) in the axillary lymph node [117,134,144,145], Metastases seem to be more frequent in cases with chondro- or osteosarcoma features [146-149] and are more infrequent in cases of liposarcomatous stromal metaplasia [150-153]. The most common sites of metastases are the lungs, bone, and heart [154-157], the central nervous system has also been described [158-160].

Therapy comprises complete excision [161-165], with removal of a clear margin of about 1cm [53]. Mastectomy is only indicated in case of large tumors that cannot be cosmetically acceptably removed by local excision. Following wide local excision, 8% (17/212), 29% (20/68), and 36% (16/45) of patients with benign, borderline, and malignant phyllodes tumors recurred in the breast [166]. Routine axillary dissection is

usually not indicated [167]. Little is known about the therapy of distant metastases. Phyllodes tumors initially did not seem to be responsive to chemotherapy or radiotherapy [168]. Other authors described however prolonged remission or palliation with chemotherapy alone or in combination with radiotherapy [169,170]. A recent investigation failed to detect drug resistance proteins Pgp and MRP in malignant phyllodes tumor xenografts in vivo. In addition, the xenografts were sensitive to vincristine, doxorubicin and cyclophosphamide [171]. These results suggest that at least some benefit can be expected from chemotherapy in disseminated disease.

The 5-year overall survival rate of patients with phyllodes tumors is about 90% [110], and that of malignant phyllodes tumors about 65% [10,120,121,129,172]. Most deaths due to metastatic disease occur within 5 years of diagnosis [129, 173,174], and are seen in patients with high grade malignant tumors [168,169]. Especially mitotic rate seems to be important. In one series [110], all phyllodes tumors that developed metastases (8 of 100) had at least 15 mitoses per 50 HPF in the primary tumor or recurrence, but these numbers vary between different series [117,174]. Similarly, Ki67 staining has prognostic value [112,175]. Other indicators of recurrence include p53 accumulation [112,175], S-phase characteristics [175-177], CEA [178] and stromal PDGF-R positivity and epithelial PDGF/stromal PDGFR co-positivity [113]. Stromal overgrowth has been reported to predict distant failure [179]. DNA ploidy does not seem to be predictive of recurrence [110,140,176,177,180,181]. We recently found that stromal p53 overexpression and number of cell cycle aberrations were independent prognostic parameters of disease free survival [182].

2.2.5 Pathology

Macroscopy

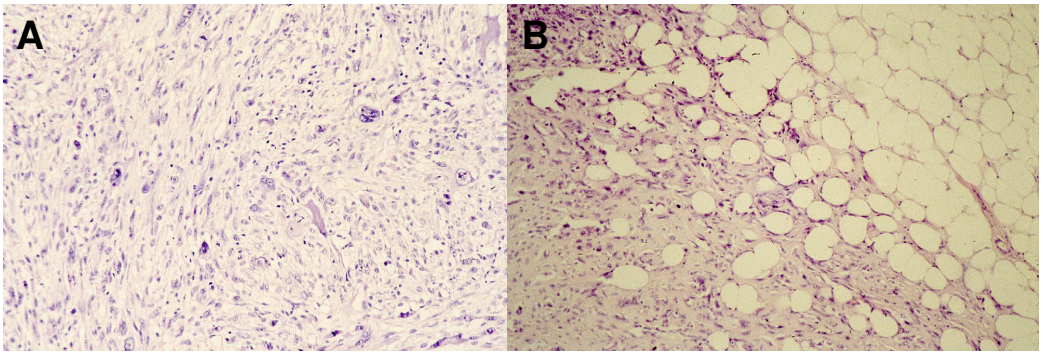
Phyllodes tumors, even if microscopically invasive, are grossly well circumscribed, but not really encapsulated. They can present as a single lesion but may be multinodular. The cut surface shows a more or less demarcated, firm grey-to-tan tumor, with a leaf-like appearance due to elongated circular clefts, where rounded fragments seem to drop off when cutting. There may be gelatinous or haemorrhagic areas due to degeneration, necrosis, and infarction, especially in malignant phyllodes tumors.

Microscopy

The classical pattern consists of a fibroepithelial tumor resembling intracanalicular fibroadenoma, with half-moon to circular shaped elongated clefts lined by a thin layer of epithelium, surrounded by and including dominant hypercellular stroma (Fig. 5). There are however many variants, and phyllodes tumors of pericanalicular type resembling their fibroadenoma counterpart certainly exist. Coexistent fibroadenomas are found in about 40% of cases [110].

The stroma of the prototypic benign phyllodes tumors often show condensation in the periductal areas where usually mitotic activity is found. The stromal cellularity may, however, be more homogeneous. Myxoid stromal changes are a common finding, whereas pseudoangiomatous stroma hyperplasia (PASH) is found in a small number of phyllodes tumors. Rare changes include lipomatous, leiomyomatous, cartilagenous and osseous stromal metaplasias [109,150], and intracytoplasmic inclusion bodies resembling those found in infantile digital fibromatosis [183]. Stromal mitotic activity, degree of stromal cellularity and atypicality vary and these features are important for grading (see below). In malignant phyllodes tumors, the stromal compartment often resembles that of fibrosarcoma (Fig 5), but there may be heterologous sarcomatous differentiation such as liposarcoma or myosarcoma [110,111,150,184-186] chondro- [110,146,185] or osteosarcoma [110,111,147-149,185,187,188].

Figure 5. A. Malignant phyllodes tumor showing little epithelium and bizarre nuclei, abundant mitotic figures and strong atypia and pleomorphism in the stroma. B. Malignant phyllodes tumor showing tumor infiltration in the surrounding adipose tissue. (haematoxylin and eosin, original magnification x100).



The epithelial component in phyllodes tumor is usually sparse with only elongated ducts and few lobular structures. The ductal spaces may be dilated. Classically, the epithelium is composed of an attenuated layer of glandular epithelium usually surrounded by myoepithelium. Apocrine metaplasia is rare [189]. Usual ductal hyperplasia may be seen [110], and atypical ductal hyperplasia [4,110] lobular [190,191] and ductal carcinoma in situ and even invasive carcinoma [64,78,110,190,192-197] may rarely be present [110]. Myoepithelial hyperplasia is not uncommon [10].

Ductal structures may be found in locally recurrent phyllodes tumors in the breast or chest wall, but metastatic deposits show the stromal component [173] which is usually fibrosarcoma-like, and may also contain heterologous stromal

metaplasia independent of the differentiation in the primary [110,150,147,148,149,184-186].

Grading

Several systems for grading of phyllodes tumors exist [9,117,143,174]. Most authors use a three tiered system and distinguish between benign, borderline and malignant cases, whereas some omit the intermediate category. Grading is based mainly on stromal cellularity, stromal overgrowth, atypia of stromal cells, mitotic activity, and the microscopic character of the tumor border, but slightly different thresholds have been defined. Since intra-tumor heterogeneity is a characteristic of phyllodes tumors, grading should be performed on excisional biopsies to avoid undergrading due to sampling error. An automated texture analysis system of tissue architecture has been developed resulting in good discrimination between benign, borderline and malignant cases [198].

Benign phyllodes tumor is characterized by less than four stromal mitoses per 10 HPF (corresponding to 1.6-2 mm^2), and modest cellular overgrowth with little pleomorphism [174]. The stromal expansion is in general uniformly distributed. The tumor is usually well circumscribed, but infiltrating margins may be present, sometimes forming secondary peripheral fibroepithelial nodules. Benign phyllodes tumors comprise approximately 64% of all phyllodes tumors [53]. Borderline phyllodes tumors more often have an invasive border, between five to nine mitoses per 10 HPF and moderate cellularity, resembling fibromatosis or low grade fibrosarcoma [174]. Epithelial hyperplasia is more often found than in benign phyllodes tumors [110], and microvessel density is also increased compared to benign phyllodes tumors [199]. Malignant phyllodes tumors comprise about 28% of all phyllodes tumors [53], show marked stromal overgrowth with ten or more mitoses per 10 HPF, and an invasive tumor border [174]. Nuclear atypicality is often marked in the stroma. The most common stromal pattern is that of fibrosarcoma, but there may be heterologous mesenchymal metaplasia [150,184,187]. Since heterologous elements and necrosis are found only in tumors which are clearly malignant by other criteria they are not decisive for diagnostics [143]. Epithelial hyperplasia is often found [110]. Malignant phyllodes tumors comprise approximately 28% of all phyllodes tumors [53].

Pietruszka and Barnes regard mitotic activity as the most important single variable [174]. Grading according to Moffat's criteria is less rigid, only the combination of all features will assign a certain grade to a tumor [143]. Benign tumors are characterized by less than 10% infiltrating margins and low to moderate stromal cellularity, atypia and overgrowth. There are fewer than 10 mitotic figures per 10 HPF. Malignant tumors show infiltration at 50% or more of the margin, moderate or high stromal cellularity, pleomorphism and overgrowth, with at least one classified as

high. There are more than 10 mitoses per 10 HPF. Borderline tumors show some but not all features of tumors of malignant grade.

Ki67 expression parallels traditional grading of phyllodes tumors [54,112,175,182,200-202].

Cytology

The cytologic appearance of phyllodes tumors is much like fibroadenomas, but stromal cells with cytoplasm rather than naked bipolar nuclei [203], individual long spindle nuclei [204,205], and hypercellular stromal fragments [204-207] and large stromal fragments [209] are more frequent in aspirates from phyllodes tumors. The aspirate from a malignant phyllodes tumor is likely to contain cellular stromal fragments composed of atypical cells and mitotic figures [10,210], and rarely liposarcomatous elements [211]. Fine needle aspiration cytology is however not reliable for the diagnosis of phyllodes tumor [167,204,212,213]. Cystic degeneration may make the diagnosis more difficult [214]. Large folded epithelial clusters also seem to be characteristic for phyllodes tumors in contrast with fibroadenomas [203,205].

2.2.6 Immunohistochemistry

The epithelial cell layers show Ck5/14 and Ck8/18/19 positivity, and CEA immunoreactivity is present in most phyllodes tumors [178]. The expression of ER- and PR in the epithelial cells of phyllodes tumors has been shown to be increased compared to that in normal breast epithelium [112,215]. Further, an inverse relation between epithelial PR and ER expression and degree of malignancy was found [216]. Stromal expression of PR and ER on the other hand was rarely seen. PR seems to be expressed more frequently in phyllodes tumors as compared to ER [54,216]. Expression of the androgen receptor seems to be low [216], although others concluded the complete opposite [54].

Furthermore, there is a marked production of endothelin-1 in the epithelium of phyllodes tumors [217], probably involved in a paracrine stimulation of proliferation of stromal cells. Indeed, stromal mitoses tend to be more frequent close to the epithelium than in more distant stroma [13]. The myoepithelial layer is Sm-actin and Ck5/14 positive. The basic immunoreaction of the stroma is that with vimentin antibody. Depending on the metaplasias present additional markers are expressed such as Sm-actin in myoid differentiation [218-220]. Contrasting with malignant adenomyoepithelial tumors, phyllodes tumors are Ck5/14 negative which may thus help to distinguish difficult cases.

Furthermore, excess perivascular deposition of type IV collagen has especially been observed in the stroma of malignant phyllodes tumors [221], and tenascin is more diffusely present in the stroma of phyllodes tumors than in the normal breast and fibroadenomas [222].

Ki67 expression parallels traditional grading of phyllodes tumors [54,112,175,182,200,201,223,224] and has prognostic value [112,175].

p53 accumulation is [113] more often observed in borderline and malignant tumors [83,86,112,113,200,201,224-228], associated with increased proliferation [113,200,224] and had prognostic value in some studies [112,175] but not in all [113,182,224]. The stromal component of phyllodes tumors displays an increasing level of cell cycle deregulation with higher tumor grade [182]. On the other hand, cell cycle aberrations are not found in the epithelial compartment.

CD34 [84,229] and bcl-2 [84] staining in the stroma is more conspicuous than in the normal breast, and tends to be decreased adjacent to the epithelium in contrast to the normal breast. As CD34 is absent in spindle cell carcinomas, CD34 may help in the distinction from malignant phyllodes tumors in difficult cases [84]. CD34 was reported to be more frequent in benign than malignant phyllodes tumors [230]. Contrary, c-kit (CD117) and Sm-actin are more frequent in the stroma of malignant phyllodes tumors [230]. Likewise, Sawyer et al found stromal c-kit and c-myc expression more frequently in malignant phyllodes tumors [231].

In their studies on the Wnt-APC-β catenin pathway in phyllodes tumors it became clear that epithelial Wnt5a expression and stromal IGF-I expression cause stromal nuclear β catenin accumulation in early stages of phyllodes tumor development [87,114]. In malignant tumors these mechanism are lost and the stromal component proliferates autonomously. Like in fibroadenomas, IGF-II overexpression is frequently seen in phyllodes tumors [87].

It seems that there is a positive relation between numbers of microvessels and tumor grade [86,199], reflecting adjustment to increased metabolic demands. Not much is known on the angiogenic mechanisms by which phyllodes tumors accomplish this. PDGF [16], FGF and VEGF [86] are expressed in phyllodes tumors but their relation to microvasculature has not been evaluated. We recently studied hypoxia-inducible factor 1 (HIF-1), which has a pivotal role in the adaptive response to hypoxia [232]. Stromal HIF-1α expression was positively correlated with grade, proliferation, p53 accumulation, and the number of microvessels and was predictive of clinical behavior. No relation to necrosis was found pointing to hypoxia independent pathways of HIF-1acitvation in stroma.

2.2.7 Genetics

One case that demonstrated progression from benign to malignant phenotype showed a p53 mutation [200]. No allelic loss of 3p was found in one study [201]. However, another found that 10 of 42 phyllodes tumors showed allelic imbalances on one or more markers on 3p and 14 of 46 on chromosome 1q. Five tumors had changes in both the epithelium and stroma, and 8 tumors had changes only detectable in the stroma and 8 changes in the epithelium only. Three tumors exhibited low-level microsatellite instability in the epithelium but not in the stroma

[234]. The authors raise the possibility that in some phyllodes tumors the epithelium may be neoplastic.

Initial reports assessing clonality based on X-chromosome inactivation revealed the epithelial component of phyllodes tumors to be polyclonal but stromal component was found to be monoclonal [11,55]. Our own study largely confirmed this, but showed that stroma of (benign) phyllodes tumors can sometimes be polyclonal, and that epithelium can be monoclonal [4]. Although the HUMARA technique has some inherent pitfalls (e.g. related to “patch size” [4]), this may suggest that both the stromal and epithelial components may be (potentially) neoplastic.

Cytogenetic studies have uncovered complex and varying karyotypic changes in benign and malignant phyllodes tumors [234-237]. Gain of 1q and structural changes of 10q emerged as frequent chromosomal changes in one study [238].

Mutations in the juxtamembrane region of the c-kit (CD117) proto-oncogene have been found in two malignant phyllodes tumors [230]. Mutation of p53 has been found occasionally as well [226]. There is yet little information on telomerase activity of phyllodes tumors [239].

In a study applying comparative genomic hybridization [240], phyllodes tumors showed no evidence of genomic amplification, but frequent changes were gain of 1q (7/18) and loss of 3p (6/18), followed by gain of 7q (4/18) and loss of 6q (4/18) and 3q (3/18). Gain of 1q material was significantly associated with histologically defined stromal overgrowth. All cases with gain of 1q, without 1p gain, had a clinical history of recurrence and 1q gain might therefore be an indicator of local aggressiveness requiring more radical treatment. A recent study confirmed that gain of 1q is frequently found, but failed to relate it to clinical behavior or tumor grade [241].

2.2.8 Differential diagnosis

The main differential diagnosis is that with fibroadenoma of intracanalicular type and adenomyoepithelial tumors. Compared to fibroadenoma, phyllodes tumors show overgrowth of the stromal compartment which shows increased cellularity, especially in the periductal stromal areas. Mitotic activity is low in the stroma of fibroadenomas, and may be substantial in phyllodes tumors. The epithelial clefts are more elongated in phyllodes tumors. A particular problem is presented by fibroadenomas with focal areas with phylloidal features [10,12,53]. Marked atypia and sarcomatous differentiation are features of phyllodes tumors. In cases with few epithelial elements, it may be difficult to discriminate phyllodes tumors from fibromatosis, and primary sarcoma of the breast. Fibromatosis shows a bland infiltrative spindle cell with no or very few mitoses, primary sarcomas lack the typical epithelial clefts. Another difficult differential diagnosis includes poorly differentiated adenomyoepithelial tumors and metaplastic carcinomas. The latter, however, contain usually Ck5/14 and Ck8/18/19 positivity in both the epithelial and sarcomatous areas. The tumor cells of phyllodes

tumors, however, lack cytokeratins. The differential diagnosis to adeno-myoepithelial tumors will be discussed elsewhere in more detail.

2.2.9 Core and vacuum assisted biopsy

Some phyllodes tumors can well be diagnosed on core biopsies due to marked cellularity, atypicality and mitotic activity of the stromal component. However, the distinction between phyllodes tumor and fibroadenoma on a core biopsy can at times be difficult [88]. In case of purely stromal fragments in a biopsy, there may also be problems with the differential diagnosis from sarcomatoid carcinoma (cytokeratin positive) or myofibroblastoma/solitary fibrous tumor (CD34 positive).

2.3 Sclerosing lobular hyperplasia

2.3.1 Synonyms

Sclerosing lobular hyperplasia is sometimes also called fibroadenomatoid mastopathy [10]. The former term is to be preferred as it more accurately reflects the histology.

2.3.2 Definition

Benign proliferative, usually reasonably well-demarcated tumor, histologically characterized by enlarged lobules with an increased number of acini, and variable interlobular fibrosis.

2.3.3 Conceptual approach

Sclerosing lobular hyperplasia is to be placed within the spectrum of fibroepithelial breast lesions as it is composed of both a stromal and an epithelial component. It can be viewed as a separate entity as it lacks the more tumourous demarcation and strict relationships between stroma and epithelium of a fibroadenoma, the more tumourous demarcation and the normal lobular architecture of a hamartoma, and the clonal stromal overgrowth of a phyllodes tumor. The finding that sclerosing lobular hyperplasia is present in the surrounding breast tissue of about 50% of fibroadenomas (with a ratio of sclerosing lobular hyperplasia to fibroadenoma of 9.3) suggests that fibroadenoma may arise from sclerosing lobular hyperplasia, or that the same or related factors contribute to the pathogenesis of both lesions [2].

2.3.4 Clinical features

Clinical presentation

This benign proliferative tumor presents between ages 14 and 41 years [2] as a localized palpable tumor up to 5 cm in diameter, usually in the upper outer quadrant

of the breast. The clinical picture is unspecific, most patients suspected of having fibroadenoma or fibrocystic disease. There are no evident risk factors.

Treatment and prognosis

Although probably not strictly indicated, most lesions will be excised and the diagnosis is made postoperatively. There are no adequate follow-up studies documenting the frequency of recurrence, but it has been suggested that these lesions may recur as a fibroadenoma [10].

2.3.5 Pathology

Macroscopy

The cut surface shows nodular, tan tissue with a granular appearance, usually lacking the sharp demarcation and whiteness of a fibroadenoma.

Microscopy

The lobules are enlarged and are composed of an increased number of acini. The intralobular stroma is collagenised, and there is variable interlobular stromal sclerosis (Figure 8). Individual lobules and/or groups of lobules may have the appearance of miniature fibroadenomas. There may be resemblance to tubular adenomas because of the prominent acinar component, but the packing of acinar structures is less tight than in tubular adenomas.

The acini have the normal breast architecture with distinct single layers of epithelial and myoepithelial cells. Secretory activity is minimal or absent. Sclerosing lobular hyperplasia is found in breast tissue surrounding about 50% of fibroadenomas [2], and the presence of a fibroadenoma may cause overlooking the accompanying component of sclerosing lobular hyperplasia.

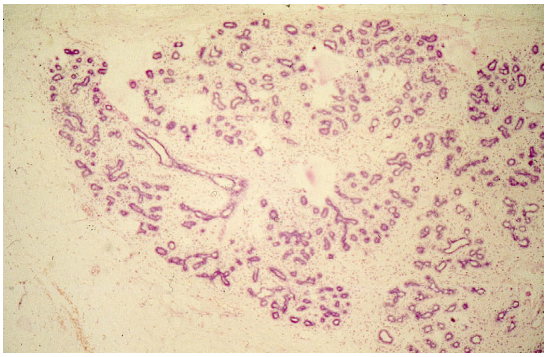


Figure 7. Sclerosing lobular hyperplasia: enlarged lobule with increased number of acini and increased collagenised intralobular stroma (haematoxylin and eosin, original magnification x100).

Cytology

There are no published studies on cytology of sclerosing lobular hyperplasia, and we have ourselves little experience with this. Probably, the cytological presentation would be somewhere in between that of normal breast and fibroadenoma.

2.3.6 Immunohistochemistry

No immunohistochemical studies have been performed on sclerosing lobular hyperplasia.

2.3.7 Genetics

No molecular or cytogenetic studies have been performed on sclerosing lobular hyperplasia.

2.3.8 Differential diagnosis

The differential diagnosis comprises fibroadenoma, hamartoma, tubular adenoma and sclerosing adenosis. In our own series [12], 7% of tumors that were originally classified as fibroadenoma were on revision diagnosed as sclerosing lobular hyperplasia. Fibroadenomas are more clearly demarcated than sclerosing lobular hyperplasia, have a more regular distribution of epithelial and stromal components, the epithelial component is often hyperplastic or may show apocrine metaplasia, show elongated ducts and intracanalicular growth patterns, the stroma is more often edematous or myxoid and cellular and may show some mitotic activity, and lack the (although sometimes vague and often enlarged) lobular architecture of sclerosing lobular hyperplasia. Hamartomas are also more clearly demarcated than sclerosing lobular hyperplasia, lack the enlarged lobular architecture of sclerosing lobular hyperplasia and instead shows normal lobular structures, and may contain a fatty or myoid component. Also tubular adenomas are more clearly demarcated than sclerosing lobular hyperplasia, and show a tight packing of acinar (and no or few ductal) structures, with little intervening stroma. However, these lesions basically form a spectrum. Fibroadenomas may be surrounded by areas of sclerosing lobular hyperplasia and contain areas of tubular adenoma, and areas within sclerosing lobular hyperplasia may resemble tubular adenoma.

2.3.9 Core and vacuum assisted biopsy

No studies have been performed. Diagnosis on core biopsy is expected to be difficult.

2.4 Hamartoma

2.4.1 Synonyms

The term choristoma [242] and mastoma are used by some, but the preferred term is hamartoma [10,53].

2.4.2 Definition

Breast mass clinically and macroscopically presenting as a tumor, but microscopically composed of almost normal breast parenchyma with distinct lobular arrangement, with sometimes a conspicuous lipomatous stromal component.

2.4.3 Conceptual approach

Although the architecture of hamartoma is much like the normal breast ("breast in breast"), is it probably not a developmental abnormality in view of the middle age at which it often presents and the association with Cowden's syndrome, but a true neoplasm albeit with a very low tendency of epithelial or stromal progression. Little is however known about the pathogenesis of mammary hamartomas. The myoid component of has been suggested to arise in a milieu of myoepithelial hyperplasia [243].

2.4.4 Clinical features

Clinical presentation

Hamartomas of the breast are rare. In one large series, hamartomas accounted for 1.2% of benign lesions and 4.8% of benign breast tumors [244]. Hamartoma most often presents in premenopausal women, and an association with pregnancy has been observed [245,246]. Growth rate is variable as is size, but they may be as big as 17 cm [10]. Hamartomas usually present as a painless well demarcated palpable mass, but may not be palpable in case of macromastia. A few cases arising in ectopic mammary tissue in the inguinal region have been described [247,248].

Most hamartomas occur sporadically, but they are seen in high frequency in Cowden's (multiple hamartoma) syndrome. This is an autosomal dominant disorder, caused by mutation in the PTEN gene, associated with benign skin tumors, breast hamartomas, and also an increased risk of breast cancer [249].

Treatment and prognosis

Hamartomas of the breast are almost always benign; malignant transformation is very rare [250-254]. They can therefore well be treated by local excision, usually resulting in complete removal of the tumor without risk of recurrence [246,255]. The rare cases with carcinoma in situ or invasive cancer within the hamartoma should be treated as usual.

2.4.5 Pathology

Macroscopy

The cut surface shows a soft well demarcated, sometimes lobulated mass surrounded by a thin fibrous pseudocapsule, composed of a mixed pattern of fat and fibrous breast parenchyma. If the fatty component is extensive, the lesion may macroscopically resemble a lipoma, and plate-like foci of cartilage have occasionally been noted [256,257].

Microscopy

The tissue basically consists of breast parenchyma with normal lobular architecture (Figure 9), although the lobular arrangement may be irregular. On higher magnification, the lesion therefore looses its tumourous impression when the surrounding pseudocapsule of compressed breast tissue is no longer within the field of vision. Fibrocystic changes are common but hyperplastic epithelium is rare in most series [244,258-260]. One series however reported ductal hyperplasia in 27% [261], with 12% of patients having coexistent fibroadenomas. Only a few cases with carcinomas in situ [250,251,253,254] and invasive cancer [250-252] arising from hamartomas have been described.

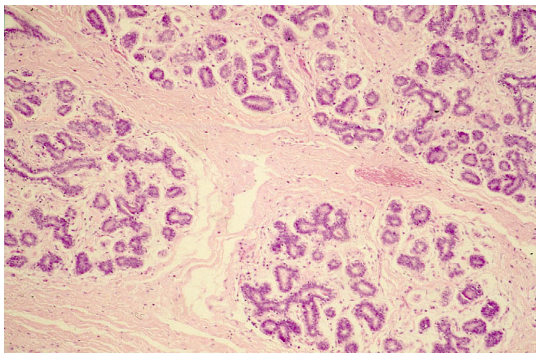


Figure 8. Typical hamartoma showing normal appearing breast lobules confined by a well-demarcated border (haematoxylin and eosin, original magnification x100).

The stromal component may show varying proportions of mature fat cells, leading to the designation adenolipoma [186,262], and one case with brown fat has been described [263]. Further, there may be sharply defined islands of hyaline cartilage [257], and smooth muscle [242,264-267] that may be cellular and show some mitotic figures [268] and show epithelioid features [269]. Glandular elements were completely absent in two lesions [270,271]. Areas of pseudoangiomatous stromal hyperplasia are not uncommon [259,272].

2.4.6 Immunohistochemistry

The immunohistochemical pattern resembles that of the normal breast tissue. [275,276]. Smooth muscle areas tend to show a myoepithelial staining pattern [257] and one case with CD34 positivity has been reported [277]. The epithelial cells usually show occasional positive nuclei for the estrogen and progesterone receptors [259,275]. Stromal cells are negative for estrogen and progesterone receptors, except in case of myoid differentiation [259,278]. Positive staining for c-erb-2 and p53 is not seen in hamartomas [276].

2.4.7 Genetics

In view of its association with Cowden's syndrome, mutations in the PTEN most likely play a role in the pathogenesis of hamartomas in patients with Cowden's syndrome. It is unclear whether the PTEN gene also plays a role in sporadic hamartomas. A first hamartoma case with involvement of 6p21 and rearrangement of the HMGIY gene has been described that awaits confirmation in bigger series [279]. One case of hamartoma of the breast that was cytogenetically analyzed revealed a 12q12-15 aberration. FISH showed the chromosome 12 translocation breakpoint to be mapping within the Multiple Aberration Region (MAR). MAR is known to be a major cluster region of chromosome 12 breakpoints of benign solid tumors such as uterine leiomyoma, lipoma, and pleomorphic salivary gland adenomas, suggesting that the same gene is involved in hamartoma of the breast as in these three benign solid tumors [90].

2.4.8 Differential diagnosis

The differential diagnosis comprises fibroadenoma and sclerosing lobular hyperplasia. Fibroadenomas lack the normal lobular arrangement of hamartomas, have more cellular and edematous/myxoid stroma. In contrast to fibroadenomas, hamartomas do not usually show epithelial hyperplasia and fibrocystic changes. Sclerosing lobular hyperplasia is less well demarcated than hamartoma, show more vague and often enlarged lobular architecture, and the stromal component is more sclerotic.

One study using dissecting microscopy of thick sections revealed ducts of penetrating or arcuate configuration, discrete lobules, Herati-style nodules composed of concentric rings of epithelium, and drifts of caraway seed-like fibrocytes, encasement of adipocytes by hyaline collagen, or spider-naevus vascular abnormalities in the hyaline interlobular connective tissue were found to be characteristic of hamartomas [280].

2.4.9 Core and vacuum assisted biopsy

Core biopsies from hamartomas will in general show histologically normal breast tissue, so the diagnosis can be missed. However, when it has been confirmed

by e.g. sonography that the lesion has been hit, presence of normal breast tissue might trigger the correct diagnosis. Also the presence of the margin, myoid elements or many fat cells may be of help.

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3

Chapter 3

Histopathology of Fibroadenoma of the Breast

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Abstract

Fibroadenoma of the breast is associated with an elevated risk for invasive breast cancer, especially in case of complex changes and epithelial proliferations in adjacent tissue. The aim of this study was, therefore, to make a thorough inventory of the histologic features of epithelium and stroma within and adjacent to breast fibroadenomas in a group of 396 cases.

Breast fibroadenomas appeared to display a wide spectrum of proliferative and non-proliferative histologic changes. Hyperplasia (excluding mild hyperplasia) within the fibroadenoma was found in 32.3% of cases. Carcinoma *in situ* (CIS; five ductal, three lobular) was found in eight fibroadenomas (2.0%) removed from six patients (1.7%), the youngest being 40 years of age. In three cases CIS was not confined to the fibroadenoma, but also involved the adjacent parenchyma. No invasive carcinoma was present within this series of fibroadenomas. Complex histology was seen in 40.4% of cases, mostly at higher age (mean age 35.4 years; $p = 0.009$). Hyperplasia in adjacent tissue was found in 8.8% of cases, usually at higher age (mean age 45.5 years; $p < 0.001$).

In conclusion, known risk-elevating lesions in and around breast fibroadenomas occur frequently and mostly above the age of 35 years. These findings may have consequences for the clinical management of a subgroup of patients with fibroadenoma.

Introduction

Fibroadenoma of the breast is a relatively frequently occurring tumor. Women can present with fibroadenoma at any age, but the peak incidence is in the second and third decade [1]. Although often considered a benign tumor, several reports describe a higher risk of subsequent breast carcinoma in patients diagnosed with fibroadenoma [2-5]. Dupont et al found relative risks (RR) ranging from 2 to 4 depending on presence of complex changes within the fibroadenoma, benign proliferative disease in the surrounding parenchyma and a positive family history for breast cancer [2]. For hyperplasia in the surrounding tissue, this was previously demonstrated by McDivitt and coworkers.4 In addition, the development of invasive carcinoma within fibroadenoma has been well documented in literature [6-10].

Fibroadenoma is a biphasic tumor, i.e. it is composed of an epithelial and a stromal component. The epithelial component of fibroadenoma can display similar aberrations as the epithelial component of the normal breast. In a series of 70 tumors, Deschenes et al found 2 carcinomas (one invasive, one *in situ*) arising within a fibroadenoma [7]. Ozello and Gump reported a combined incidence of 0.3% for *in situ* and invasive carcinoma arising within fibroadenomas [9]. The incidence of apocrine metaplasia and sclerosing adenosis inside fibroadenoma has been reported to be 14% and 6%, respectively [11]. Comprehensive studies describing the histologic features of fibroadenomas are not available in literature. Only limited data can be found on the incidence of changes such as squamous metaplasia [12], focal tubular adenoma [13], smooth muscle [14] and, although often mentioned in textbooks, hyperplasia within fibroadenomas [11,15,16]. If hyperplasia behaves in a similar way as in the otherwise normal breast [17], it may contribute to the higher risk of subsequent invasive breast carcinoma.

Since there are clues that fibroadenoma indicates a higher risk of subsequent carcinoma and little is known about lesions occurring within and adjacent to fibroadenomas, the aim of this study was to make a thorough inventory of the histologic features of the epithelium and stroma within and around breast fibroadenomas in a large group of cases.

Materials and Methods

Patients

Excluding consultation and revision cases, a total of 426 lesions originally diagnosed as fibroadenoma between 1984 and 1999 were retrieved from the archives of the Department of Pathology, Free University Hospital Amsterdam, the Netherlands. All these lesions had been removed in our Hospital. A total of 30 cases (7.0%) were on revision not classified as fibroadenoma, since another diagnosis seemed more appropriate (Table 1), leaving 396 fibroadenomas in 358 patients. The average age of the patients was 33.4±12.1 years (range 12-81 years). Size of the

tumors varied between 0.1 and 22 centimeters, with a mean of 1.5±1.4 centimeters. In 7.8% of patients, multiple tumors were found. In 59.3% of patients with multiple tumors the fibroadenomas were located ipsilaterally, in 40.7% the tumors were found in both breasts.

Diagnosis	No. of cases	%
Fibroadenoma	396	93.0
Sclerosing lobular hyperplasia	5	1.2
Phyllodes tumor	5	1.2
Hamartoma	4	0.9
Tubular adenoma	3	0.7
Pseudoangiomatous stromal hyperplasia	6	1.4
Adenomyoepithelioma	1	0.2
Normal tissue	6	1.4

Table 1. Revised diagnosis of 426 cases originally diagnosed as fibroadenoma.

Histopathology

All available hematoxylin and eosin (H&E) stained slides (on average 4) were thoroughly reviewed by two observers, either AK or EM and PvD, an experienced breast pathologist. Fibroadenomas were screened for proliferative epithelial changes (hyperplasia, carcinoma *in situ* [CIS], invasive carcinoma), fibrocystic epithelial changes (apocrine metaplasia, cysts, squamous metaplasia, sclerosing adenosis, microglandular adenosis, papilloma, lactational changes, calcifications), stromal changes (foci of pseudoangiomatous stromal hyperplasia, presence of smooth muscle), and various other changes such as foci of tubular adenoma and phyllodes tumor.

Complex fibroadenomas were, according to Dupont et al [2], defined as fibroadenomas harboring one or more of the so-called complex features: epithelial calcifications, apocrine metaplasia, sclerosing adenosis and cysts larger than 3 mm.

At least 0.5 cm^2 of tissue had to be present around the fibroadenoma in order to be evaluable for changes in the surrounding breast parenchyma [2].

In diagnosing hyperplasia and CIS, the criteria as described by Page et al [18] and Holland et al [19] were used. Only the most advanced lesion of the so-called usual ductal hyperplasias (mild, moderate or florid) was scored, i.e. if moderate and florid ductal hyperplasia were both present, only florid ductal hyperplasia was scored. Because the distinction between hyperplastic epithelium and tangential sectioning can be difficult to make, the appearance of myoepithelial cells throughout a duct was used as an additional criterion in favor of tangential sectioning (Fig 1). A pitfall previously described by Rosen [20] is an artificial hyperplasia like pattern caused by detachment of the epithelium with subsequent curling up, leading to a widened duct filled with epithelial strands (Figure 2).

Figure 1. Dispersed myoepithelial cells in this seemingly increased amount of epithelium indicate tangential sectioning and not hyperplasia in a breast fibroadenoma (hematoxylin and eosin, original magnification x200).

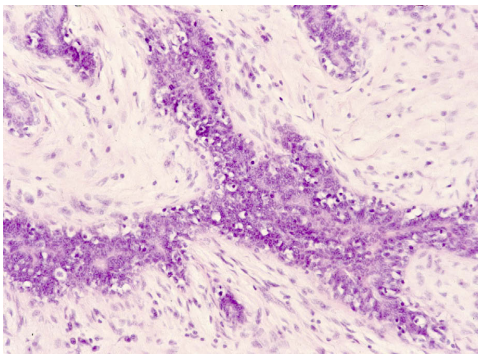
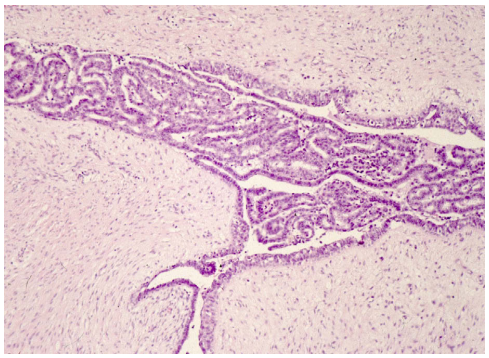


Figure 2. The epithelial pattern observed in this fibroadenoma is due to detachment and curling up of the epithelium and should not be classified as hyperplasia (hematoxylin and eosin, original magnification x100).



Another difficulty was sometimes the distinction between normal stroma and smooth muscle. When in doubt, immunohistochemical staining for smooth muscle actin was performed.

Phyllodes tumor was distinguished from fibroadenoma using Rosen's criteria, i.e. expansion and increased cellularity of the stromal component with often a leaf-like stromal growth pattern [15]. In phyllodes areas of fibroadenomas, stromal mitoses were counted per 10 high power fields (HPF; 400x magnification, ± 1.6mm^2).

Finally, fibroadenomas were classified as pericanalicular or intracanalicular when 90% of the tumor displayed that particular type of growth pattern. If neither type could be assigned to a tumor, we diagnosed it as mixed histological type.

Data analysis

Relations between age and histologic findings were investigated using the Student's *t*-test. The chi-square test was used to investigate relations between hyperplasia within the fibroadenoma, hyperplasia in adjacent parenchyma and complexity of the fibroadenoma. *P*-values below 0.05 were regarded as significant.

Table 2. Frequency of histopathological changes in 396 cases of fibroadenoma.

Lesion	No. of cases	%
Proliferative epithelial changes		
Mild ductal hyperplasia	46	11.6
Moderate ductal hyperplasia	106	26.8
Florid ductal hyperplasia	21	5.3
Atypical ductal hyperplasia	1	0.3
Atypical lobular hyperplasia	0	0
Lobular carcinoma in situ	3	0.8
Ductal carcinoma in situ	5	1.3
Invasive carcinoma	0	0
Fibrocystic epithelial changes		
Apocrine metaplasia	111	28.0
Cysts	20	5.1
Sclerosing adenosis	49	12.4
Calcifications	15	3.8
Microglandular adenosis	1	0.3
Papilloma	7	1.8
Pseudolactational changes	2	0.5
Squamous metaplasia	1	0.3
Stromal changes		
Pseudoangiomatous changes	15	3.8
Smooth muscle	11	2.8
Other		
Foci of tubular adenoma	2	0.5
Focal phyllodes tumor	3	0.8

Results

Changes within the fibroadenoma

The frequencies of histopathological changes found within the fibroadenomas are shown in Table 2. 60.2% of fibroadenomas were of the pericanalicular type, 20.8% were classified as intracanalicular and 19.0% were of the mixed histological type.

In this series, hyperplasia was a common feature of fibroadenoma. Mild ductal hyperplasia was found in 11.6% of cases. Moderate ductal hyperplasia was seen in 26.8% and florid ductal hyperplasia in 5.3% of cases (Fig 3). Atypical ductal hyperplasia (ADH) was detected once (Fig 4). All together, in 43.9% of fibroadenomas some form of hyperplasia can be found. However, since in the otherwise normal breast an elevated risk for invasive carcinoma has been proven only for moderate, florid and atypical hyperplasia, we excluded mild ductal hyperplasia from further considerations. Within fibroadenomas, hyperplasia of higher grade than mild was found in 32.3% of fibroadenomas, and was present in all age groups (mean age 32.9 years; n.s.). No relation with hyperplasia in adjacent tissue could be detected. However, complexity of fibroadenomas was significantly associated with the presence of hyperplasia within the fibroadenoma ($p=0.005$).

Figure 3. Usual ductal hyperplasia within a fibroadenoma (haematoxylin and eosin, original magnification x100).

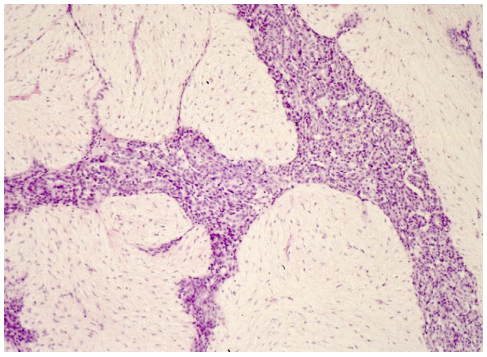
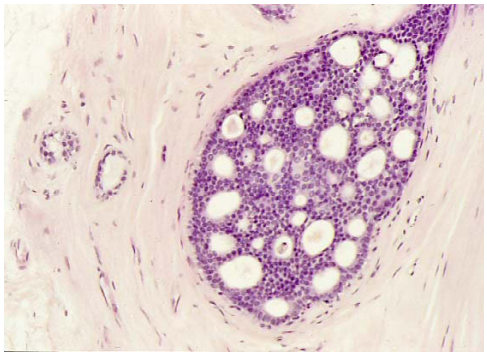


Figure 4. Atypical ductal hyperplasia found in a heavily sclerosed fibroadenoma (haematoxylin and eosin, x200).



Lobular carcinoma *in situ* (LCIS) was found three times (0.8%) (Fig 5). Ductal carcinoma *in situ* (DCIS) was seen five times (1.3%) (Fig 6). Mean age of these patients was 51.7 years, which is significantly older than those without this lesion ($p<0.001$). The youngest patient with this lesion was 40 years of age. CIS arising within fibroadenoma was accompanied by CIS in adjacent tissue in three of eight cases (37.5%). Invasive carcinoma within fibroadenoma was not seen in this series.

Figure 5. Fibroadenoma with extensive lobular carcinoma *in situ* in a 46-year-old patient (haematoxylin and eosin, original magnification x200).

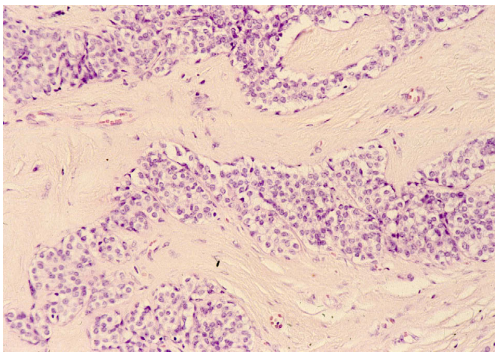
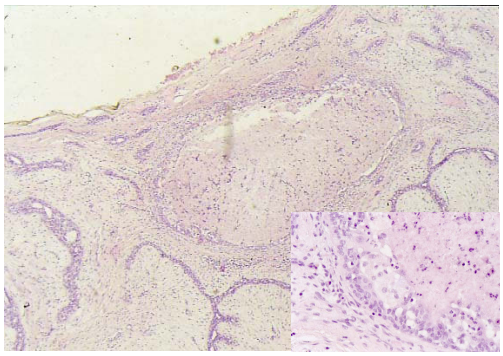


Figure 6. A small focus of poorly differentiated ductal carcinoma *in situ* detected in a fibroadenoma removed from a 40 year old patient (haematoxylin and eosin, original magnification x50; insert: cellular details at original magnification x400).



The so-called complex features were frequently seen, apocrine metaplasia being most frequent (28.0%). Taken together, 40.4% of fibroadenomas in this series were complex. 18.4% of the complex fibroadenomas harbored more than one complex feature, 2.5% harbored more than two complex features. Complex

fibroadenomas were seen more often at higher age (mean age 35.4 years; p=0.009). No relation between complexity and hyperplasia in adjacent tissue was detected.

Focal pseudoangiomatous stromal changes could be detected in 3.8% of cases. In two cases we observed foci of tubular adenoma. In three fibroadenomas we detected a part of the tumor which had to be classified as a focal phyllodes tumor. Two of these foci were benign, but one had a mitotic count of 8 per 10 HPF (see Figure 2, Chapter 2).

Table 3. Frequency of histopathological changes in the adjacent parenchyma of 317 cases of fibroadenoma.

Lesion	No. of cases	%
Proliferative epithelial changes		
Mild ductal hyperplasia	16	5.1
Moderate ductal hyperplasia	22	6.9
Florid ductal hyperplasia	3	1.0
Atypical ductal hyperplasia	2	0.6
Atypical lobular hyperplasia	2	0.6
Lobular carcinoma in situ	1	0.3
Ductal carcinoma in situ	6	1.9
Invasive carcinoma	3	1.0
Fibrocystic epithelial changes		
Apocrine metaplasia	75	23.7
Cysts	8	2.5
Sclerosing adenosis	46	14.5
Calcifications	11	3.5
Microglandular adenosis	6	1.9
Papilloma	1	0.3
Pseudolactational changes	4	1.3
Squamous metaplasia	0	0
Stromal changes		
Pseudoangiomatous changes	0	0
Smooth muscle	0	0
Other		
Foci of tubular adenoma	0	0
Focal phyllodes tumor	0	0

Changes in the adjacent parenchyma

Lesions found in the adjacent parenchyma of the fibroadenoma are displayed in Table 3. Seventy-nine fibroadenomas had less than 0.5 cm² of surrounding tissue, leaving 317 cases with sufficient adjacent breast tissue to be evaluable.

Mild ductal hyperplasia was seen in 5.1% and moderate and florid ductal hyperplasia in 6.9% and 1.0% of cases, respectively. ADH and atypical lobular hyperplasia (ALH) were both seen twice (mean age 43.8 years; n.s.). Therefore, in 13.9% of cases, some form of hyperplasia could be observed in the surrounding tissue (one ALH coexisted with a moderate ductal hyperplasia). To be consistent with Dupont et al [2], we excluded mild hyperplasia from further considerations, leaving 8.8% of fibroadenomas with hyperplasia in surrounding tissue. Hyperplasia in adjacent parenchyma was seen significantly more often at higher age (mean age

45.5 years; $p<0.001$). There were no significant correlations with hyperplasia within the fibroadenoma and complexity.

LCIS was detected once, synchronous with a lobular invasive carcinoma. DCIS was seen six times, twice synchronous with an invasive ductal carcinoma. Mean age of cases with CIS in adjacent tissue was 43.3 years. Invasive carcinoma was seen three times in the surrounding parenchyma of the fibroadenoma, without involvement of the fibroadenoma itself (mean age 48.3 years).

As within the fibroadenoma, apocrine metaplasia was the most common of the fibrocystic epithelial changes in the surrounding tissue (23.7% of cases).

Discussion

This is the first study reviewing in detail the histologic features of a large group of 396 fibroadenomas. Although fibroadenoma is associated with a higher risk for invasive breast cancer [2-5], few data on histological changes adjacent to and inside fibroadenomas were available in literature. We found that the fibroadenoma displays a large variety of histological changes, some of which are expected to be of importance.

In some cases, the diagnosis fibroadenoma can be difficult to make (Table 1). Various benign lesions had been mistaken for fibroadenomas, such as pseudoangiomatous stromal hyperplasia (6x) and sclerosing lobular hyperplasia (5x). This is of little practical consequence. However, 5 phyllodes tumors were originally classified as fibroadenomas. This distinction can be of importance, particularly if the tumor was only partially excised. Phyllodes tumors have the tendency to recur and may then be of a higher grade than the primary tumor [21]. Histopathological criteria for the distinction between fibroadenoma and phyllodes tumor can be difficult to apply [15,16]. On several occasions, we experienced difficulty in differentiating between benign phyllodes tumor and fibroadenoma. Therefore, in case of doubt one has to make sure that resection was complete. In addition, in three cases part of the fibroadenoma could only be interpreted as phyllodes tumor. This has been described by Rosen [15] and Elston and Ellis [16]. Further, progression of fibroadenoma to phyllodes tumor was demonstrated by Noguchi and coworkers by means of clonal analysis [22]. This underlines that phyllodes tumors may derive from fibroadenomas by clonal expansion of the stromal compartment.

Hyperplasia within fibroadenoma was frequently seen in this series, even when excluding mild ductal hyperplasia, it was present in 32.3% of cases. It can be found at all ages. We cannot exclude the possibility of observer subjectivity, but we have been strict in using Page's criteria for hyperplasia and included an additional feature (dispersed myoepithelial cells) which, in our opinion, denies hyperplasia. Further, we excluded a hyperplasia like pattern which is caused by curling up of the epithelium of larger ducts when it is disrupted and detached. Dupont et al gave relative risks for

hyperplasia in breast parenchyma ranging from 2 to 5 [17]. It is tempting to apply these relative risks to hyperplasia found within fibroadenomas, but nothing has been proven regarding this matter. However, if hyperplasia within fibroadenoma behaves in the same way as in the otherwise normal breast, it could make a contribution to the increased relative risk associated with fibroadenoma and may be a reason for excision. It is conceivable that part of the increased relative risk associated with complexity of the fibroadenoma, as described by Dupont et al [2], can be attributed to hyperplasia within the fibroadenoma since the presence of the former was correlated with presence of the latter in our study. No correlation with hyperplasia in the surrounding tissue was found. Therefore, the meaning of hyperplasia within fibroadenomas in terms of progression risks remains to be determined.

8.8% of fibroadenomas were associated with hyperplasia in the adjacent parenchyma. Dupont et al found an incidence of 13.7%, which was associated with a relative risk of 3.9 [2]. Likewise, McDivitt et al reported an odds ratio of 3.7 [4]. Mean age of patients with fibroadenomas with hyperplasia in the adjacent parenchyma was significantly older than mean age of those without this feature. Frequencies of both usual ductal and atypical hyperplasia (7.9% and 1.2%) are somewhat lower in our study than those found by Dupont et al (13.7 % and 1.7%) [2]. Although in 80.1% of cases the requirement of 0.5 cm^2 of surrounding tissue was met, in most cases not much more tissue was present. Possibly, minimal surgery provided us with less surrounding tissue and therefore lower chances of finding epithelial proliferations compared with Dupont et al, who studied fibroadenomas which were removed four decades ago [2]. In order to identify women with this risk factor, it would be preferable to include, if possible, a small rim of surrounding tissue when resecting a fibroadenoma.

A frequency of 2.0% for CIS within fibroadenoma was found. Five cases of DCIS and three cases of LCIS were detected in our series. However, from one patient three CIS lesions arising within three fibroadenomas were removed. Thus, CIS arising within fibroadenoma was detected in six patients (1.7%). Few heterogeneous figures describing the occurrence of *in situ* and invasive carcinoma within fibroadenoma exist. Ozello and Gump [9]. found an incidence of 0.3% for invasive and *in situ* carcinoma taken together and Deschenes et al [7] found one carcinoma *in situ* and one invasive carcinoma in 70 fibroadenomas (1.4% each). Further, Buzanowski et al reported five cases of LCIS in 4000 fibroadenomas (0.1%) [6]. Our percentage seems somewhat higher, but this figure is nevertheless realistic in view of the fact that we excluded revision and consultation cases. Three of eight cases also had CIS in the adjacent breast tissue; two fibroadenomas with DCIS were accompanied by DCIS, one fibroadenoma with DCIS by LCIS. Therefore, if CIS is detected in an enucleated fibroadenoma, the surrounding tissue should be explored as well. It is hard to say if these CIS lesions occur synchronously or metachronously. The former, however, seems more likely and may reflect the genetic relationships

(and thus susceptibility to progression) between fibroadenoma epithelium and adjacent epithelium. In our series, mean age of patients with CIS within fibroadenoma was relatively high (51.7 years), comparable to the study by Diaz et al [8].

In seven cases, CIS (six ductal, one lobular) was found in the surrounding tissue. Invasive carcinoma was found three times in the adjacent parenchyma, all three without involvement of the fibroadenoma. Therefore, there is no reason to assume that the relationship between both lesions is anything but coincidental. Again, both lesions were found in elder women.

We could classify more than twice as many fibroadenomas as complex in comparison with Dupont et al [2]. This is mainly due to the high incidences found for sclerosing adenosis and apocrine metaplasia. This may be contributed to the large amount of slides available per case, as Azzopardi already stated; "no doubt more extensive sampling would reveal its (apocrine metaplasia) presence even more (i.e. more than 14%) frequently" [11]. There is a tendency for complex fibroadenomas to occur at higher age. Since no correlation with hyperplasia in the surrounding tissue was found, it seems that the elevated risk associated with complexity cannot be explained by a higher incidence of epithelial proliferation in the surrounding breast.

Several authors have opted for conservative management of the fibroadenoma below a certain age. 25 years [23], 35 years [24] and 40 years [25] have been suggested as age thresholds. Another study demonstrated that a large proportion of fibroadenomas in women under 20 years of age will resolve [26]. In dealing with fibroadenoma, two problems need to be acknowledged. First, stroma and epithelium of the fibroadenoma itself can undergo malignant transformation. As underlined by this study, CIS arising within fibroadenoma is found mostly at older age. In our study the youngest patient with this lesion was 40 years of age. Therefore, removal of fibroadenomas in women over the age of 35 tackles the problem of epithelial progression. However, no relation with age was found in the four cases of stromal expansion (mean age 31 years; n.s.). A criterion to distinguish between fibroadenoma and phyllodes tumor is rapid growth. Therefore, rapid growth in a tumor previously diagnosed as fibroadenoma should raise suspicion of stromal transformation (and possibly epithelial transformation). Malignancy arising within fibroadenoma should be treated as in the otherwise normal breast [9,10]. Second, fibroadenoma is associated with a long-standing increased risk of invasive breast cancer [2]. Depending on presence of hyperplasia in adjacent tissue, complexity of the fibroadenoma and a positive family history for breast cancer the RR may rise to 4, nearly twice the RR for women with a first degree relative with breast cancer [27]. Since 45.0% of fibroadenomas harbor either complex features or hyperplasia in adjacent tissue, it would be ideal to remove all fibroadenomas in order to identify all women at increased risk for breast cancer. However, since fibroadenoma is a frequently occurring tumor often only seen on ultrasonography, this has major clinical

implications. As long as it is unclear whether the indicated RR is high enough to have clinical consequences such as intensive follow-up or chemoprevention, there are no clear-cut arguments to advise removal of all fibroadenomas.

Of course, the diagnosis of fibroadenoma first needs to be established before a wait-and-see approach can be advised in individual cases. To this end, a triple diagnostic procedure including clinical investigation, mammography/ sonography and fine needle aspiration (FNA)/ needle core biopsy (NCB) can be useful. The advantage of NCB over FNA may be that it more easily reveals complex changes and epithelial proliferations. Excision of fibroadenomas above the age of 35 years will remove all malignant lesions arising within fibroadenomas. Surveillance may be warranted for women with a known family history for breast cancer diagnosed with fibroadenoma with complex features or hyperplasia in adjacent tissue on NCB or excision. For this group, removal may not be necessary since the RR associated with such lesions appears to be bilateral and not specific to the site or the fibroadenoma.

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Chapter 4

Multiple Fibroadenomas Harboring Carcinoma in Situ in a Woman with a Familty History of Breast/ Ovarian Cancer

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J Clin Pathol 2002;55:795-797

Abstract

A 46-year-old woman with a family history of breast and ovarian cancer presented with multiple fibroadenomas in both breasts. From three fibroadenomas removed from the left breast carcinoma *in situ* (CIS) had developed. One fibroadenoma gave rise to ductal CIS (DCIS), whereas the other two harbored lobular CIS (LCIS). This is the first report of three fibroadenomas simultaneously giving rise to CIS. In addition, synchronous fibroadenomas harboring different types of CIS from one fibroadenoma to the other have never been described. Direct sequencing revealed a mutation (5075G>A) in the BRCA1 gene, but retention of BRCA1 immunohistochemical staining and no loss of heterozygosity (LOH) at the BRCA1 locus by polymerase chain reaction (PCR) made a pathogenic mutation in BRCA1 unlikely. Further, in this family we found no cosegregation of breast cancer with this BRCA1 mutation. Indeed, this mutation is now regarded as a polymorphism.

This case stresses the need for histologic evaluation of all breast masses in women with a strong positive family history for breast and/or ovarian cancer.

Introduction

Fibroadenoma of the breast is relatively frequent. Women can present at any age, but the peak incidence is in the second and third decade [1]. Fibroadenoma is a biphasic tumor, composed of a stromal and an epithelial component. Although generally considered benign there is evidence for an association with an increased risk of invasive breast cancer [2-4]. Dupont et al. describe a relative risk (RR) from 2 to 4 depending on complex histology, hyperplasia in adjacent tissue and family history [5].

Besides being associated with an increased RR for invasive breast cancer, fibroadenoma itself can display malignant progression. Epithelial hyperplasia is frequently found in fibroadenomas [6]. Furthermore, progression of the epithelial component to carcinoma *in situ* (CIS) and invasive carcinoma has been documented in the literature. Ozello and Gump found an incidence of 0.3% for CIS and invasive carcinoma taken together [7], and Buzanowski et al. found 5 cases of lobular carcinoma in situ (LCIS) arising within fibroadenoma in a series of 4000 tumors [8]. This progression is usually found in women over 40 years of age, which is nearly a decade older than those with usual fibroadenoma [7-9]. We report a rare finding of three in situ carcinomas (1 ductal, 2 lobular) arising simultaneously within three fibroadenomas.

Case report

A 46-year-old woman, with one first-degree family member with breast cancer and one with both breast and ovarian cancer, presented at our hospital with multiple palpable masses in both breasts. Fine needle aspiration of one of the lesions in the left breast was inconclusive, a subsequent core biopsy was compatible with fibroadenoma. Because of clinical and radiological suspicion, excisional biopsy was performed of both breasts. On pathological examination of the haematoxylin and eosin (H&E) sections the breast masses were found to be fibroadenomas. Three fibroadenomas removed from the left breast showed CIS. Well-differentiated (low grade) ductal carcinoma in situ (DCIS) (Fig1) had developed from one of the fibroadenomas, whereas the other two had given rise to LCIS (Fig 2). Immunohistochemical staining for E-cadherin (Zymed Laboratories, clone HECD-1; working dilution 1:200) was performed to confirm these diagnoses [10]. As expected, the LCIS cases showed complete loss of E-cadherin expression which was retained by the DCIS case. Two fibroadenomas in the right breast showed no conspicuous changes. Because of the positive family history of breast and ovarian cancer, a suspicion for a BRCA mutation existed. Routine mutation screening (BRCA1: protein truncation test on exon 11, single strand conformation analysis on exon 2, 20 and 24, polymerase chain reaction {PCR} screening for genomic deletions of exon 13 and 22. BRCA2: protein truncation test on exon 10 and 11) did not reveal mutation in BRCA1

Figure1. Well-differentiated DCIS in a sclerosed fibroadenoma (H&E stained, original magnification x25).

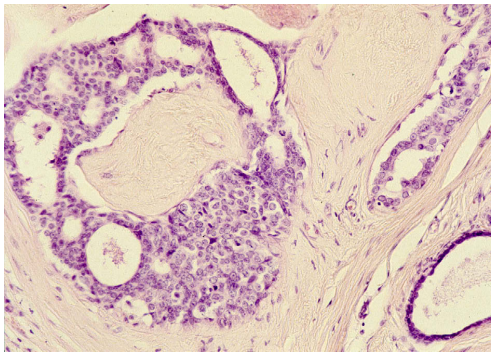
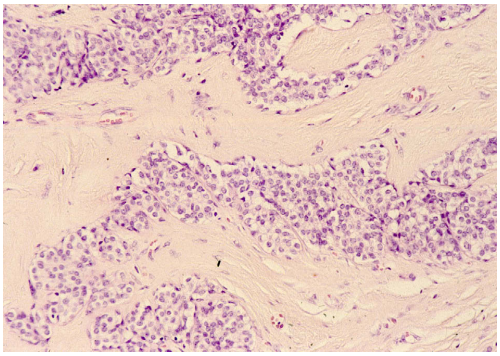


Figure 2. LCIS arising within a fibroadenoma (H&E stained, original magnification x25).



or BRCA 2 genes. Because of the multiple fibroadenomas with CIS, the strong positive family history and our patient's wish, a bilateral mastectomy was performed. 14 Blocks from the right breast and 21 blocks from the left breast were examined. Besides foci of usual ductal hyperplasia and fibrocystic changes in both breasts, the H&E sections revealed no other aberrations. No residual CIS was present and no further fibroadenomas were identified. Later, by direct sequencing of the full BRCA1 and BRCA2 genes, we found a missense germline mutation in exon 16 of the BRCA1 gene. This mutation, 5075G>A, leads to an amino acid substitution of methionine for isoleucine. The mother of our patient, herself a breast cancer patient, did not carry this mutation. Expression of the BRCA1-protein in the fibroadenomas was demonstrated by immunohistochemical staining (Oncogene, Ab-1; working dilution 1:500). Loss of heterozygosity (LOH) for BRCA1 could not be demonstrated by PCR of 3 polymorphic markers (D17S855, D17S1323, D17S1322).

Discussion

To the best of our knowledge this is the first report of three fibroadenomas synchronously giving rise to CIS. In addition, synchronous fibroadenomas harboring different types of CIS (DCIS or LCIS) from one fibroadenoma to the other have never been described. Complete sequencing of BRCA1 and 2 revealed a BRCA1 mutation. This mutation, 5075G>A in exon 16 was first described by Couch et al [11]. Later, Wagner (ASHG 1999, Breast Cancer Linkage Consortium 1999) reported that this mutation is a polymorphism occurring in the Indo-European population with a frequency of about 3%. In the family of our patient we found no cosegregation of this mutation with breast cancer predisposition, which indicates that this mutation is not responsible for the breast cancer predisposition in this family. However, since even complete sequencing does not reveal all mutations and can especially miss larger

genomic deletions and promoter mutations, it is still possible that this patient and her family members carry some undetected BRCA mutation. For BRCA1 this was made unlikely by presence of the BRCA1 protein and by absence of LOH of BRCA1. However, for BRCA2 we cannot exclude this, and another gene implicated in familial breast/ ovarian cancer may also be affected.

It is not known whether fibroadenomas occur more often in women with a strong family history for breast cancer or a BRCA mutation or if these tumors are more prone to malignant transformation. Searching the archives of the Department of Pathology, Free University Medical Centre, Amsterdam, The Netherlands revealed two more fibroadenomas removed from two women with a BRCA1 mutation. Both these fibroadenomas lacked lesions associated with an increased relative risk for breast cancer, i.e. no complex changes or CIS.

Two reports exist on the simultaneous occurrence of CIS arising within multiple fibroadenomas. In both reports a patient is described with two fibroadenomas containing LCIS [4,7]. Our report is the first describing CIS arising in three fibroadenomas. In addition, synchronous fibroadenomas harboring different types of CIS from one fibroadenoma to the other have never been described. Reviewing 400 fibroadenomas at the Free University Medical Center yielded 8 fibroadenomas with CIS [6], with the patient described here being the sole one with multiple fibroadenomas with CIS. Therefore, CIS within multiple fibroadenomas seems to be an extremely rare event. In the otherwise normal breast CIS is associated with a RR for invasive breast cancer of approximately 10 [11]. The exact relative risk associated with CIS arising within fibroadenoma is not known, but Ozello and Gump advise to treat it as if it arose from an otherwise normal breast [5].

Although malignant transformation of a fibroadenoma is infrequent, the presence of this tumor in a woman with a positive family history may be of a greater clinical significance compared to fibroadenomas arising in women without any additional risk factors. Detection of malignancy developing within a fibroadenoma can be difficult. Clinical and radiological signs may be masked until breach of the false capsule [12]. Physicians should be aware of the progression capabilities of breast fibroadenomas, in particular in women with a known BRCA mutation or a strong family history for breast/ovarian cancer. This case report supports the need for a more aggressive diagnostic approach towards solid benign appearing breast lesions in women with a strong positive family history of breast and/or ovarian cancer.

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Chapter 5

Analysis of Progression of Fibroepithelial Tumors of the Breast by PCR Based Clonality Assay

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Abstract

Fibroadenoma and phyllodes tumor of the breast are both fibroepithelial tumors. Although progression to epithelial malignancy has been described, the behavior of most fibroadenomas is benign. Phyllodes tumor on the other hand can display locally destructive growth and can even metastasize. A relation between the two tumors has been suggested in literature. We investigated the clonality of both stroma and epithelium of these fibroepithelial tumors and attempted to construct a model in which fibroadenoma can progress in both epithelial and stromal direction.

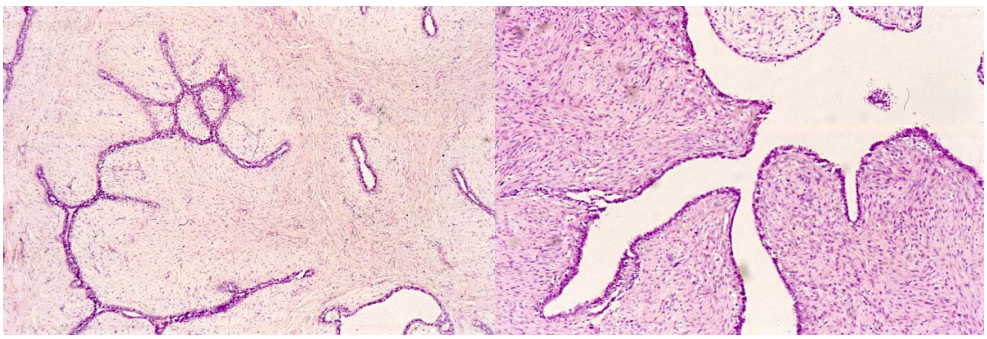
25 fibroadenomas and 12 phyllodes tumors were selected for analysis. Tissue was microdissected and analyzed for clonality using a polymerase chain reaction-based assay targeted at an X-linked polymorphic marker, the human androgen receptor gene (HUMARA).

19 fibroadenomas and 9 phyllodes tumors could be analyzed. Normal appearing epithelium, hyperplastic epithelium and stroma removed from fibroadenomas was polyclonal. As expected, carcinoma in situ (CIS) removed from 4 fibroadenomas was monoclonal. Three areas of apparent stromal expansion inside fibroadenoma were monoclonal suggesting stromal progression. Mostly, stroma of phyllodes tumors was monoclonal and epithelium polyclonal. In two cases however epithelium seemed to be monoclonal whereas in three other cases the stromal component was polyclonal. These findings indicate that fibroadenoma can progress in epithelial direction to CIS and in stromal direction to phyllodes tumor.

Introduction

Fibroadenoma of the breast is one of the most frequent causes of a breast mass. There is evidence for an associated increased relative risk for breast cancer [1]. Hyperplastic changes are frequently found in fibroadenomas and a considerable amount of reports exist on the development of malignancy from within the fibroadenoma [2-5]. Fibroadenoma is related to phyllodes tumor, both are fibroepithelial tumors, i.e. composed of an epithelial and a connective tissue component (Fig 1).

Figure 1. Typical fibroadenoma (left) and phyllodes tumor (right). Although both biphasic tumors, phyllodes tumor is characterized by a more cellular stroma, nuclear atypia, leaf-like stromal overgrowth and more mitotic figures as compared to fibroadenoma (haematoxylin and eosin, original magnification 100x).



However, the behavior of fibroadenomas is benign, in contrast with the unpredictable character of phyllodes tumors which can recur and even metastasize [6]. Progression of the stromal compartment of fibroadenoma to phyllodes tumor has been suggested [7-9], but direct evidence is lacking. A sharp line between fibroadenomas and phyllodes tumors cannot be drawn based upon histological criteria [7]. The connective tissue component of phyllodes tumors is more prominent, displaying a higher cellularity, more mitoses and nuclear atypia compared to fibroadenomas, as well as a more prominent leaf-like growth pattern [7, 8]. Indeed, the connective tissue compartment of the phyllodes tumor is thought to be the neoplastic component. Surprisingly, a recent study has suggested that at least in a subset of phyllodes tumors the epithelial component is neoplastic as well [10]. In addition, a case of phyllodes tumor has been described in which the metastasis was composed of both stroma and epithelium [11]. Fibroadenoma is regarded by some as a hyperplastic lesion based on clonality data [12]. However, in several cases of fibroadenoma cytogenetic studies after short-time culture detected clonal aberrations in connective tissue alone and in both connective tissue and epithelium after short-time cell culture [13-15].

Usually, a neoplasm is considered to descend from a single transformed progenitor cell (monoclonal) [16,17]. In contrast, a reactive process is characterized by a proliferation of cells of multiple origins (polyclonal). Analysis of clonality in females takes advantage of the silencing of one of both X-chromosomes by methylation [18], allowing to make the distinction between both X-chromosomes. In a polyclonal cell population, in fifty percent of cells the maternal X-chromosome is inactivated, in the remaining half the paternal X-chromosome (random inactivation). In a neoplasm, being monoclonal in nature, the same X-chromosome is inactivated in all cells (non-random inactivation). A second requirement for clonal analysis is polymorphism of an X-linked gene. The human androgen-receptor gene is a highly polymorph locus with a heterozygosity rate of 90% [19]. Methylation of its HhaI and HpaII restriction sites is correlated with X-inactivation [19]. After treatment of DNA with a methylation sensitive restriction enzyme only the methylated, inactive X-chromosome will be available as a polymerase chain reaction (PCR) template. Since in a monoclonal cell population in all cells either the short or the long allele is inactivated, this will result in one PCR product. In contrast, in a polyclonal cell population both alleles will be available for amplification.

It has been described for only a limited number of cases, that epithelium in both fibroadenomas and phyllodes tumors is polyclonal and the connective tissue compartment in fibroadenomas is polyclonal whereas in phyllodes tumors it is monoclonal [12]. However, several reports suggest that this may not be so straightforward. We therefore applied the human androgen receptor based clonality assay to a larger, histologic diverse group of these fibroepithelial tumors. We tested the hypothesis that fibroadenomas can progress in both epithelial (hyperplasia, carcinoma in situ [CIS], invasive carcinoma) and stromal direction (phyllodes tumor).

Materials and methods

Sample selection and microdissection

Formalin fixed and paraffin embedded blocks of a total of 37 cases, 25 fibroadenomas and 12 phyllodes tumors, were collected from the archives of the Department of Pathology, VU University medical center Amsterdam, and the Pathology Laboratory East Netherlands, Enschede, The Netherlands. Most fibroadenomas were usual with elongated two-layered epithelial ducts and low cellular stroma without nuclear atypia and no more than one mitoses per 10 high power fields (HPF). 4 fibroadenomas contained an area of apparent stromal expansion with increased cellularity and/or more than 1 mitoses per 10 HPF [7]. Further, 6 fibroadenomas harbored epithelial hyperplasia and 5 contained CIS (3 ductal CIS [DCIS] and 2 lobular CIS [LCIS]). The phyllodes tumors comprised 6 benign tumours, 3 of borderline malignancy and 3 malignant tumors. Histologic diagnoses were made according to the criteria of Moffat [6]. Normal tissue of 28

cases could be retrieved and was screened for zygosity. For selective microdissection of epithelial and stromal compartments, 10 sections of 8µm were cut and haematoxylin stained. A 4µm adjacent section was haematoxylin and eosin (H&E) stained to facilitate orientation. Epithelium and connective tissue were microdissected with a laser [20,21] and harvested with a needle under an inverted microscope. We aimed at obtaining a minimum of 2000 cells dissected from at least two different ducts or areas of stroma. The microdissected tissue was placed in 75µl of digestion buffer containing 50 mM Tris, 1 mM EDTA, 0.5% Tween 20 and 200µg/ml Proteinase K at a pH of 8.5 and incubated overnight at 55°C. If residual material was present, additional Proteinase K was added until complete digestion was achieved. 7.5µl of the DNA mixture were digested overnight at 37°C in 10U of HpaII (Roche) in a total volume of 15µl. A separate aliquot of DNA was prepared leaving out the restriction enzyme. Prior to PCR samples were heated to 94°C for 4 minutes in order to deactivate the restriction enzyme.

nPCR for clonality assay

The first round of the nested PCR (nPCR) was performed in a total volume of 20µl containing 10pmol of each outer primer AR1 (5'-TGTGGGGCCTCTACGATG-3') and AR3 (5'-CCGTCCAAGACCTACCGA-3') [22], 200µM of each dNTP, 0.5U Taq DNA polymerase (Perkin Elmer), 1.25mM $MgCl_2$, 1x PCR-reaction buffer and 2µl of the DNA mixture. After initial denaturation at 94°C for 4 minutes in a thermal cycler (Perkin Elmer), 25 cycles using cycling parameters of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 30 seconds were performed. As a negative control water, instead of DNA digestion mixture was used, whereas purified female DNA served as positive control. The second round of amplification was performed with inner primers 2 (5'-CCGAGGAGCTTTCCAGAATC-3') and 4 (5'-TACGATGGGCTTGGGGAGAA-3') [23], using 10 pmol of each primer. Primer 4 was fluorescently labelled with FAM. The total reaction volume of 20 µl consisted of 1x PCR-reaction buffer, 0.5U of Taq DNA polymerase (Perkin Elmer), 1.25mM $MgCl_2$, 200µM of each dNTP, 5 µg bovine serum albumin and 2 µl of PCR product of the first round. PCR parameters used were 94°C for 5 minutes and 25 cycles of 94°C for 30 seconds, 57°C for 45 seconds, 72°C for 45 seconds and a final extension period of 7 minutes at 72 °C. Efficiency of amplification was tested on a 2% agarose gel after ethidium-bromid staining.

PCR products were mixed with a commercial size standard (GS350 Rox, ABI-Perkin-Elmer) and electrophoresed on a 6% 24-cm well-to-read denaturating polyacrylamide gel using an ABI373A automatic sequencer (ABI-Perkin-Elmer). All samples were PCR amplified and analyzed in duplicate.

Interpretation of results

Interpretation of results was performed according to Lucas et al with minor modifications [24]. The total PCR product of an allele was calculated by combining the area of the peak of the highest molecular weight stutter band and the primary band. To obtain an amplification ratio (AR), the total PCR product for the long allele was divided by that of the short allele. The clonality ratio (CR) for a sample was calculated by dividing the undigested AR by the restriction digested AR. This method corrects for preferential amplification of one of both alleles. The clonality ratio gives the change in amplification after methylation sensitive restriction digestion and is an indication of methylation preference. Theoretically, a polyclonal cell population is characterized by a CR of 1 and a monoclonal population by a CR of 0. However, a CR<0.4 is regarded as being indicative of monoclonality because of unavoidable contamination of non-tumorous cells and technical limitations [24]. Since we work with archival tissues, blood is not available to correct for unequal lyonization [25,26], but blood does not necessarily reflect the X-inactivation status in breast tissue [27]. We therefore prefer using normal adjacent breast tissue as normal control. Still, we were not able to obtain sufficient normal adjacent breast tissue in all cases because the tumor had been enucleated. Since patch size in normal breast tissue is about one lobule with a variable stretch of duct [28], using too little tissue for correction for constitutive skewing might lead to distorted results. Therefore, if sufficient adjacent normal tissue was present, a CR<0.5 of the normal tissue was reason to exclude the patient for risk of constitutive skewing [25]. If normal tissue lacked or was scarce, a case was only included if a CR ≥0.6 was found in one of the compartments.

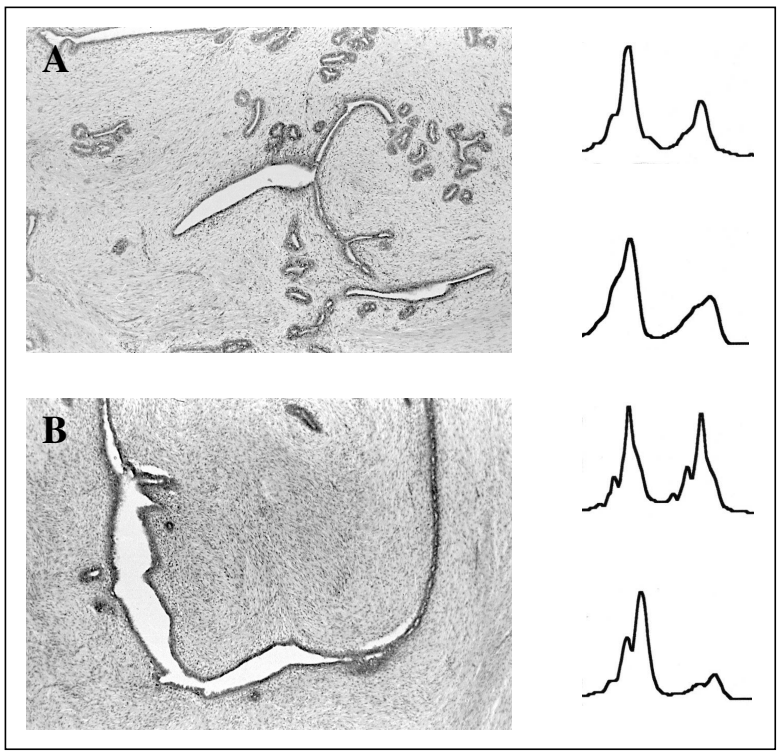
Results

Of the 28 samples with normal tissue available 3 were homozygous for the human androgen receptor gene and were excluded. In addition, 1 phyllodes tumor and 1 fibroadenoma with DCIS had to be excluded because of suspected constitutive skewing. All 9 samples without normal tissue displayed polyclonal heterozygosity in at least one compartment and could therefore be included in the study. Therefore, 91% of our samples were informative for this study. This is in agreement with the heterozygosity rate of 86% reported in literature [19]. Four further cases gave results that could not be confirmed on repeat analysis and were excluded from the study. A total of 28 cases, 19 fibroadenomas and 9 phyllodes tumors, remained.

A total of 19 fibroadenomas were analyzed. Because of near-total replacement of the normal epithelium by CIS or hyperplasia, normal epithelium was not analyzed in 8 cases. In all of the remaining cases the normal epithelium displayed a polyclonal pattern. Hyperplastic enlarged ducts could be microdissected from six cases of fibroadenomas, which were all polyclonal. All four fibroadenomas with CIS eligible for this study (2 DCIS and 2 LCIS) showed non-random X-inactivation. As expected, the

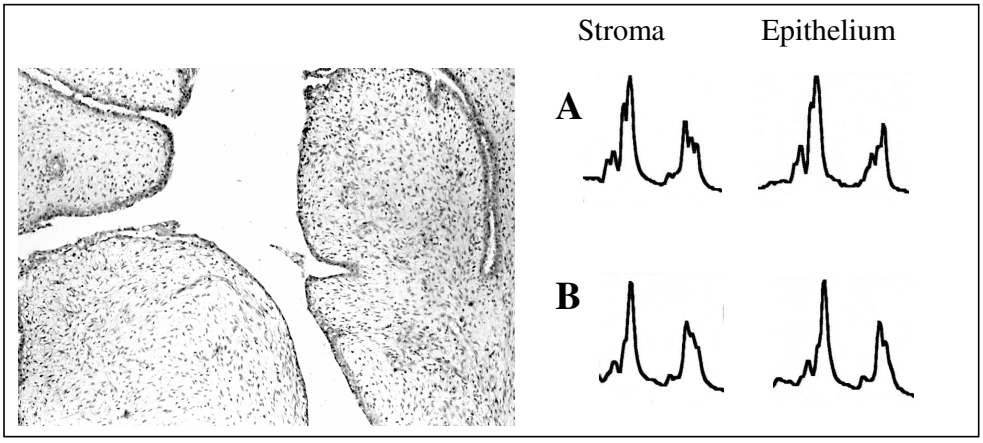
connective tissue component of most fibroadenomas was polyclonal. However, in three fibroadenomas a part of the tumor with phylloid like stromal expansion was monoclonal, whereas the normal appearing stroma was polyclonal (Fig 2).

Figure 2. Fibroadenoma with an area of stromal expansion. In an otherwise unsuspicious fibroadenoma an area of increased stromal cellularity was found with a maximum of 8 mitoses per 10 high power fields. On analysis, normal appearing stroma (A) was characterized by the same pre- (top) and postdigestion (bottom) allelic amplification ratios (CR=1). The area of stromal expansion (B), however, showed a strong decrease in amplification of the high molecular weight allele after restriction digestion. The corresponding CR was 0.26 (haematoxylin and eosin, original magnification 100x).



Digestion of microdissected epithelium from phyllodes tumors resulted in sufficient DNA in 7 of 9 cases. The two cases which lacked amplifiable amounts of epithelial DNA were malignant phyllodes tumors with very little epithelium. In 5 cases the epithelium was polyclonal, but two cases displayed a non-random pattern of inactivation. In these two cases the connective tissue compartment was monoclonal too, with methylation preference for the same allele as in the epithelium. In all 9 cases amplification of stromal DNA was successful. Stroma of phyllodes tumors was monoclonal in 6 cases. Surprisingly, in three cases stroma gave a polyclonal pattern (Fig 3). Two of these cases were benign, one was of borderline malignancy.

Figure 3. Phyllodes tumor with identical pre- (A) and postdigestion (B) sequencergraphs. Therefore, both epithelium and stroma are polyclonal (haematoxylin and eosin, original magnification 100x).



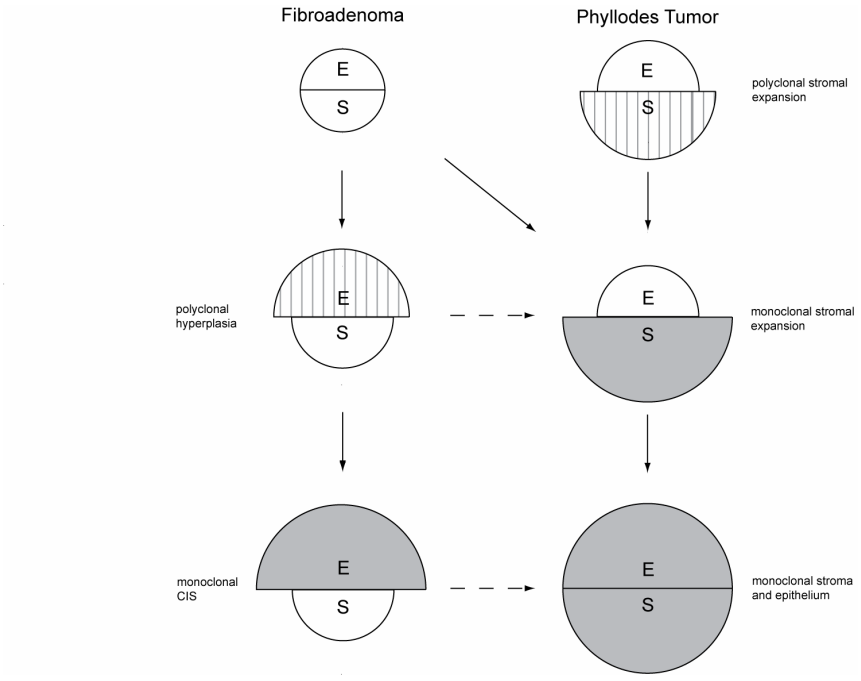
Discussion

Although an argument in favor, monoclonality is not equal to neoplasia. Indeed, a recent study demonstrated that up to a third of breast carcinomas may be multiclonal and not monoclonal [29]. Moreover, some monoclonal proliferations are quite indolent and do not display malignant behavior [22,30,31]. In the present study we were not able to produce consistent results in four cases. A reason for these problems may be the poor quality of DNA obtained from archival formalin fixed tissue [32]. Further, a low amount of template DNA will result in an increase of variability [32]. However, we aimed at harvesting at least 2000 cells, which is much more than the amounts recommended as lower limits in previous studies [33,34]. Diaz-Cano and co-workers recommend microdissection from two different tumor areas [34]. We therefore harvested the target cells from at least two separate ducts or stromal areas. This strategy also reduces the influence of patches. Variable methylation patterns at the HpaII or HhaI restriction sites may also hinder obtaining reproducible results [35]. We therefore tested all samples in duplicate and included only those with consistent results [32]. In addition, we performed a nested PCR in order to raise sensitivity and specificity. As described before, in several cases a preference in amplification was seen for the short allele, which can also be attributed to reduced template quality [32].

In the present study we propose a model for progression of fibroadenoma in both stromal and epithelial directions to phyllodes tumor and carcinoma in situ, respectively (Fig 4). This model is based on well-documented morphological observations of the epithelial progression capabilities of breast fibroadenomas to usual hyperplasia, atypical hyperplasia and DCIS and LCIS [36]. Incidence of usual

hyperplasia of higher grade than mild in fibroadenomas is approximately 30% [2], and that of CIS arising within fibroadenoma ranges from 0.1%-2.1% in the literature [2,4]. In phyllodes tumors epithelial hyperplasia and even CIS may be seen as well [7]. In some lesions with the overall aspect of fibroadenoma, areas of phylloid stromal expansion with increased cellularity, nuclear atypia and mitoses can be seen [2,7,8], suggesting that eventually the overgrowth of these areas in such a lesion may lead to full blown phyllodes tumor. Further, pronounced intracanalicular growth of fibroadenomas may mimic the leaf like growth pattern of phyllodes tumors. All this explains that the distinction between fibroadenoma and phyllodes tumor can be difficult [7,8], and that these lesions in fact form a morphological continuum.

Figure 4. Progression model for breast fibroepithelial tumors. Fibroadenoma may clonally progress in epithelial direction (to CIS) and in stromal direction (to phyllodes tumor). Phyllodes tumor may evolve from a polyclonal stage to total monoclonality.



In accordance with the proposed progression model, we detected polyclonality in morphologically normal stroma and epithelium of fibroadenomas. Also the hyperplastic epithelium of fibroadenomas was polyclonal. These results seem to be in agreement with an earlier report, finding polyclonality in 10 out 12 benign ductal hyperplasias [37]. In contrast, Diallo et al detected monoclonality in all ductal hyperplasias analyzed [22]. These hyperplasias seemed to originate from a patch

whereas our hyperplasia cases arose from the polyclonal background of the normal fibroadenoma epithelium. CIS in fibroadenoma was monoclonal as is the case in the non-fibroadenomatous breast [22,38]. The fact that phylloidic stromal areas of 3 fibroadenomas displayed monoclonality supports the potential neoplastic nature of these areas and suggests that these areas could progress to phyllodes tumor. Monoclonality in the stromal component of breast fibroadenomas has been detected in three fibroadenomas that recurred as phyllodes tumor [9]. In addition, monoclonal stroma was found in one complex fibroadenoma and one fibroadenoma with phylloidal features [39]. We are the first to advance molecular evidence supporting the hypothesis that phyllodes like stromal expansion in fibroadenomas may be an early tumor progression event. Still, monoclonal progression of breast fibroadenomas in epithelial or stromal direction seems to be an infrequent event. Based on earlier work we estimate its frequency to be approximately 4% [2].

In most phyllodes tumors connective tissue was monoclonal and epithelium was polyclonal. However, in two cases epithelium was monoclonal, suggesting that also this component may be neoplastic. In addition, allelic imbalances have been detected in both stroma and epithelium, implying that both are neoplastic [10]. The histopathology of our two cases was that of mild hyperplasia and normal epithelium. A possible explanation for the apparent monoclonal epithelium might be inclusion of an epithelial patch by expansive growth of the phyllodes tumor. However, by harvesting a relatively large amount of target cells from different ducts we reduced the influence of this mechanism. If a phyllodes tumor would arise from a patch, both stroma and epithelium would be monoclonal with methylation preference for the same allele. Indeed, this was observed in both cases. If not originating from a patch, the question arises if a tumor with similar X-inactivation patterns in both compartments originates multiclonally or monoclonally [40]. The possibility of the epithelial and stromal component sharing the same ancestor cell may exist, though [40-43]. The stromal component of three phyllodes tumors displayed a polyclonal pattern. Two were benign, one was of borderline malignancy. These results are in conflict with those of Noguchi et al. who detected monoclonality in stroma of all five phyllodes tumors analyzed [12]. However, they did not specify the histology of the tumours and possibly analyzed only borderline and malignant tumors with lower chances of finding polyclonality. The observed clonal patterns in phyllodes tumors may be explained by Nowell's clonal evolution theory [17]. It suggests that a tumor evolves from a polyclonal stage into an oligoclonal phase and finally to a monoclonal tumor. This model seems to exist in several types of tumours [43-46]. Since polyclonality was found in two benign and one borderline phyllodes tumor but not in full-blown malignant phyllodes tumors this model of clonal evolution may suit these fibroepithelial tumors, as has been suggested previously by a cytogenetic study [47]. Appearance of a monoclonal stromal expansion from the polyclonal stroma of a fibroadenoma also seems to fit this model. An alternative explanation for the

observed polyclonality would be the gain of an Xq-arm or a whole X-chromosome. However, by CGH analysis no gain of Xq or the whole X chromosome was observed [48]. Although clonal analysis seems to tolerate a considerable amount of contamination by non-tumorous elements [39,49,50], we minimized this factor by careful microdissection.

These results provide evidence for our hypothesis that fibroadenomas may progress in epithelial direction to fibroadenomas with epithelial hyperplasia (polyclonal) or CIS (monoclonal). Likewise, fibroadenoma may progress in stromal direction by clonal expansion of phylloidic areas to phyllodes tumor and in both directions to phyllodes tumors with both clonal epithelial and connective tissue compartments. Further, our results suggest existence of clonal evolution in the progression of phyllodes tumors of the breast.

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Chapter 6

Progressive Deregulation of the Cell Cycle with Higher Tumor Grade in the Stroma of Breast Phyllodes Tumors

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Abstract

Breast phyllodes tumors are rare fibroepithelial lesions with largely unknown tumorigenesis. As cell cycle derailment is a common phenomenon in carcinogenesis of different types of tumors, we studied the involvement of several key cell cycle regulating proteins in the pathogenesis of phyllodes tumors.

Immunohistochemistry for Ki67, cyclin A, cyclin D1, pRb, p53, $p16^{INK4A}$, bcl-2, and $p21^{waf1}$ was performed on 40 primary and 7 recurrent phyllodes tumors of different grades. Immunoreactivity was assessed in epithelium and stroma.

Twenty-one benign, 8 borderline and 11 malignant phyllodes tumors were identified. In the vast majority of cases the epithelium did not show altered expression of cell cycle regulators. Stromal overexpression of $p16^{INK4A}$, p53, cyclin A, pRb and $p21^{waf1}$ all were significantly correlated with tumor grade. The total number of altered proteins in stroma displayed an increase with higher grade and was accompanied by an increase in proliferation. Stromal cyclin A expression was found to be the single best separating marker between tumor grades. Correlations existed between stromal overexpression of G1-S checkpoint proteins $p16^{INK4A}$ and $p21^{waf1}$, $p16^{INK4A}$ and p53, and p53 and pRb. No differences in immunostainings were detected between primary tumors and 7 corresponding recurrences. Four or more altered proteins and p53 expression in the stromal component were independent negative prognosticators for disease free survival.

Taken together, our results show that the stromal component of mammary phyllodes tumors displays an increasing level of cell cycle deregulation with higher tumor grade, whereas the epithelial compartment mostly remains inconspicuous. Several combinations of aberrantly expressed cell cycle proteins seem important in the stromal progression of phyllodes tumors. Further, the number of stromal cell cycle aberrations and stromal p53 expression may predict clinical behavior.

Introduction

Phyllodes tumors are fibroepithelial breast tumors, composed of epithelial and stromal components. We previously demonstrated that, mostly, the stroma of phyllodes tumors is monoclonal and the epithelium polyclonal [1]. Morphologically, phyllodes tumor is characterized by overgrowth of stroma in a pericanalicular or leaf-like intracanalicular pattern, increased stromal cellularity, stromal cellular atypia and increased numbers of stromal mitoses [2]. Phyllodes tumors are graded as benign, borderline or malignant [2,3]. Recurrence occurs in 8 to 65% of cases, depending on grade of the primary tumor [4]. Metastases are encountered in up to 22% of malignant tumors [2]. Several studies suggested that a positive surgical margin is a major prognosticator for recurrence [2,5], but others failed to confirm this [6,7]. Similar contradictory reports exist with regard to stromal overgrowth, mitotic activity and flow cytometric features [8-15]. Therefore, there is a need for better prognostic criteria. Few studies have addressed the role of tumor suppressor genes in phyllodes tumors. Immunohistochemical accumulation of p53 correlated with tumor grade but not with clinical outcome in some studies [16-18], while another larger study found a relation with disease free survival [13]. p53 accumulation was detected in several phyllodes tumors, but $p21^{waf1}$ and mdm2 expression were absent [19]. No comprehensive studies however exist on the role and prognostic value of cell cycle regulators in phyllodes tumors.

Distortion of the cell cycle machinery is a major phenomenon in carcinogenesis. The cell cycle is regulated by cyclins, cyclin-dependent kinases (CDK) and two groups of cyclin-dependent kinase inhibitors (CDKIs) [20,21]. The CIP/KIP family members, including $p21^{waf1}$, p27 and p57, are universal CDKIs. The INK4 family members p15, $p16^{INK4A}$, p18 and p19 exert a negative regulatory function on CDK4/CDK6. The cyclin:CDK complexes phosphorylate the retinoblastoma protein, pRb, which allows cells to enter the S-phase. Distortion within the proliferation controlling cyclin D1/CDK4/$p16^{INK4A}$/Rb-pathway leads to loss of control at the G1-S checkpoint. The role of p53 as a major regulatory protein in the cell cycle is well established. Through promotion of expression of $p21^{waf1}$, p53 can halt cellular growth and induce apoptosis in response to DNA damage or other types of cellular stress.

By studying primary tumors of different grades and their recurrences we attempted to gain insight in the role of cell cycle regulators in the development, progression and prognosis of breast phyllodes tumors.

Methods

Tissue samples

Forty primary formaldehyde fixed/paraffin embedded phyllodes tumors were retrieved from our archives, as well as seven recurrences of these tumors. Tumors were graded as benign, borderline or malignant based on the degree of stromal cellularity, stromal overgrowth, cellular atypia, invasiveness of tumor margin and mitotic activity index (MAI) as described previously in detail by Moffat et al [2]. Mitoses were counted using established criteria in an area of $1.6mm^2$ at a 400x magnification [22]. Tumors were graded in the most unfavorable areas provided they

comprised at least 10% of the total tumor area [2]. Clinical data was gathered from medical charts.

Immunohistochemistry

Four µm sections were cut and mounted on coated slides. To stop endogenous peroxidase activity, deparaffinized and rehydrated sections were submersed in 0.3% hydrogen peroxide. Subsequently, antigen retrieval was performed by autoclaving the slides in a pressure cooker (120°C, 20') in 0.01M citrate buffer (ph=6.0) or EDTA (ph=8.0) when staining for $p16^{INK4A}$. Normal rabbit serum was used to block non-specific staining. Slides were incubated overnight at 4°C with the following mouse monoclonal antibodies: cyclin D1 (Neomarkers DCS-6, 1:400), $p16^{INK4A}$ (Pharmingen G175-405, 1:500), pRb (Pharmingen G3-245, 1:1000), $p21^{waf1}$ (Oncogene Science Ab-1, 1:50), p53 (DAKO DO-7, 1:500), cyclin A (Novocastra 6E6, 1:100), Ki67 (DAKO MIB-1, 1:40) and bcl-2 (DAKO 124, 1:50). A secondary biotinylated rabbit anti-mouse antibody diluted 1:500 was applied for 30' at room temperature. Thereafter, slides were incubated with avidin-biotin-peroxidase complex (DAKO) at 1:200 dilution for 1 hour at room temperature. 3,3'diaminobenzidine-tetrahydrochloride was used as chromagen and haematoxylin as counterstain. Between steps, slides were rinsed in phosphate buffered saline. Negative (primary antibody omitted) and appropriate positive controls were included throughout.

Immunoreactivity was scored both in the epithelial and stromal components. The percentage of positive cells was estimated on a semi-continuous scale by AK and PvD in the area displaying the highest (Ki67, cyclin A, cyclin D1, p53, $p21^{waf1}$) or lowest (bcl-2) density of positive cells, believed to represent the biologically most relevant areas. These areas had to involve at least 10% of the total tumor area (see above). Since both accumulation and loss of pRb reactivity might be of importance [23-25], staining for pRb was estimated for the whole slide. $p16^{INK4A}$ was assessed similarly, as scoring of $p16^{INK4A}$ immunoreactivity varied between different studies [26-28]. For statistical analysis, high expression was defined as 10% or more positive staining cells in accordance with previous studies [19,27,29,30]. Further, loss of expression of pRb was indicated by less than 1% nuclear immunostaining.

Statistical analysis

P-values (SPSS, Chicago, IL, USA) below 0.05 were regarded as significant. The chi-square test was used to test for differences in expression of cell cycle proteins between tumor grades and between primary and recurrent tumors. Stepwise discriminant analysis was applied to detect the best separating features between tumor grades. Immunohistochemical expression of cell cycle regulators and proliferation markers were correlated among themselves by Fisher's exact test. Differences in number of altered proteins between grades were investigated by the Kruskall-Wallis test. The clinical endpoint for survival analysis was local or distant recurrence (disease free survival [DFS]). Patients treated by mastectomy were excluded from survival analysis. For univariate survival analyses, Kaplan-Meier curves were plotted and evaluated with the log-rank test. Cox regression was performed to identify independent prognostic variables.

Results

Patient characteristics

21 benign, 8 borderline and 11 malignant primary phyllodes tumors were identified. Epithelial hyperplasia, mostly focal, was found in 15 cases (37.5%) and was not related to grade. Mean ages of patients with benign, borderline and malignant phyllodes tumors were 45.5±16.8 years, 57.9±12.8 years and 54.3±12.9 years, respectively (p=0.080). Mean tumor sizes were 4.5±2.5 cm, 7.1±7.0 cm and 4.8±2.5 for benign, borderline and malignant grade, respectively (p=0.855). Five patients were treated by mastectomy, the remainder by local excision. Excision was incomplete for 13 primary tumors, whereas for 6 tumors this information could not be retrieved.

Differences between tumor grade and cell cycle expression

Altered expression of cell cycle proteins was seldomly found in the epithelial component. Only 3 cases of $p21^{waf1}$ overexpression, 3 of altered pRb expression, 1 overexpressing cyclin A and 3 with cyclin D1 overexpression were found in the epithelium, all without relation to tumor grade or epithelial hyperplasia. In contrast, aberrant expression was frequent in the stromal component. Differences in stromal overexpression of cell cycle regulators for the different grades are summarized in Table 1.

Table 1. Relation between altered expression of cell cycle regulators in stroma and tumor grade. Numbers and percentages (between brackets) of tumors with aberrant expression are shown (chi-square test). [a] ns, statistically not significant

	Grade			
Marker	Benign (n=21)	Borderline (n=8)	Malignant (n=11)	*P* value[a]
Ki67	1 (5)	5 (63)	11 (100)	<0.001
p53	1 (5)	1 (13)	5 (46)	0.015
$p16^{INK4A}$	2 (10)	3 (38)	8 (73)	<0.001
cyclin A	0 (0)	0 (0)	8 (73)	0.001
cyclin D1	2 (10)	2 (25)	2 (18)	0.583
$p21^{waf1}$	0 (0)	1 (13)	4 (36)	0.013
pRb <1%	3 (15)	0 (0)	1 (9)	0.466
pRb ≥10%	0 (0)	0(0)	4 (36)	0.003
bcl-2	0 (0)	0 (0)	1 (9)	0.271

All malignant phyllodes tumors displayed high Ki67 expression in the stromal component. Stromal p53 overexpression correlated significantly positively with tumor grade (p=0.015) and was found in nearly half of malignant grade tumors. Stromal overexpression of cyclin A showed a striking increase from borderline to malignant tumors, from 0 to nearly 75% of tumors. In contrast with negative staining for pRb, high expression correlated positively with grade (p=0.003) and was exclusively seen in malignant tumors. $p16^{INK4A}$ immunostainings displayed both nuclear and cytoplasmic staining. Cytoplasmic staining seemed to parallel nuclear staining, both were positively correlated with tumor grade (p=0.005 and p<0.001, respectively).

Stromal cyclin A expression was found to be the single best discriminating factor between tumor grades (p<0.001, 77.5% accuracy; Fig 1).

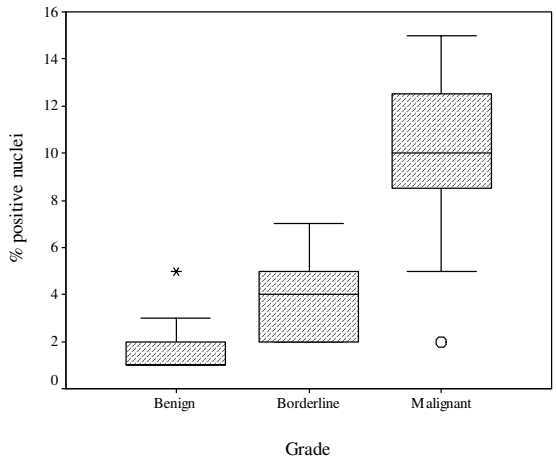

Figure 1. Differences in percentages of stromal cyclin A positive nuclei between breast phyllodes tumors of different grades.

Concerted expression of cell cycle regulators

No inter-relations existed between studied markers in the epithelial component. Table 2 displays correlations among cell cycle regulators and proliferation markers in stroma. In the stroma, several combinations of aberrantly expressed cell cycle regulators were found, rendering both pathways of cell cycle control ineffective (Table 2). $p16^{INK4A}$ stromal overexpression correlated with stromal overexpression of p53 (p<0.001) and $p21^{waf1}$ (p=0.031), hereby affecting both pathways. Likewise, stromal pRb overexpression was positively correlated with stromal overexpression of p53 (p=0.013).

Table 2. Interrelations between altered expression of cell cycle regulators and proliferation markers in stroma of breast phyllodes tumors (Fisher's exact test). [a] Cases were divided in high and low MAI using a cut-off of 10 mitoses.

	$p16^{INK4A}$	cyclin A	cyclin D1	$p21^{waf1}$	p53	pRb	pRb	bcl-2	MAI [a]
Ki67	0.005	<0.001	1.000	0.144	0.029	1.000	0.026	0.416	<0.001
$p16^{INK4A}$		0.008	1.000	0.031	<0.001	1.000	0.092	0.333	0.002
cyclin A			0.583	0.257	0.002	1.000	0.001	0.205	<0.001
cyclin D1				1.000	1.000	0.574	1.000	1.000	0.205
$p21^{waf1}$					0.204	0.507	1.000	1.000	0.007
p53						0.565	0.013	0.179	0.011
pRb <1%								1.000	1.000
pRb ≥10%								0.103	0.020
bcl-2									0.410

Number of events

The number of proteins with altered expression (further denoted “events”) in the stroma displayed a steady increase with grade. Excluding Ki67 expression, benign, borderline and malignant phyllodes tumors displayed a mean of 0.7, 1.6 and 3.5 events in the stromal compartment, respectively ($p<0.001$). The distribution of the number of stromal events was as follows: 14/40 (35%) tumors displayed no aberrations, 8/40 (20%) showed 1 event, 2 altered proteins were found in 6/40 (15%) tumors, both 3 and 4 events were found in 5/40 tumors (12.5%) and 5 altered proteins were seen in 2/40 tumors (5%). The number of events was correlated with proliferation as measured by Ki67 expression ($p<0.001$; figure 2) and MAI ($p<0.001$). In the majority of phyllodes tumors (30/40, 77.5%) no epithelial events were found, 9/40 (22.5%) showed altered expression of one protein and only 1 tumor (2.5%) had 2 abnormalities. No differences in number of epithelial events were detected between tumor grades ($p=0.542$).

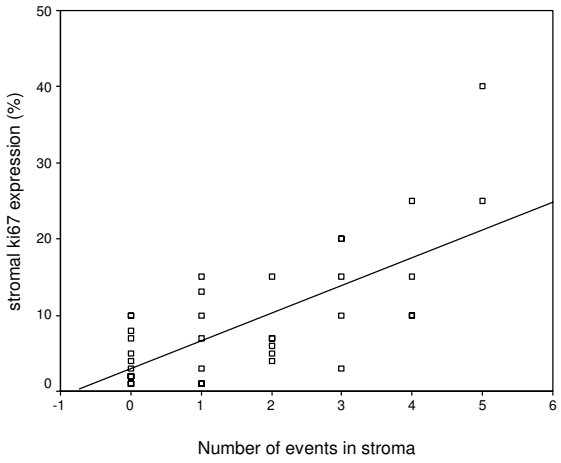

Figure 2. Relation between number of aberrantly expressed proteins (“events”) in the stromal component of breast phyllodes tumors and proliferation as measured by Ki67 expression ($r=0.680$; $p<0.001$).

Recurrent disease and prognosis

Three patients were lost to follow-up. The median follow-up time of the remaining 37 patients was 93 months (range 4-215). Ten (27%) tumors recurred; five (50%) malignant, one (12.5%) borderline and four (19.1%) benign tumors. The median time to recurrence was 38 months (range 4-162). Most were local recurrences, but one malignant tumor metastasized to the lung.

The corresponding local recurrences of three benign and three malignant tumors were recovered. One malignant tumor recurred twice, resulting in 7 available recurrent tumors. Two tumors had progressed to a higher grade, and one malignant tumor recurred with borderline grade. No statistically significant differences in expression of cell cycle regulators could be detected between primary and recurrent tumors.

Univariate survival analysis (Table 3) showed an inverse relation between DFS and stromal overexpression of p53 ($p<0.001$; Figure 3), cyclin A ($p=0.002$), Ki67 ($p=0.015$) and pRb ($p=0.030$), four or more stromal events ($p<0.001$), tumor diameter of ≥5cm ($p=0.042$) and tumor grade ($p=0.023$). Margin status had no prognostic

significance. However, recurrent tumors from incompletely excised tumors recurred earlier than those from completely excised tumors (Fig 4). When entering all variables that showed a univariate correlation with DFS in Cox regression, stromal p53 overexpression and four or more stromal events were independent prognosticators for recurrent disease.

Table 3. Prognostic variables in phyllodes tumors as determined by univariate analysis of disease free survival. Data given as number (percentage).

Variable		No. of	With	Disease	*P* value	Log-rank
p53 stroma	< 10%	27	5 (18)	21 (82)	<0.001	12.54
	≥10%	6	5 (83)	1 (17)		
cyclin A stroma	<10%	26	6 (23)	20 (77)	0.002	9.70
	≥10%	7	4 (57)	3 (43)		
Ki67 stroma	<10%	19	4 (21)	15 (79)	0.015	5.91
	≥10%	14	6 (43)	8 (57)		
pRb stroma	<10%	30	8 (27)	22 (73)	0.030	4.74
	≥10%	3	2 (67)	1 (33)		
No of stromal events	<4	28	6 (21)	21 (79)	<0.001	14.27
	≥4	5	4 (80)	1 (20)		
Grade	benign	18	4 (22)	14 (78)	0.023	7.57
	borderline	5	1 (20)	4 (80)		
	malignant	10	5 (50)	5 (50)		
Size	<5 cm	19	2 (11)	17 (89)	0.042	4.12
	≥5 cm	12	6 (50)	6 (50)		

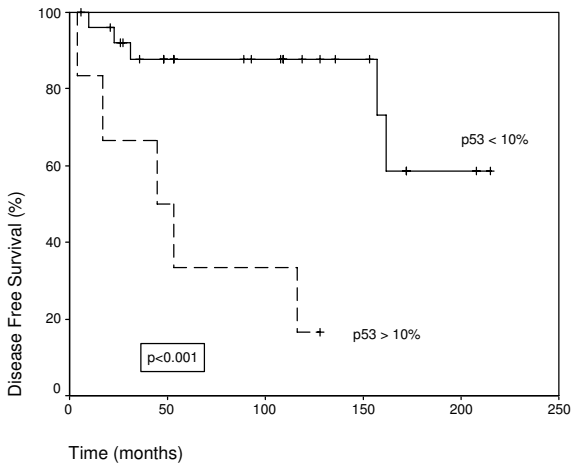

Figure 3. Kaplan-Meier survival curve illustrating disease free survival for patients with breast phyllodes tumors with high (≥10%) versus low (<10%) expression of p53 in the stromal component.

Discussion

This is the first study evaluating two major cell cycle control pathways in phyllodes tumors of different grades. With higher tumor grade, the stromal compartment of phyllodes tumors showed progressive cell cycle derailment. Although

recent studies have suggested more than a passive role for the epithelium [31,32], the epithelial component did not display conspicuous cell cycle changes.

$p16^{INK4A}$ is a tumor suppressor which blocks cell cycle progression at the G1 restriction point by inhibition of cyclin:cdk4 and cyclin:cdk6 activity [33]. Surprisingly, high stromal expression of $p16^{INK4A}$ was positively correlated with stromal p53 overexpression, mitotic count, tumor grade and stromal Ki67 overexpression. Although initial reports in breast cancer showed that loss of $p16^{INK4A}$ expression indicated adverse prognosis [26], recent studies related high $p16^{INK4A}$ protein and mRNA levels to a more malignant phenotype and poor outcome [28,34,35]. Further, a relation between cytoplasmic overexpression of $p16^{INK4A}$ and aggressive features has been described [27,28]. In the present study, cytoplasmic $p16^{INK4A}$ overexpression seemed to parallel nuclear overexpression (data not shown). The mechanisms behind up-regulation of $p16^{INK4A}$ are poorly understood, but possible explanations include loss of pRb [36], accumulation due to high proliferation and long half-life of the protein [33] and cellular stress such as replicative senescence, ultraviolet radiation or hyperthermia [36,37]. No inverse relation was present between pRb and $p16^{INK4A}$ in our group, but $p16^{INK4A}$ was related to proliferation.

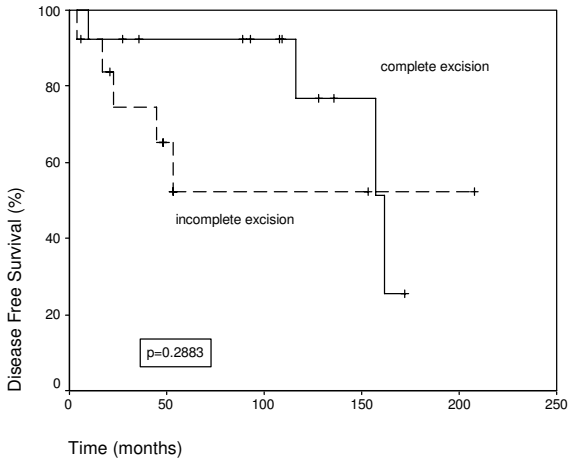

Figure 4. Kaplan-Meier survival curve for disease free survival of breast phyllodes tumors according to margin status.

Stromal cyclin D1 overexpression was not correlated with established prognostic markers and its role in phyllodes tumors, therefore, seems limited. A recent study detected overexpression of cyclin D1 in epithelium in 46% and in stroma of 19% of phyllodes tumors [31]. We detected stromal cyclin D1 overexpression in 15% of tumors, but in epithelium in only 3 (7.7%) cases. Cyclin D1 may be overexpressed in ductal hyperplasia [38,39], but like Sawyer et al, epithelial cyclin D1 overexpression was not related to the presence of hyperplasia. Although comparisons are hard to make since the cut-off point for cyclin D1 overexpression used in their study was not specified, the reason for this difference in frequency of epithelial cyclin D1 expression is unknown.

The p53 protein is involved in DNA damage control. In keeping with previous reports [13,16-19,40-44], stromal p53 overexpression was correlated with grade and was found in nearly 50% of malignant cases. Although not all immunohistochemically

detectable p53 accumulation is caused by mutation of the gene, p53 mutations have been detected in phyllodes tumors [19,42,45,46]. A defective p53 protein might lead to uncontrolled progression through the cell cycle and associated uncontrolled growth and tumor progression. Indeed, stromal p53 accumulation was correlated with increased proliferation. Further, we found that stromal p53 accumulation was an independent prognostic factor, confirming the results of a large study by Niezabitowski et al [13]. p53 seems to play an important role in the progression of phyllodes tumors.

Recently, cyclin A has been implicated as an important prognosticator in breast cancer [30,47]. Cyclin A is expressed in the late S, G2 and M phases of the cell cycle, shortly before mitosis, and is required to initiate DNA replication [48]. Not surprisingly therefore, stromal cyclin A expression was correlated with MAI and stromal Ki67 overexpression. Benign and borderline tumors on the one hand did not display stromal cyclin A overexpression, whereas 73% of malignant tumors showed overexpression. Stromal cyclin A overexpression may therefore reflect a final step towards uncontrolled proliferation in many cells. By stepwise discriminant analysis, stromal cyclin A expression turned out to be the best discriminating marker between tumor grades (Fig 1) and therefore seems a useful aid in accurately grading phyllodes tumors. Further, stromal cyclin A expression had prognostic power in univariate DFS analysis, but this was not an independent effect.

$p21^{waf1}$ is a transcriptional target of p53 and blocks cell cycle progression by interaction with CDKs [49], but p53 independent $p21^{waf1}$ activation pathways exist [50,51]. In breast cancer, increased $p21^{waf1}$ expression has been associated with decreased DFS [23], but others failed to detect this [52,53]. In our study $p21^{waf1}$ stromal overexpression was positively correlated with adverse prognosticators grade and MAI, but did not possess prognostic value. Overexpression of $p21^{waf1}$ was not seen in a previous report [19], but malignant tumors were strongly underrepresented in this study. Indeed, stromal $p21^{waf1}$ overexpression was seen pre-eminently in malignant phyllodes tumors (36%). Two of five $p21^{waf1}$ overexpressing phyllodes tumors were also p53 positive, indicating that $p21^{waf1}$ induction may occur independent of p53, e.g. due to specific growth factors [50]. Although $p21^{waf1}$ overexpression was found in epithelium of three tumors, no relation with other variables was found.

Since mitotic counts are part of almost all grading systems one can expect a positive correlation between tumor grade and Ki67 expression. Indeed, like the present study, all previous articles could confirm this [13,18,42,43,54-57]. In our group, all malignant tumors expressed Ki67 in more than 10% of stromal cells. Therefore, malignant grade may be denied by a finding of less than 10% positive stromal Ki67 staining. Further, stromal Ki67 overexpression was predictive of DFS, in part agreeing with a previous paper which found it to be predictive of overall survival but not of DFS [13]. Another paper did not detect any prognostic power, though [17].

The intratumor heterogeneity of phyllodes tumors is reflected in pRb staining, areas with complete loss and with moderate or over-expression coexisted within the same tumor. Since both loss of and over-expression of pRb may be of importance in carcinogenesis [23-25], immunoquantification was problematic and pRb results should be interpreted with caution. Stromal pRb overexpression was correlated with

grade and high proliferation rate. Although most papers focus on loss of pRb expression, the relation between proliferation and accumulation of pRb has been described previously in breast cancer [58]. In fibrosarcomas, phenotypically related to stroma of malignant phyllodes tumors, overexpression of pRb was associated with decreased DFS [24]. These observations were attributable to dominance of hyperphosphorylated forms of the protein. In our group, pRb overexpression was associated with decreased DFS, but pRb was not an independent prognosticator. Possible explanations for accumulation of this tumor suppressor gene product include abnormalities of upstream regulators of pRb expression or stabilization of pRb by binding to other proteins or viral oncoproteins [25]. Furthermore, pRb overexpression might provide protection against apoptosis and growth inhibition [59]. Loss of stromal pRb was not correlated with grade or other variables.

Like Kuenen et al [19], loss of the anti-apoptotic protein bcl-2 in stroma was found only in one primary phyllodes tumor and therefore seems of minor importance. Conflicting bcl-2 data is found in literature. Loss of bcl-2 in a recurrent tumor and metastasis in contrast with the primary tumor indicates a potential role for loss of bcl-2 [19]. We did not observe loss of bcl-2 in recurrent tumors. Another study found progressive loss of bcl-2 expression in both compartments with higher grade [57]. Moore et al found loss of bcl-2 in the stromal component of malignant tumors, but not in the epithelial compartment [60]. The epithelial component in the present study invariably expressed bcl-2 as does normal breast epithelium [39].

We evaluated 6 pairs of primary and recurrent tumors. Two tumors progressed to a more malignant phenotype, whereas one malignant primary recurred as a tumor of borderline grade. On comparison with the initial lesion, this recurrence showed a strong histologic and immunophenotypic resemblance with a small peripheral area of the primary which was located outside the high grade area. Therefore, the apparent “regression” was most likely due to the incomplete excision of the peripheral lower grade area. In general, no large differences in immunophenotype were found between primary and recurrent tumors. This suggests that the recurrent tumors were the result of residual disease and that wide local excision is the treatment of choice in phyllodes tumors. However, in survival analysis, a positive surgical margin was not predictive of recurrence. This is at variance with some previous reports [2,5]. As shown in Figure 4, a short follow-up time may result in an inverse correlation between positive margins and DFS. Therefore, differences in length of follow-up may in part explain these discrepancies. Like most previous reports [3,5,8,9,13], we found that tumor grade was predictive of recurrent disease. In agreement with a recent report [61], tumor size was inversely related to DFS in our series. Others found size to be predictive of death, not of DFS [5,16]. Besides being accompanied by an increase in proliferation, the number of events in the stromal component was of prognostic value as well. Four or more stromal events in a tumor turned out to be an independent prognosticator for DFS.

Although a paracrine role has been suggested for the epithelium [31,32,62,63], the epithelial compartment did not show cell cycle alterations in the vast majority of the tumors. Previously, a model was proposed in which growth of stroma in early stages of tumorigenesis is under control of the epithelium [31,63]. Uncoupling of this interaction in later stages would then lead to autonomous growth of stroma. It was

unclear however which mutations would initiate this process and in which compartment they would occur. Our findings suggest that cell cycle abrogation in the stromal component might be responsible for this loss of stromal-epithelial communication and progression towards a malignant phenotype. The transition from borderline to malignant grade seems to be of particular importance. The number of tumors with stromal overexpression of p53, $p16^{INK4A}$, $p21^{waf1}$, pRb and cyclin A is sharply increased during this final step towards a malignant phenotype. It is thought that multiple derailments in one pathway, i.e. the proliferation controlling Rb pathway or the apoptotic p53 pathway, do not add to a greater growth advantage. Indeed, in the stromal component, overexpression of p53 was not correlated with overexpression of $p21^{waf1}$, and overexpression of pRb not with overexpression of $p16^{INK4A}$. Further, overexpression of $p16^{INK4A}$ was correlated both with overexpression of p53 and $p21^{waf1}$, whereas overexpression of pRb was related to p53, assigning these three combinations of abnormally expressed G1-S checkpoint regulators a role in the progression of phyllodes tumors.

Taken together, our results suggest that the stromal component of mammary phyllodes tumors is characterized by an increasing level of cell cycle deregulation with higher tumor grade. The epithelial compartment does not display aberrant expression of cell cycle regulators. Several defined combinations of defective cell cycle regulators seem responsible for malignant progression of the stromal component of breast phyllodes tumors. Further, the number of stromal events and stromal p53 expression may predict clinical behavior.

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7

Chapter 7

Expression of HIF-1α and its Downstream Targets in Fibroepithelial Tumors of the Breast

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Abstract

Hypoxia-inducible factor-1α (HIF-1α) and its downstream targets CAIX and VEGF are key factors in the survival of proliferating tumor cells in a hypoxic microenvironment. We studied expression and prognostic relevance of HIF-1α and its downstream targets in phyllodes tumors and fibroadenomas of the breast.

Expression of HIF-1α, CAIX, VEGF and p53 was investigated by immunohistochemistry in a group of 37 primary phyllodes tumors and 30 fibroadenomas with known clinical follow-up. Tumor microvasculature was visualized by immunohistochemistry for CD31. Proliferation was assessed by Ki67 immunostaining and mitotic counts. Being biphasic tumors, immunoquantification was performed in stroma and epithelium.

Only two fibroadenomas displayed low-level stromal HIF-1α reactivity in the absence of CAIX expression. Stromal HIF-1α expression was positively correlated with phyllodes tumor grade ($p=0.001$), proliferation as measured by Ki67 expression ($p<0.001$) and number of mitoses ($p<0.001$), p53 accumulation ($p=0.003$), and global ($p=0.015$) and hot-spot ($p=0.031$) microvessel counts, but not to CAIX expression. Interestingly, concerted CAIX and HIF-1α expression was frequently found in morphologically normal epithelium of phyllodes tumors. The distance from the epithelium to the nearest microvessels was higher in phyllodes tumors as compared to fibroadenomas. Microvessel counts as such did not differ between fibroadenomas and phyllodes tumors, however. High expression of VEGF was regularly found in both tumors, with only a positive relation between stromal VEGF and grade in phyllodes tumors ($p=0.016$). Stromal HIF-1α overexpression in phyllodes tumors was predictive of disease free survival ($p=0.032$).

These results indicate that HIF-1α expression is associated with diminished disease free survival and may play an important role in stromal progression of breast phyllodes tumors. In view of the absence of stromal CAIX expression in phyllodes tumors, stromal upregulation of HIF-1α is most likely caused by hypoxia independent pathways, with p53 inactivation as one of possible causes. In contrast, coexpression of HIF-1α and CAIX in the epithelium in phyllodes tumors points to epithelial hypoxia, most likely caused by relatively distant blood vessels. On the other hand, HIF-1α and CAIX seem to be of minor relevance in breast fibroadenomas.

Introduction

After reaching a critical volume of several mm^3, a growing tumor becomes increasingly depleted of oxygen and nutrients and needs to adapt to its changing microenvironment. In order to survive, tumor cells must develop a vascular system and adapt their metabolism. A key regulator in this process is the transcription factor hypoxia-inducible factor-1 (HIF-1) [1], which controls the expression of several target genes. The protein product of *HIF-1* is a heterodimer and consists of two subunits, HIF-1α and HIF-1β. Under normoxic conditions the HIF-1α protein is rapidly degraded. O_2-dependent hydroxylation of proline residues in HIF-1α causes binding of the von Hippel-Lindau (VHL) tumor suppressor protein, which leads to ubiquitilation and subsequent degradation by the proteasome [2,3]. Hypoxia inhibits this process, resulting in upregulation of HIF-1α and its downstream target genes [4]. On the other hand, hypoxia-independent upregulation of HIF-1α may be accomplished by loss of tumor suppressor genes such as *PTEN* [5] and *VHL* [6], activation of oncogenes like *v-Src* [7] and stimulation by growth factors such as insulin-like growth factors [8,9] and epidermal growth factor [10]. Recently, it has been demonstrated that HIF-1α expression is of prognostic value in several types of cancer, including breast cancer [11]. A well-known target of HIF-1α is the vascular endothelial growth factor (*VEGF*) gene [12]. VEGF is a potent endothelial cell specific mitogen and is a major participant in the process of angiogenesis, resulting in formation of microvessels. Both VEGF expression [13,14] and microvessel density [15,16] are established prognosticators in many types of cancer. Upregulation of carbonic anhydrase 9 (*CA9*) gene expression was found to be dependent on HIF-1α [17]. The protein product of the *CA9* gene, CAIX, catalyses the hydration of carbon dioxide to carbonic acid and contributes to acidification of the surrounding microenvironment. CAIX is constitutively expressed in cells lining the alimentary tract [18]. Because of its correlation with lowered pO_2 in carcinoma of the cervix, CAIX expression is regarded as an intrinsic marker of hypoxia [19]. Further, CAIX expression has recently been related to poor outcome in breast cancer [20]. It seems therefore that HIF-1α and its downstream targets play pivotal roles in development and progression of cancer.

Breast fibroadenoma and phyllodes tumor are both fibroepithelial tumors, i.e. they are composed of an epithelial and stromal component. The distinction between both tumors may be difficult [21]. However, the behavior of fibroadenomas is benign, in contrast to phyllodes tumors which can recur and even metastasize. In a small number of cases, fibroadenoma may progress to phyllodes tumor [22,23]. Little is known on the mechanisms by which these tumors maintain a steady supply of nutrients as they grow. Few studies have addressed expression patterns of angiogenic growth factors in fibroepithelial breast tumors. Expression of basic fibroblast growth factor (bFGF), fibroblast growth factor receptor (FGFR) and VEGF were found at higher levels in stroma of phyllodes tumors as compared to

fibroadenomas [24]. Unfortunately, no information on the epithelial component was provided in this work. Expression patterns of platelet derived growth factor (PDGF) and PDGF-receptor (PDGF-R) suggest the presence of autocrine loops in stroma and paracrine stimulation of stroma by the epithelium [25]. A similar auto- and paracrine loop has been described for acidic FGF and FGFR-4 in fibroadenomas [26]. These studies suggest that stromal proliferation may be stimulated by secretion of mitogens by the epithelial compartment. Interestingly, hypoxia may stimulate both FGF and PDGF expression [27,28]. It would therefore be interesting to evaluate the role of HIF-1α and its downstream effectors in the tumorigenesis of biphasic breast tumors.

Therefore, we studied expression of HIF-1α and its downstream targets VEGF and CAIX in breast phyllodes tumors of various grades and fibroadenomas. Further, since HIF-1α seems to play a major role in the process of angiogenesis we evaluated the microvascular network by counting of CD31 positive microvessels. Proliferation, as important functional end point of various carcinogenic processes, was assessed by mitotic counts and Ki67 expression. Since HIF-1α degradation may be promoted by wild type p53 [29], we evaluated the relation between p53 and HIF-1α expression in phyllodes tumors. Finally, prognostic value of HIF-1α, its downstream effectors and microvessel counts was assessed for phyllodes tumors as well.

Materials and Methods

Tissue samples

Formaldehyde fixed and paraffin embedded tissue samples were retrieved from the archives of our hospitals. A total of thirty-seven primary phyllodes tumors and thirty fibroadenomas were acquired. The presence of epithelial proliferative changes was noted. Phyllodes tumors were graded as benign, borderline or malignant based on the degree of stromal cellularity, stromal overgrowth, cellular atypia, invasiveness of the tumor margin and mitotic activity index (MAI) as described previously in detail by Moffat et al [30]. Mitotic figures were counted using established criteria in ten consecutive high power fields at a 400x magnification [31]. As grading of phyllodes tumors may be complicated by intratumoral heterogeneity, tumors were graded in the most unfavorable areas provided they comprised at least 10% of the total tumor area [30]. Clinical data was gathered by studying medical records.

Immunohistochemistry

Four μm sections were cut and mounted on coated slides. After deparrafinization and rehydration, sections were immersed in methanol containing 0.3% hydrogen peroxide to stop endogenous peroxidase activity. No antigen retrieval was necessary for CA IX staining. Antigen retrieval for HIF-1α was performed in target retrieval solution (DAKO, Glostrup, Denmark) in a water bath at 97°C for 45

minutes, the remaining antigens were unmasked by microwaving the slides for 10-15 minutes in citrate buffer (pH=6.0). The following panel of mouse monoclonal antibodies was used; VEGF (Labvision JH121, 1:10), HIF-1α (BD Pharmingen, 1:500), CAIX (kind gift of Dr. S. Pastorekova, M75, 1:50), CD31 (DAKO JC/70 A, 1:40), Ki67 (DAKO MIB-1, 1:40) and p53 (DAKO DO-7, 1:500). VEGF, Ki67 and p53 were incubated overnight at 4°C, incubation time for HIF-1α and CAIX was 30 minutes at room temperature and CD31was incubated for one hour at room temperature. VEGF, Ki67 and p53 were detected by application of a secondary biotinylated rabbit anti-mouse antibody (DAKO) diluted 1:500 followed by incubation with avidin-biotin-peroxidase complex (DAKO) at 1:200 dilution. The Catalyzed Signal Amplification system (DAKO) was used to detect HIF-1α, CD31 was visualized with the Ultravision system (Labvision) and CAIX was detected with the Envision system peroxidase (DAKO). All stainings were developed with 3,3'diaminobenzidine tetrahydrochloride. Counter staining was performed with haematoxylin. Negative controls were obtained by omitting the primary antibody and appropriate positive controls were included throughout.

As fibroadenomas and phyllodes tumors are biphasic tumors, immunostainings were scored in both stroma and epithelium. Immunoquantification was performed simultaneously by two observers (AK and PvD) behind a double-headed microscope. The percentage of positive staining nuclei for HIF-1α, Ki67 and p53 was estimated on a continuous scale, regarding only homogeneously and darkly stained nuclei as positive. CAIX expression was scored as positive or negative, scoring a case positive when membranous staining in any amount was present. Cytoplasmic VEGF staining was scored semi-quantitatively in four categories from 0 to +++, with category 0 expressing no positive staining, category + showing focal or diffuse weak staining, and categories ++ and +++ displaying respectively focal or widespread strong staining. Counting of CD31 positive microvessels was performed in the microvessel hotspot in four adjacent fields of vision at 400x magnification as described before [15,16]. In addition, a global microvessel density was acquired by counting of vessels in 10 diagonally adjacent fields at a magnification of 400x from a random starting point generated semi-automatically by the QPRODIT interactive digitizing video overlay system (Leica, Cambridge, UK).

For statistical analysis, stainings for Ki67 and p53 were divided into high and low using 10% positive staining as cut-off [32], categories 0 and + staining for VEGF were grouped as low expression and ++/+++ as high, and microvessel counts were dichotomized using the median value. As determined by staining of normal breast tissue, preinvasive breast lesions and invasive breast cancer, HIF-1α overexpression was defined as ≥1% positive staining nuclei [33].

Mean shortest distances from microvessels to epithelial basal membrane were determined with the QPRODIT system. Using CD31 stained sections, distances of epithelium to nearest microvessel were measured between manually placed markers.

Means were calculated from a minimum of 50 measurements per case with fields of view selected according to a systematic random sampling method [34].

Statistical analysis

All statistical analyses were performed with SPSS-software (SPSS, Chicago, IL, USA). Differences in expression of HIF-1α, Ki67, VEGF and CAIX and microvessel density between fibroadenomas and different grades of phyllodes tumors were investigated by the chi square test. Correlations between markers were evaluated using Fisher's exact test. The clinical endpoint for survival analysis of phyllodes tumors was local or distant recurrence (disease free survival [DFS]). Wide local excision is the preferred treatment for phyllodes tumors. Therefore, patients treated by primary mastectomy were excluded from survival analysis since these would bias results due to strongly reduced chances of recurrence. Kaplan-Meier curves were plotted and differences between the curves were evaluated with the log-rank test. P-values below 0.05 were regarded as significant.

Results

Patient characteristics

Thirty fibroadenomas were analyzed. Mean age of patients was 33.7±10.5 years and mean tumor size was 1.6±0.6 cm. 10 fibroadenomas (33%) harbored epithelial proliferative changes. A total of 18 benign, 8 borderline and 11 malignant primary phyllodes tumors were identified. Epithelial hyperplasia of ductal type, mostly focal, was found in 16 cases (43%) and was not related to grade. Mean age of patients with benign, borderline and malignant phyllodes tumors was 44.4±17.4, 57.9±12.8 and 54.3±12.9 years, respectively (p=0.073). Mean tumor sizes were 4.8±2.4 cm, 7.1±7.0 cm and 4.8±2.5 for benign, borderline and malignant phyllodes tumors, respectively (p=0.924). Patients with phyllodes tumors were older and had larger tumors (p<0.001 both) as compared to those with fibroadenomas. Five patients with phyllodes tumors were treated by primary mastectomy, the remainder by local excision. Excision was incomplete for 13 phyllodes tumors, whereas for 6 phyllodes tumors this information could not be retrieved.

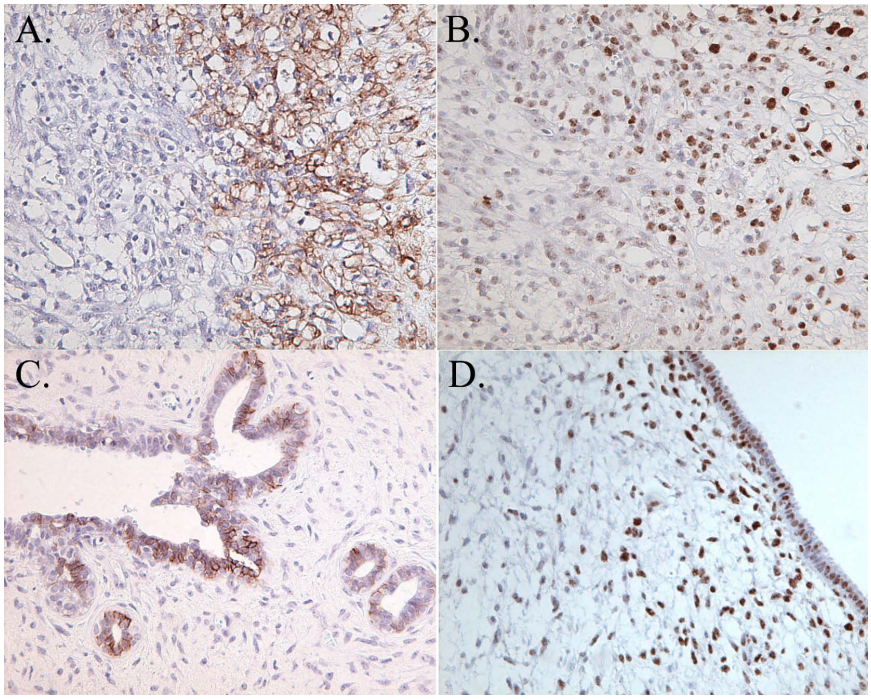
Differences between fibroadenomas and phyllodes tumors.

Table 1 summarizes the differences in immunostaining between fibroadenomas and phyllodes tumors. Stromal and epithelial overexpression of HIF-1α were found almost exclusively in phyllodes tumors (p=0.001 and p<0.001 respectively; Fig 1B, D). Only two fibroadenomas with low level stromal HIF-1α immunoreactivity (1% and 2% positive nuclei) were found without concerted CAIX expression. Stromal and epithelial CAIX expression were only seen in phyllodes tumors (Fig 1A, C).

Table 1. Differences in expression of HIF-1α, CAIX, VEGF and p53, microvasculature and proliferation between fibroadenomas and phyllodes tumors. Due to empty blocks, repeated unsuccessful staining attempts or too little epithelium in a malignant tumor, numbers varied slightly for some stainings. Dichotomized values were used. Abbreviation; MVC, microvessel count. [a] χ^2-test, *P* values below 0.05 were regarded as significant.

Marker	Fibroadenoma n=30	Phyllodes tumor n=37	*P* value[a]
Stroma			
HIF-1α	2 (7%)	19 (51%)	0.001
CAIX	0 (0%)	2/36 (6%)	0.497
VEGF	7 (23%)	17 (47%)	0.074
Ki67	1 (3%)	17 (47%)	<0.001
Hot-spot MVC	13 (43%)	22/35 (63%)	0.140
Global MVC	13 (43%)	20/35 (57%)	0.324
p53	0(0%)	7/36(19%)	0.014
Epithelium			
HIF-1α	0 (0%)	15/35 (43%)	<0.001
CAIX	0 (0%)	9/35 (26%)	0.003
VEGF	22 (73%)	22/36 (61%)	0.432
Ki67	2 (7%)	2/36 (6%)	1.000
p53	0(0%)	0(0%)	-

Figure 1. Examples of immunostaining for HIF-1α and CAIX in breast phyllodes tumors. Malignant phyllodes tumor with stromal CAIX expression (A); Same tumor as A with topographically overlapping HIF-1α overexpression (B); Benign phyllodes tumor with CAIX positive staining epithelium (C); Borderline phyllodes tumor with HIF-1α overexpression in normal appearing epithelium and in subepithelial stroma (D). For color plate see page 187.



High expression of VEGF was regularly found in both fibroadenomas and phyllodes tumors. As a result, no differences in stromal and epithelial VEGF expression were found between both tumors. Many small microvessels were concentrated around the epithelium of fibroadenomas. In phyllodes tumors, microvessels lacked such a peri-epithelial preference and were distributed more evenly throughout the tumor. The number of microvessels as such counted by both methods did not differ significantly between fibroadenomas and phyllodes tumors. Indeed, when regarding microvessel counts on a continuous scale, a large overlap was observed between fibroadenomas, benign and borderline phyllodes tumors (Fig 2). In 5 fibroadenomas and 10 phyllodes tumors, of which 5 had stretches of HIF-1α positive epithelium, mean shortest distance between microvessels and the epithelial basal membrane was 50±11 μm for fibroadenomas and 83.3±16 μm for phyllodes tumors (p=0.005). Because of the focal staining patterns resulting in methodological problems and low numbers, phyllodes tumors with HIF-1α negative and positive epithelium were not separately analyzed here. Due to these methodological problems the results should be interpreted with caution.

Fibroadenomas and phyllodes tumors did not differ with regard to the presence of epithelial proliferative changes (p=0.458).

Table 2. Differences between different grades of phyllodes tumors in expression of HIF-1α, CAIX, VEGF and p53, microvasculature and proliferation. Due to empty blocks, repeated unsuccessful staining attempts or too little epithelium in a malignant tumor, numbers varied slightly for some stainings. Dichotomized values were used. Abbreviation; MVC, microvessel count. [a] χ^2-test, *P* values below 0.05 were regarded as significant.

	Phyllodes tumor			
Marker	Benign (n=18)	Borderline (n=8)	Malignant (n=11)	*P* value[a]
Stroma				
HIF-1α	5 (31%)	3 (38%)	11 (100%)	0.001
CAIX	0 (0%)	0 (0%)	2 (18%)	0.090
VEGF	5 (31%)	3 (38%)	9 (82%)	0.016
Ki67	1 (6%)	5 (63%)	11 (100%)	<0.001
Hot-spot MVC	9/16 (56%)	2 (25%)	11 (100%)	0.003
Global MVC	8/16 (50%)	2 (25%)	11 (100%)	0.002
p53	1/18(6%)	1/8(13%)	5/11(46%)	0.025
Epithelium				
HIF-1α	7/17 (42%)	2 (25%)	6/10 (60%)	0.323
CAIX	5 (32%)	1/7 (14%)	3/10 (30%)	0.735
VEGF	12 (67%)	5 (63%)	5/10 (50%)	0.684
Ki67	1 (6%)	0 (0%)	1/10 (10%)	0.655
p53	0(0%)	0(0%)	0(0%)	-

Differences between phyllodes tumor grades

Correlations between grade of phyllodes tumor and HIF-1α and its downstream targets, microvessels and proliferation are summarized in Table 2. Stromal HIF-1α overexpression in phyllodes tumors was strongly correlated with tumor grade (p=0.001), with all malignant tumors displaying overexpression. Necrosis could be detected in only 1 malignant phyllodes tumor, with no typical perinecrotic HIF-1α overexpression pattern. Epithelial HIF-1α overexpression was not related to grade (p=0.323). Only 2 malignant phyllodes tumors displayed stromal CAIX expression (p=0.090). Epithelial CAIX expression was seen more often than stromal CAIX expression, but was not correlated to grade (p=0.735). Overexpression of HIF-1α and expression of CAIX in the epithelial component were both not related to hyperplasia and were mostly found in normal appearing, two-layered epithelium. A statistically significant difference in number of microvessels was found between grades, both when counted in the hot-spot and by the global method (p=0.003 and p=0.002 respectively). The number of microvessels was strongly increased in malignant tumors, as compared to benign and borderline tumors (Fig 2). High VEGF expression in the stromal component displayed a positive relation with tumor grade (p=0.016).

HIF-1α expression in both compartments of phyllodes tumors was not significantly related to tumor size, both with size cut-off at the median and when used as a continuous variable (data not shown).

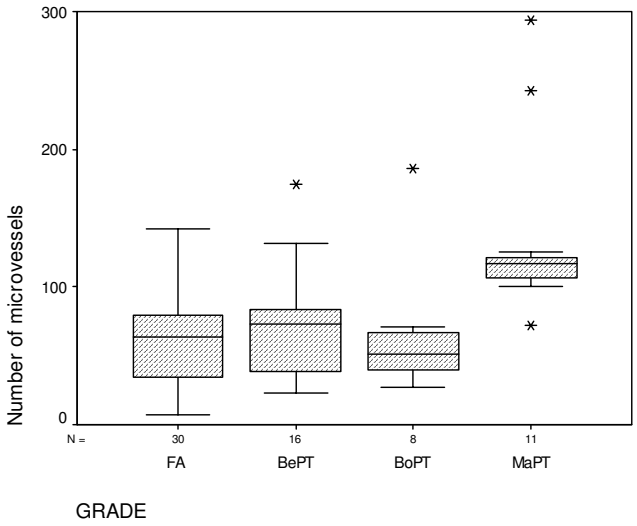


Figure 2. Boxplot showing large overlap in numbers of microvessels between fibroadenoma, benign and borderline phyllodes tumors when counted in the hot-spot. Abbreviations; FA, fibroadenoma; BePT, benign phyllodes tumor; BoPT, borderline phyllodes tumor; MaPT, malignant phyllodes tumor.

Coexpression of markers

The relation between HIF-1α expression and its downstream targets, microvessels and proliferation markers in the stromal component of fibroepithelial tumors are displayed in Table 3. Since only two cases showed stromal CAIX expression, the relation between stromal CAIX and stromal HIF-1α overexpression did not reach statistical significance (p=0.098). The relation between HIF-1α overexpression and strong VEGF expression in stroma reached borderline significance (p=0.098). Global (p=0.015) and hotspot (p=0.031) microvessel counts were both related to stromal HIF-1α overexpression. Overexpression of HIF-1α in stroma was correlated with proliferation as measured by high stromal Ki67expression (p<0.001) and high MAI (p<0.001). Stromal overexpression of HIF-1α and p53 were strongly associated as well (p=0.003).

Epithelial HIF-1α overexpression was related to epithelial CAIX expression (p=0.014). There was no relation between HIF-1α overexpression in the epithelial component and VEGF or Ki67 expression. Epithelial HIF-1α overexpression was related to increased stromal proliferation (Ki67 p=0.006; MAI p=0.004).

		<1%	≥1%	[a] *P* value
p53	<10%	45	15	0.003
	≥10%	1	6	
VEGF	Weak	33	10	0.098
	Strong	13	11	
Hot-spot MVC	<71	25	5	0.031
	≥71	20	15	
Global MVC	<86	27	5	0.015
	≥86	18	15	
CAIX	Negative	45	19	0.098
	Positive	0	2	
Ki67	<10%	42	7	<0.001
	≥10%	4	14	
MAI	<10	43	8	<0.001
	≥10	3	13	

Table 3. Association with HIF-1α with its downstream effectors, microvessel counts and proliferation markers in stroma of fibroepithelial tumors. Dichotomized values were used. Abbreviations; MVC, microvessel count; MAI, mitotic activity index. [a] Fisher's exact test, *P* values below 0.05 were regarded as significant.

Survival analysis.

As mentioned above, patients treated by mastectomy were excluded from survival analysis. Two patients were lost to follow-up. Ten of the remaining 30 tumors recurred. Most were local recurrences, but one malignant tumor metastasized to the lung. By univariate analysis of survival we found an inverse correlation between stromal HIF-1α overexpression (p=0.032; Fig 3), tumor grade (p=0.039), stromal Ki67 overexpression (p=0.028) and stromal p53 overexpression (p<0.001) and DFS, as shown in Table 4. HIF-1α expression did not add to the prognostic power of p53

expression alone. Tumor size (median 3cm; p=0.88), age (p=0.36), MAI (p=0.09), microvessel counts (hot-spot p=0.54; global p=0.25), margin status (p=0.23), VEGF expression (stromal p=0.96; epithelial p=0.11), CAIX expression (stromal p=0.46; epithelial p=0.16) and epithelial HIF-1α expression (p=0.76) were not of prognostic relevance. Surprisingly, epithelial overexpression of Ki67 had prognostic power as well (p=0.011). However, this is of no relevance since only two cases showed epithelial overexpression of Ki67.

Table 4. Prognostically significant variables in phyllodes tumors as determined by univariate analysis of disease free survival (DFS). [a] DFS in months (95% CI).

Variable	No. of patients	With disease (%)	Disease free (%)	[a]Mean DFS	*P* value	Log-rank
p53 stroma						
< 10%	24	5 (21)	19 (79)	173 (142-204)	<0.001	10.97
≥10%	6	5 (83)	1 (16)	61 (23-98)		
HIF-1α stroma						
<1%	15	3 (20)	12 (80)	180 (146-214)	0.032	4.59
≥1%	15	7 (47)	8 (53)	103 (64-142)		
Ki67 stroma						
<10%	16	4 (25)	12 (75)	176 (145-207)	0.028	4.85
≥10%	14	6 (43)	8 (57)	91 (55-127)		
Grade						
Benign	15	4 (27)	11 (73)	165 (130-200)	0.039	6.48
Borderline	5	1 (20)	4 (80)	140 (86-194)		
Malignant	10	5 (50)	5 (50)	71 (32-109)		

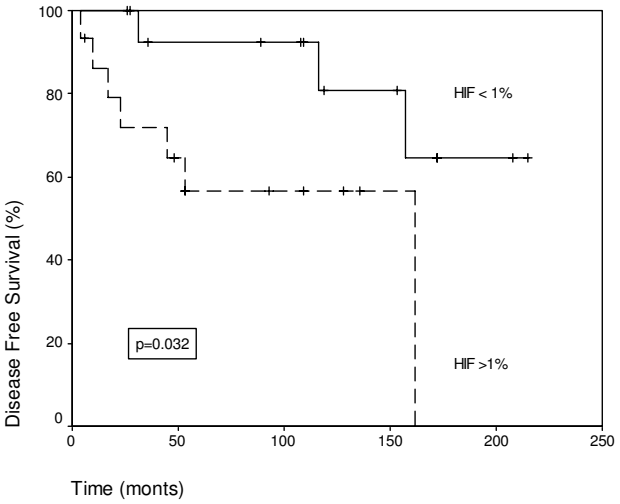


Figure 3. Kaplan-Meier survival curve illustrating disease free survival for patients with breast phyllodes tumors with high (≥1%) versus low (<1%) expression of HIF-1α in the stromal component. Numbers of patients at risk at different time points are displayed below the horizontal axis.

Discussion

Evidence is mounting that HIF-1α and its downstream targets play pivotal roles in development and progression of many types of human cancers. This is the first study evaluating HIF-1α and CAIX expression in breast phyllodes tumors. We found that stromal HIF-1α overexpression predicts prognosis and may play an important role in the stromal progression of phyllodes tumors. Surprisingly, we regularly found concerted HIF-1α and CAIX expression in normal appearing epithelium of phyllodes tumors. Since only two fibroadenomas displayed low level stromal HIF-1α immunoreactivity and none expressed CAIX, HIF-1α and CAIX seem to be of little relevance in these tumors.

HIF-1α overexpression is induced by hypoxia and by oxygen independent mechanisms. In our study, stromal HIF-1α overexpression was related to grade of phyllodes tumors with a marked increase from borderline to malignant grade. Benign and borderline tumors showed comparable levels of HIF-1α overexpression. We only found two fibroadenomas with low level expression of HIF-1α in the stroma, in agreement with Zhong et al, who found all their fibroadenomas to be negative [35]. By clonality analysis we previously demonstrated that fibroadenomas may progress to phyllodes tumors by clonal expansion of stroma [22]. Perhaps, positive stromal HIF-1α staining in a fibroadenoma may reflect an increased intrinsic capacity to progress to phyllodes tumor. The fibroadenomas with stromal HIF-1α positivity were microscopically inconspicuous, though. In several types of cancer, hypoxia induced HIF-1α expression seems to be characterized by a perinecrotic distribution, whereas oxygen independent overexpression results in a diffuse pattern of HIF-1α immunoreactivity [11,36,37]. Necrosis was found in the stromal component of only one malignant phyllodes tumor. Further, stromal HIF-1α overexpression was not accompanied by an increase in CAIX expression, which is regarded as a marker of hypoxia [19]. Indeed, our group recently showed that, in contrast to homogenous/ diffuse HIF-1α expression, necrosis-related/ focal HIF-1α expression is accompanied by CAIX expression [38]. All this suggests that HIF-1α upregulation in stroma of phyllodes tumors is normoxic and may be caused by changes in stromal expression of oncogenes, tumor suppressor genes or growth factors. Wild-type p53 has been shown to promote MDM2 mediated ubiquitination of HIF-1α [29]. Therefore, p53 inactivation by gene mutation has been implicated in increased HIF-1α expression. We showed that aberrant expression of cell cycle protein p53 only occurred in the stromal component. The transition from borderline to malignant phyllodes tumors was accompanied by a strong increase in stromal p53 expression, similar to stromal HIF-1α expression. Further, we found a significant relation between stromal p53 and HIF-1α overexpression. Another candidate for hypoxia independent upregulation of HIF-1α in phyllodes tumors is PDGF. PDGF overexpression was found in 24% of phyllodes tumors, but not in fibroadenomas [25]. Interestingly, PDGF may induce HIF-1α expression [39]. Stromal HIF-1α and p53 expression in phyllodes tumors

were predictive of disease free survival, underlining the importance of the p53-HIF-1α axis in its progression and clinical behavior.

Epithelial HIF-1α overexpression in phyllodes tumors was associated with CAIX expression. This suggests a causative role for epithelial hypoxia. Fibroadenomas were negative for CAIX, confirming the results of Bartosova et al [40]. Mean distance from microvessels to the epithelium was significantly higher for phyllodes tumors as compared to fibroadenomas, suggesting that the hypoxic microenvironment in the epithelial component of phyllodes tumors is caused by relatively distant microvessels. However, most vessels in phyllodes tumors were within 150μm of the epithelial basal membrane, which is a critical distance for necrosis [41]. Still, it is conceivable that rapidly growing stroma stretches the two-layered epithelium in such way that its oxygen supply does not keep up, resulting in a state of mild hypoxia. Indeed, a previous report detected CAIX expression 80μm from the nearest blood vessel [42], which is comparable to the distance we found for phyllodes tumors. It is possible that the decreasing oxygen gradient between 80 and 150μm from nearest vessel to epithelium is sufficient to induce HIF-1α and CAIX expression. The scattered foci of positive staining for HIF-1α and CAIX suggest the presence of focal mild hypoxia in the epithelial component. Microenvironmental disturbance of normal tissue by adjacent malignant disease had been described previously for HIF-1α and CAIX [40,43,44]. On the other hand, several other possible causes for epithelial HIF-1α overexpression exist. Epithelial PDGF expression which is found in most phyllodes tumors [25] may cause HIF-1α overexpression [39]. Shpitz et al found expression of HER-2*/neu* in epithelium of 61% of phyllodes tumors [45]. Recently, it was demonstrated that enhanced HER-2*/neu* signaling induces HIF-1α protein expression [46]. Numerous factors interact with HIF-1 and other as yet unidentified changes in epithelium of phyllodes tumors may cause HIF-1α overexpression. The tumor biological significance of increased epithelial HIF-1α and CAIX expression is unclear. Morphologically, the epithelial component was mostly two-layered epithelium without atypia. Further, clonality studies mostly found the epithelial component of phyllodes tumors to be polyclonal [22,23]. Moreover, phyllodes tumor metastases are composed of stroma, with only one case described in which the epithelial component disseminated as well, leading to a biphasic metastasis [47]. Finally, epithelial HIF-1α and CAIX expression are not predictive of prognosis. It seems therefore that HIF-1α and CAIX expression in the epithelium of phyllodes tumors merely reflect a physiological adaptation to microenvironmental disturbance by rapidly proliferating stroma with lagging peri-epithelial angiogenesis. However, possible upregulation of growth factors in the epithelium by HIF-1α may exert an additional stimulatory force on the stromal component. Indeed, it has been suggested that in early stages of phyllodes tumor development the epithelium secretes mitogens stimulating the stromal component [48,49]. The presence of such autocrine and paracrine loops have been described for PDGF/PDGFR [25]. Further, stimulation of the stromal

component by the epithelium was suggested previously by studying endothelin-1 expression in epithelium of phyllodes tumors [50]. Interestingly, endothelin-1 turned out to be a target of HIF-1α [51].

An abrupt increase in number of microvessels was observed in malignant phyllodes tumors, coinciding with a strong increase in stromal overexpression of HIF-1α from borderline to malignant grade. At variance with a previous report [52], we found no difference in microvessel counts between benign and borderline phyllodes tumors. In addition, when regarding hot-spot and global counts we found that there was no difference between fibroadenomas, benign phyllodes tumors and borderline phyllodes tumors. This is in contrast with a previous report claiming a difference between fibroadenomas and phyllodes tumors [24]. On the other hand, Weind et al found a large overlap in microvessel counts between fibroadenomas and invasive breast cancers demonstrating that fibroadenomas are capable of producing large numbers of microvessels [53]. We found large numbers of small peri-epithelial microvessels in fibroadenomas responsible for its high numbers of microvessels. It seems that microvessel counts are not helpful in differentiating between fibroadenoma and benign phyllodes tumor, which poses the biggest diagnostic problem.

VEGF expression in fibroadenomas did not differ from that in phyllodes tumors. Several growth factors such as FGF-4 [54], PDGF [55] and transforming growth factor β (TGF- β) [56] may stimulate VEGF expression. Expression of FGF [26], PDGF [25] and TGF- β [57] have been described in fibroadenomas and may contribute to HIF-1α independent expression of VEGF. In phyllodes tumors, the relation between VEGF expression and HIF-1α reached borderline significance. Although HIF-1α most likely contributes to VEGF expression in phyllodes tumors, VEGF expression seems to be, at least in part, independent from HIF-1α.

In view of its annual incidence of 2.1 per 1 million women [58], we feel that our group of phyllodes tumors is reasonably sized and well composed with all grades present. Still, future investigations confirming our results in larger series are warranted. Further, experiments covering a variety of molecular elements such as DNA microarray techniques may unravel the complex mechanisms underlying the presumed non-hypoxic upregulation of HIF-1α in the stromal component of phyllodes tumors and its subsequent influence on tumor progression.

This is the first report on HIF-1α and CAIX expression in breast phyllodes tumors. Our results show that HIF-1α is related to diminished DFS and may play an important role in stromal progression of phyllodes tumors. The significant relation between tumor grade and stromal HIF-1α overexpression underlines its importance, with all malignant tumors showing HIF-1α overexpression. Stromal HIF-1α overexpression in phyllodes tumors is most likely caused by hypoxia independent pathways, with p53 inactivation as one of possible causes. Surprisingly, normal-appearing epithelium in phyllodes tumors frequently displayed HIF-1α and CAIX

expression. The distance from nearest vessels to epithelium was higher in phyllodes tumors as compared to fibroadenomas and a hypoxic effect seems plausible here, although of doubtful biological significance. In contrast to phyllodes tumors, HIF-1α seems of minor relevance in tumorigenesis of fibroadenomas. Considering its possible role in progression of the stromal component of phyllodes tumors and the fact that metastases are composed of stroma, novel therapies targeting HIF-1α [59] may contribute to treatment of disseminated phyllodes tumor, which is poorly responsive to conventional chemo- and radiotherapy.

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Chapter 8

Genomic profiling by array comparative genomic hybridization reveals novel DNA copy number changes in phyllodes tumors and lack of alterations in fibroadenomas

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Abstract

Fibroadenoma and phyllodes tumor (PT) are fibroepithelial tumors with morphological similarities but different clinical behavior. Cytogenetic data on both tumors is sparse. Array comparative genomic hybridization (CGH) was performed to obtain genome-wide copy number profiles of both tumors.

DNA was isolated from fresh-frozen tissue samples. 11 PT and 3 fibroadenomas were analyzed. Arrays composed of 2464 genomic clones were used providing a resolution of ~1.4Mb across the genome. Each clone contains at least one STS for linkage to the human genome sequence.

No copy number changes were detected in fibroadenomas. On the other hand, 10 of 11 PT (91%) showed DNA copy number alterations. The mean number of chromosomal events in PT was 5.3 (range 0-16) per case. A mean of 2.0 gains (range 0-10) and 2.8 losses (range 0-7) was seen per case of PT. Three cases showed amplifications. DNA copy number change was not related to grade. We observed recurrent losses on chromosome 1q, 4p, 10, 13q, 15q, 16, 17p, 19 and X. Copy number gains were seen on 1q, 2p, 3q, 7p, 8q, 16q, 20.

This is the first study using array CGH for genomic profiling of fibroepithelial breast tumors. Whereas most PT showed genomic instability, fibroadenomas lacked copy number alterations. Some copy number aberrations observed in PT had not previously been associated with this tumor. Several well-known cancer related genes, such as *TP53* and several *Cadherin* family members, are located within the recurrent regions of copy number alteration.

Introduction

Phyllodes tumors (PT) and fibroadenomas are fibroepithelial tumors of the breast, i.e. composed of an epithelial and a stromal component. Fibroadenoma itself is typically benign, but large epidemiological studies related fibroadenoma to an elevated risk for invasive breast cancer in later life [1,2]. Further, carcinoma in situ may develop from within a fibroadenoma in a small percentage of cases [3]. PT on the other hand is a rare tumor. It is graded as benign, borderline or malignant [4]. Depending on grade of the primary, local recurrence can be seen in 8 to 65% of cases [5] and metastases, mostly hematogeneous to the lung, in 25% of malignant cases [6]. Sharing a similar phenotype, the distinguishing between benign PT and fibroadenoma in particular can be difficult [3]. Further, by PCR based clonality assay we have demonstrated that fibroadenoma may progress to PT [6]. Little is known about the underlying genotype of both tumors. Conventional cytogenetic studies using short term culture and G-banding detected clonal chromosomal aberrations in several dozens of fibroadenomas, but recurrent fibroadenoma-specific changes rarely emerged due to the wide variety of alterations [7-11]. The only recurrent aberration was found by Tibiletti et al, who detected a deletion of 6q27-qter in nearly 85% of fibroadenomas [11]. Similarly, no specific alterations have been found in PT by karyotyping [12,13]. Using CGH, a technique which screens the whole genome for DNA copy number alterations, Lu et al found recurrent gain of 1q and loss of 3p in PT, with gain of 1q being predictive of clinical behavior [14]. Another group defined the minimal overlapping region as 1q21-q23, but was unable to confirm its relation to clinical behavior [15]. CGH has been used to study fibroadenomas as well, with conflicting results [16-19]. An early study by Ried et al found no copy number changes in 13 fibroadenomas [16]. Their findings were recently confirmed by Cavalli et al [17]. Two other studies however found many aberrations scattered throughout the genome [18,19].

Recently, CGH was further refined by the introduction of microarray based CGH (array CGH) [20]. In contrast to chromosomal CGH, which uses metaphase chromosomes as hybridization targets, array CGH uses genomic BAC, P1, cosmid or cDNA clones as targets resulting in superior resolution. Typically, the genomic resolution with which copy number aberrations can be detected is approximately 10 to 20 Mb for chromosome CGH, whereas the resolution of array CGH is mainly dependent on the genomic distance between the arrayed DNA elements and the size of the elements [20]. In addition, array CGH allows direct mapping of alterations to the human genome sequence [20,21]. These properties facilitate the identification of candidate oncogenes participating in tumor formation and progression, [22].

The aim of the present study was to identify candidate genes involved in tumorigenesis of fibroepithelial breast tumors by obtaining genomic profiles by array CGH. Comparing different grades of PT may result in identification of genomic regions and genes involved in PT progression. Further, since fibroadenoma and PT

may be morphologically similar but behave differently, comparing copy number profiles may reveal discriminating genes or a different degree of genomic instability.

Material and Methods

Tissue

Fresh frozen tissue samples were retrieved from the tissue banks of our hospitals. Three fibroadenomas and 11 PT, 10 primary tumors and one recurrence, were studied. Two 4µm sandwich H&E cryosections were examined to identify normal tissue and for grading of the tumor. PT was graded according to the criteria of Moffat et al [4]. In brief, based on the degree of stromal overgrowth, margin infiltration, stromal cellularity, stromal atypia and number of mitosis, tumors were graded as benign, borderline or malignant. Mitotic figures were counted using established criteria in ten consecutive fields at 400x magnification [23]. Blocks containing areas of normal tissue were trimmed. Ten µm sections were cut for DNA isolation. DNA extraction was performed using affinity columns (QIAmp Tissue Kit, QIAgen Inc) with modifications to the manufacturer's protocol [24].

Arrays

Arrays were produced as described previously [21]. In brief, bacterial artificial chromosome (BAC) DNA was amplified by ligation-mediated polymerase chain reaction (PCR) in order to obtain sufficient amounts and concentrations for spotting of the arrays. BAC DNA was printed on chromium-coated microscope slides using a custom built arrayer. A total of 2464 BAC clones were printed in triplicate (HumArray 2.0). Each clone contains at least one STS for linkage to the human genome sequence (http://genome.ucsc.edu).

Comparative genomic hybridization

Approximately 300 ng of tumor and reference DNA were labelled by random priming (BioPrime DNA labelling, Gibco BRL). Tumor DNA was labelled with Cy3 dCTP and reference DNA with Cy5 dCTP (Amersham Pharmacia Biotech). To remove non-incorporated nucleotides the DNA mixture was spun through a Sephadex G-50 column. Labeled test and reference DNA were then co-precipitated in the presence of Cot-1 DNA (~100 µg; Gibco BRL) with ethanol. The precipitated DNA was subsequently dissolved in a hybridization solution containing 50% formamide, 10% dextran sulfate, 2xSSC, 4%SDS and 500 µg yeast tRNA. To denature the DNA, the hybridization mixture was incubated at 70°C for 10-15 minutes, followed by incubation at 37°C for one hour to block repetitive sequences. A ring of rubber cement was placed around the array to form a well, in which 50 µl of blocking solution containing 500 µg of salmon sperm DNA was added and incubated at room temperature for 30 minutes. Subsequently, approximately three-quarters of

the blocking solution was removed and the denatured and re-annealed hybridization mixture was added. Hybridization was performed for approximately 48 hours at 37°C on a slowly rocking table (~1rpm). After hybridization, slides were rinsed in PN buffer (PN: 0.1M sodium phosphate, 0.1% nonidet P40, pH 8), washed once in 50% formamide, 2xSSC, pH 7, at 45°C and once in PN buffer both for 15 minutes. After draining of excess fluid, slides were mounted in a solution containing 90% glycerol, 10% PBS and 1µM DAPI to counterstain DNA targets.

Imaging and analysis

Images were acquired with a custom build CCD camera system resulting in 16 bit 1024x1024 pixel DAPI, Cy3 and Cy5 images as described previously [20]. Imaging processing was performed using two custom programs, SPOT and SPROC software packages (http://jainlab.ucsf.edu/Projects.html) [25]. The normalized $\log_2$ transformed fluorescence ratio generated for each spot is directly linked to the corresponding clone of the July 2003 freeze of the draft of the human genome sequence (http://genome.ucsc.edu). Fluorescence ratios of clones for which only one of the triplicate values remained after SPROC analysis or for which the standard deviation was >0.2 were rejected from further analysis.

DNA copy number alterations were identified with a recently described segmentation algorhytm [26]. Low-level single BAC clone alterations were not counted as real events. An amplification was defined by a single clone or group of clones with a normalized $\log_2$ transformed fluorescence ratio of 1.0 or larger, with the graph showing a peak rather than a plateau.

Table 1. Relation between phyllodes tumor grade and chromosomal events. [a] Including amplifications.

Grade	Benign	Borderline	Malignant	*P* value
Gains	2.6±4.2	1.0	1.6±2.3	ns
Losses	1.8±2.5	1.0	4.2±2.8	ns
Total copy number change[a]	4.4±6.5	2.0	6.8±4.7	ns

Results

Ten of 11 PT (91%) showed chromosomal aberrations, whereas fibroadenomas displayed no copy number changes at all. The mean number of chromosomal events, i.e. the sum of all gains, losses and amplifications, was 5.3 (range 0-16) per case of PT. A mean of 2.0 gains (range 0-10) and 2.8 losses (range 0-7) was seen per case of PT. The relation between tumor grade and DNA copy number changes is summarized in Table 1. The number of DNA copy number aberrations present in the PT was not associated with grade.

Detailed information on copy number alterations per case is displayed in Table 2. A schematic overview of the distribution of chromosomal aberrations per case is given

in Table 3. We observed recurrent losses on chromosome 1q, 4p, 10, 13q, 15q, 16, 17p, 19 and X. Recurrent copy number gains were seen on 1q, 2p, 3q, 7p, 8q, 16q, 20.

Amplifications were seen infrequently (3 of 11 PT). One malignant PT displayed a complex cluster of 3 amplicons on chromosome 5q (Fig 1). The proximal amplicon, flanked by RP11-17H13 and RP11-47P7, harbored interleukin 6 signal transducer isoform 1 (*IL6ST)* and *MAP3K1. PIK3R1* maps to the second amplicon, flanked by RP11-15P2 and RP11-267K19, whereas no known cancer-related genes map to the distal amplicon flanked by RP11-62D9 and RP11-1E10.

A primary PT and its corresponding recurrence shared the same amplification of clone RP11-22M5 at 22q11.22, flanked proximally by *TUPLE1* and distally by *BCR*. Genes mapping to this amplicon include *PPM1F*, *TOP3B*, *MAPK1*, *PIK4CA* and *PRAME.*

Table 2. Summary of DNA copy number changes in fibroepithelial breast tumors. Be: Benign PT; Bo: Borderline PT; Ma: Malignant PT; FA: fibroadenoma.

Case	Lesion	Gains	Losses	Amplifications
1	Be	2p13-11.2, 7p15.2-14, 7p13-11.2, 8q24.1-24.2, 12ptel-11.2, 15q23-qtel, 16q12.1-22, 18p12-11.21, 18q, 20	7q11.1-21.1, 10, 13q12.1-14.3, 15q11.2-21.3, 16q22-qtel, 17ptel-11.2	-
2	Be	1q, 16	-	-
3	Be	-	19p13.12-pter, 19q13.2-q13.4	-
4	Be	-	19	-
5	Be	1	-	-
6	Bo	20	6	-
7	Ma	3q23-qter, 5q35, 8q	5q11.2-q32, 5q34-q35, 8p23.1-q11.2, 16q21-q23, 16q24-qtel, 17p12-p13, 19, 21q22.1-q22.2	5q11.2, 5q13.1, 5q14.1
8	Ma	-	-	-
9	Ma	-	1q41-qtel, 4p16.2-p11, 13q14.2-q32, 16, X	22q11.2
10	Ma	-	1q42-qtel, 4ptel-p13, 13q13-qtel, 16, 22q13.2-qter, X	22q11.2
11	Ma	2, 3q, 7p, 7q21.11-21.3, 7q31.1-tel	9p21, 10, 16q	-
12	Fa	-	-	-
13	Fa	-	-	-
14	Fa	-	-	-

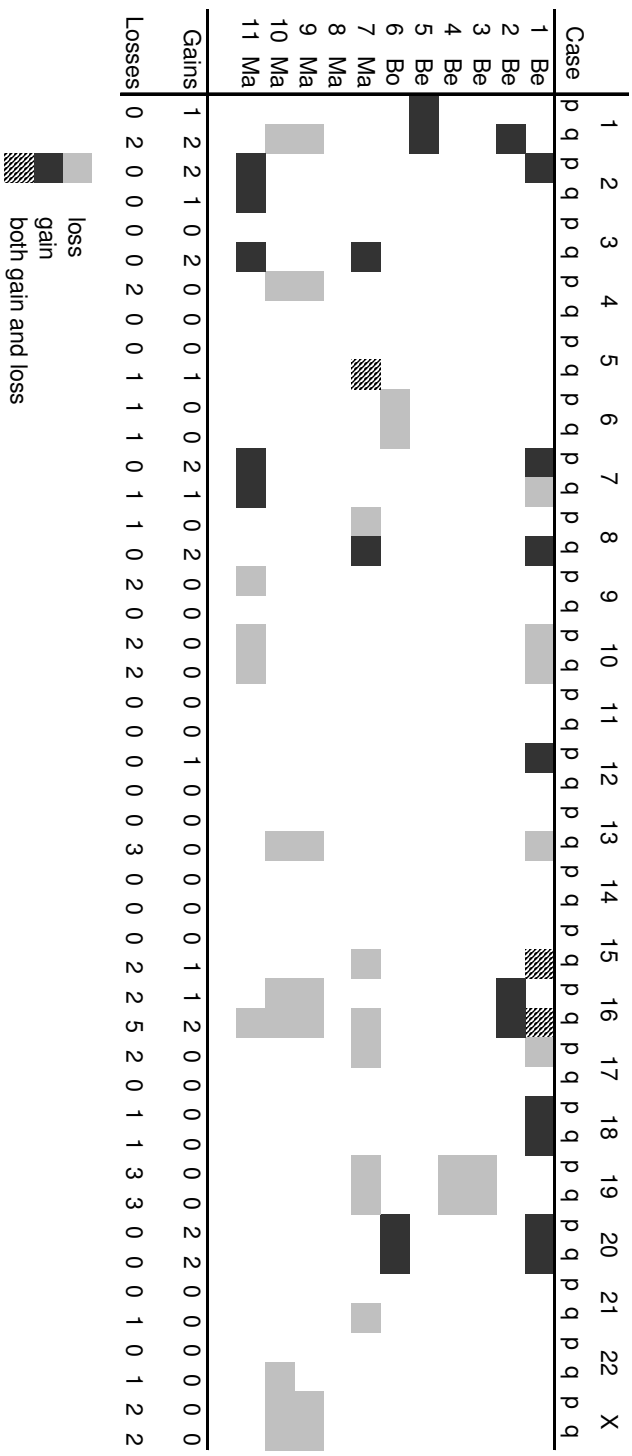
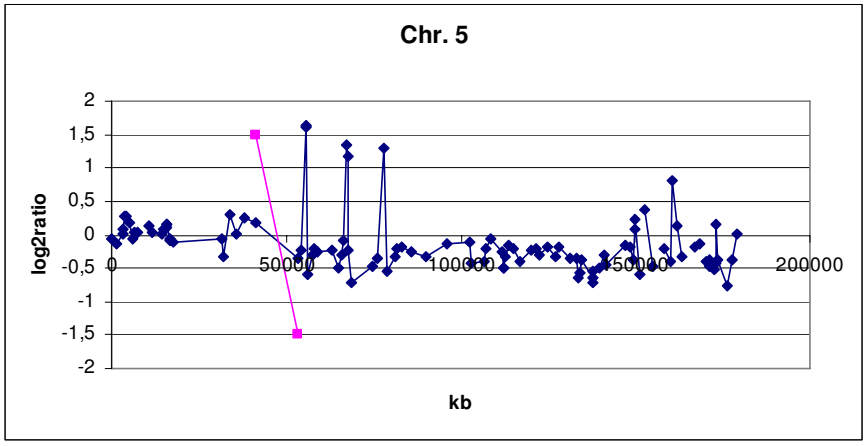


Table 3. Schematic overview of affected chromosomal regions in phyllodes tumors of the breast. Amplifications are not shown here. Be: benign grade phyllodes tumor; Bo: borderline; Ma: malignant.

Figure 1. Although amplifications are rare in PT, this malignant PT showed 3 amplifications on proximal 5q. Copy number loss was seen in between the amplifications. The distal peak on 5q was counted as a gain. The vertical bar represents the location of the centromere.



Losses at 16q were observed in 5 of 11 PT. One PT showed loss of the 16q-arm and two PTs showed loss of the entire chromosome 16. Two other tumors showed focal loss at 16q. Combining these results revealed two minimal regions of deletion; one flanked by clones RP11-84C4 and RP11-217K3, the other includes the clones distally of RP11-118F19. The proximal overlapping region harbored *CDH 1* (E-cadherin)*, CDH 3, CTCF*, *NQO1* and *TERF2*. Cancer related genes mapping to the distal region of overlap include *CDH 15* (M cadherin), *CDK10, FANCA* and *GAS8*.

Recurrent copy number loss was found at chromosome 13q (3/11 PT), overlapping at 13q13-q14.3. This region contains *RB1*. Further, loss at chromosome 17p was found in 2 of 11 PT and harbors *TP53.* Loss of chromosome 19 was detected in 3 of 11 PT. Interestingly, chromosome 19 loss never included the *CCNE1* region, which remained at normal copy number, suggesting the presence of negative selection pressures against loss of this region. Candidate tumor suppressor genes mapping to the areas of copy number loss at chromosome 19 include *XRCC1* and *BAX* (Bcl-2 associated X protein).

DNA copy number gain was detected at chromosome 7p and chromosome 8q (2/11 PT both). The region of overlap on the former chromosome contains *EGFR*. *WISP1,* a member of the Wnt signaling pathway, and *CMYC* are located within the overlapping region on 8q. In previous reports gain at 1q was a prominent alteration. In our work, two (18%) PT showed gain of the complete 1q arm. Many genes reside here, but several cancer related genes can be identified, such as *MUC1, HDGF, MDM4, WNT3A and AKT3*.

Discussion

In the present study, genomic profiling by array CGH revealed chromosomal instability in the majority of PT (91%). PT grade and copy number alterations were not related. In contrast to PT, not a single chromosomal copy number alteration was found in 3 fibroadenomas. We here describe novel regions of altered DNA copy number in PT, some of which mapped to regions harboring well known oncogenes or tumor suppressor genes.

Single BAC clone alterations may reflect genetic instability and may be counted as real events [27,28]. However, single BAC clone changes may be due to clone mismapping or inadequate hybridization. We therefore chose not to include these changes. Although some changes may have been underestimated by this stringent approach (such as 8q and 13q alterations), we believe we have focused on the most biologically relevant changes for the tumor.

Fibroadenomas and PT are biphasic tumors. Allelic imbalance of chromosome 1q and 3p has been detected in PT epithelium, suggesting that the epithelial component is more than merely reactive [29]. In this light, performing selective array CGH of the epithelial component would be interesting. However, such a study would be difficult, since in fibroepithelial tumors the epithelium mostly is one-layered (like the normal breast) making it very time consuming to microdissect and almost impossible to obtain sufficient DNA at optimal concentrations. In addition, genomic amplification techniques such as DOP-PCR need further optimalization. Since the epithelium only composes of a fraction of the whole tumor, our results reflect copy number changes of the stroma, which usually is the progressive component of phyllodes tumors and therefore most important to study. Further refinement of amplification techniques may make whole genome screening of the epithelial component feasible.

The effect of copy number change at the gene expression level determines the impact of individual chromosomal aberrations on tumor progression. This relation between gene dosage and expression is obvious in oncogenic activation by DNA sequence amplification, such as *ERBB2*. Chromosomal amplifications were rare in our group, though. Amplifications were found in three tumors. One malignant PT displayed a complex cluster of three amplicons (Fig 1), with similar DNA copy number amplitudes for all three amplicons. This suggests that these regions might be co-amplified and physically present in one amplicon. The fact that no known cancer related genes are present in the third amplicon could thus be explained. The mechanisms behind the formation of these complex amplicons remain elusive, though. Amplifications were observed exclusively in malignant tumors, in keeping with the general notion that amplifications are more prevalent at advanced tumor stage [30]. Two previous studies described no amplifications in PT [14,15], emphasizing that high-level amplifications are infrequent. On the other hand, low-level copy number changes were found relatively frequently. The relevance of these

alterations has been disputed for a long time. Recently, low-level copy number gain of *PIK3CA* has been linked to increased expression [31,32]. Further, the influence of modest copy number change on gene expression was confirmed in genome-wide experiments [33,34]. Therefore, the low-level changes described here might result in a survival advantage to the cell.

The differences in clinical behavior between both tumors seem to be reflected at the genomic level. All benign PT show copy number alterations, in contrast to the morphologically related fibroadenoma, which showed no copy number changes by array CGH, hereby confirming previous chromosome CGH studies [16,17]. Even single BAC clone changes were very rare in fibroadenomas. We cannot explain the large difference between these results and those of two recent studies, which detected scattered alterations on many different chromosomes [18.19]. Little agreement existed between the latter studies though. A possible source of bias is misclassification of benign PT for fibroadenoma [3], but this is infrequent and would not fully explain the differences. Studies using karyotype analysis found clonal aberrations in several dozens of fibroadenomas [7-11]. The number of cells containing chromosomal changes was highly variable, with as few as 2-15% of cells displaying the specific aberration in one study [35]. Aberrations of this type may be missed by array CGH, since it can not detect balanced aberrations and aberrations in a low percentage of cells. In addition, the karyotyping studies used different culture protocols and short term culture, which may select for distinct tumor clones.

Two previous studies used chromosomal CGH to obtain copy number profiles of PT [14,15]. On the whole, we found more chromosomal events per case with array CGH. Further, we added novel regions of DNA copy number changes to those of presumed importance in tumorigenesis of PT. The high resolution and increased sensitivity of array CGH allowed us to pick up small regions of altered DNA copy number, which in chromosome CGH may be masked by averaging effects. All studies, including the present, are hampered by suboptimal sample size due the low incidence of PT (2.1 per 1 million women per year [36]) and may therefore be liable to sampling bias. Previously, we detected a positive relation between tumor grade and cell cycle deregulation in the stroma of PT [37]. Deregulation of the cell cycle machinery may lead to loss of genetic integrity and accumulation of chromosomal alterations. However, like Jee et al [15], we did not find a significant relation between copy number changes and tumor grade. Until larger studies are performed it remains to be determined whether the relation between grade and copy number change is really non-existent or just too weak to be detected in limited patient groups.

Gain at 1q was not as prominent in our study (2/11, 18%) as in two previous studies [14,15]. Still, 1q gain seems an important event in PT tumorigenesis. Several cancer related genes reside on 1q. However, little is known about the role of these genes in PT. The Wnt signaling pathway was recently implicated in PT pathogenesis. It was demonstrated that deregulation of beta-catenin is caused by Wnt

overexpression and not by mutation of beta-catenin or *APC* [38]. Interestingly, WNT3A, which maps to 1q enhances levels of beta-catenin and stimulates cell growth in prostate cancer cells [39]. Therefore, *WNT3A* may be a candidate 1q gene.

Additional data on tumor suppressor and oncogene expression to which we can relate our current findings is sparse. Recently, we found that p53 accumulation is correlated to higher tumor grade and is an independent prognosticator of recurrent disease [37]. Kuenen et al previously established the importance of the p53-axis in PT [40]. Not surprisingly therefore, we found a region of common loss at 17p12-p13. The clones flanking this region included *TP53* further confirming its importance in PT development and progression. Interestingly, losses at 16q may involve *NQO1* which stabilizes p53, with lack of NQO1 leading to increased proteasomal degradation of p53 [41]. Further, individuals with NQO1 deficiency have a propensity for various malignancies [42,43]. RB1 expression was studied in our previous work as well [37]. Immunoquantification was problematic due to marked intratumoral heterogeneity, but loss of RB1 expression was not related to grade or survival. In the present study, we found loss at 13q13-q14.3 containing *RB1* in three tumors, including one malignant PT and its recurrence. In addition, isolated BAC clone loss was seen in two tumors. Losses at 13q were described previously as well [14,15]. These findings may indicate a more prominent role for *RB1* loss than suggested by immunohistochemistry.

Overall, loss at 16q was the most frequent chromosomal aberration found. Three distinct regions of copy number loss were identified on 16q. Several members of the cadherin family map to these regions, including its most well known member E-cadherin. Although loss of 16q and E-cadherin expression play major roles in the pathogenesis of lobular carcinoma of the breast [44], E-cadherin seems to be of minor relevance in PT or fibroadenomas [45]. Cadherin-family members 3, 13, 15 and 16 also map to these regions of loss. The exact role of the cadherin family in fibroepithelial tumors is unclear. Loss of cellular cohesion may however reflect an early step towards the capacity to disseminate.

CMYC expression has been demonstrated in the stroma of PT [46]. Gain at 8q24.1-qter was found in two tumors, with *CMYC* situated in the minimal region of overlap. In addition, one tumor showed isolated BAC clone loss here. Lu et al previously described gain at this region [14]. Although copy number gain may play a role in CMYC expression, Sawyer et al concluded that this is not the major mechanism of CMYC expression in PT since a minority of tumors showed additional copies of *CMYC* as determined by FISH [46]. In this light, recurrent losses at 16q22-q23 which contain the candidate tumor suppressor gene *CTCF* are interesting. CTCF was found to be a transcriptional repressor of *CMYC* [47]. Deletions at its corresponding locus have been commonly observed in breast and prostate cancer [48]. Loss of *CTCF* copy number may therefore influence CMYC expression levels.

We firstly describe recurrent losses at chromosome 19 in PT. *BAX,* one of the candidate genes here, is situated on chromosome 19q13.33 and is a pro-apoptotic

gene functioning as a tumor-suppressor gene [49]. Reduced levels of BAX mRNA have been described in invasive breast cancer as compared to normal breast tissue [50] and reduced immunostaining has been associated with shorter times to tumor progression and overall survival [51]. One report studied BAX immunostaining in PT [40]. In 3 of 19 cases strong expression of BAX was found, leading to the conclusion that BAX overexpression represents the pathological state in PT. It may be possible that in fibroepithelial tumors baseline BAX expression differs from that in epithelial malignancies and that loss of BAX is not of importance in PT genesis. Still, a possible role for loss of BAX cannot be ruled out.

In conclusion, this is the first study on genomic profiling of fibroepithelial breast tumors by array CGH. Fibroadenomas lacked copy number alterations, whereas genomic instability was found in all but one PT. Several areas of recurrent copy number change harbored well-known oncogenes or tumor suppressor genes. With the development of arrays with overlapping clones and contiguous coverage of the genome [52], it will become possible to pin-point the most relevant changes even further.

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9

Chapter 9

Amplifications of the Epidermal Growth Factor Receptor Gene (*EGFR*) are Common in Phyllodes Tumors of the Breast and are Associated with Tumor Progression

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Abstract

Phyllodes tumors of the breast are rare biphasic tumors with the potential for invasion and metastatic spread. An important role of the Epidermal Growth Factor Receptor (EGFR) in phyllodes tumors has been proposed. However, detailed pathogenetic mechanisms remained unclear.

We investigated 58 phyllodes tumors of the breast (40 benign, 10 borderline and 8 malignant) by means of *egfr* fluorescence in-situ hybridization (FISH) and gene dosage PCR for a regulatory sequence within intron 1 of *egfr*. Immunohistochemical staining was performed for EGFR, p16, p21, p27, p53, c-myc, Cyclin A, Cyclin D1, Cyclin E, c-kit and Ki67.

Immunopositivity for EGFR was detected in 19% of phyllodes tumors (75% of all malignant tumors) in stromal tumor cells, but not in the epithelial component. Whole gene amplifications were seen by FISH in 15.8% (in stromal cells only) and intron 1 amplifications by gene dosage PCR in as much as 41.8% of all phyllodes tumors. Significant correlations were seen between tumor grade on the one hand and EGFR overexpression ($p=0.001$) and intron 1 amplifications ($p<0.05$) on the other. EGFR overexpression further correlated positively with immunohistochemical staining for p53, p16, Cyclin A, Cyclin E, Ki67 and c-kit. Presence of intron 1 amplifications correlated with p16 ($p<0.01$), p21 ($p=0.009$) and p53 immunoreactivity ($p<0.001$). Neither EGFR overexpression nor whole gene amplification were observed in a control series of 167 fibroadenomas and only one of 43 (2.3%) exhibited intron 1 amplification in gene dosage PCR.

In conclusion, our results show for the first time that activating mutations in and overexpression of *egfr* are associated with the progression in grade of phyllodes tumors of the breast. The observed association between intron 1 amplification and overexpression of EGFR provides further insight into regulation mechanisms of EGFR overexpression.

Introduction

Phyllodes tumors are rare tumors characterized by a hypercellular monoclonal stroma and a usually modest polyclonal epithelial component, thereby representing the far end of the spectrum of fibroepithelial breast tumors [1]. The vast majority of phyllodes tumors are benign. However, some can also exhibit a malignant clinical behavior with local recurrences and distant metastatic spread [2]. The histologic characteristics of the overgrowing stroma alone determines whether a tumor should be called benign or malignant [3,4]. The spectrum of the stromal morphology is very broad from obviously benign with only moderately cellular stroma lacking atypia and mitoses to frankly malignant with highly cellular stroma rich in mitoses and heterologous differentiation as seen in usual soft-tissue sarcomas. There are a number of histologic characteristics which have been shown to correlate with clinical outcome and thus can be helpful in grading of phyllodes tumors [5-10]. However, no morphological feature could be presented so far which exclusively allows for definitive determination of the tumor grade by itself.

Recent studies have provided evidence on the involvement of various biological factors involved in the pathogenesis and the progression of breast phyllodes tumors such as overexpression of c-myc and c-kit and a deregulated Wnt-pathway [11]. Interestingly, mutations within these genes have not been observed. In other studies, involvement of the Epidermal Growth Factor Receptor (EGFR) in tumor progression has been postulated, even though *EGFR* mutations have not been investigated so far.

In this study we aimed to gain further insight into the biology of phyllodes tumors of the breast. We assessed the overexpression of EGFR, *EGFR* whole gene amplifications, and amplification status of a short CA repeat within intron 1 of *EGFR* which has been shown to have regulatory impact on *EGFR* transcription and expression [12,13]. Additionally, the expression profiles of a number of markers in involved cell cycle regulation, proliferation and apoptosis were analyzed by means of tissue microarrays. We show that intron 1 amplifications within the *EGFR* gene are quite common in phyllodes tumors and are clearly associated with overexpression of EGFR as well as grade of malignancy.

Material and Methods

58 phyllodes tumors and 167 fibroadenomas of the female breast were retrieved from the files of the Institute of Pathology, University of Muenster, the Institute of Pathology in Osnabrueck, Klinikum Osnabrueck and the Institutes of Pathology in Koeln-Rodenkirchen and Limburg, Germany. The cases were reviewed and graded according to the guidelines of the WHO [14] by at least two pathologists. We were aware that no single histological feature alone will in all cases provide a universally accepted diagnosis. Therefore we tried to appreciate the tumors complete

histological appearance as a whole utilizing the 6 attributes recommended by the WHO: stromal hypercellularity, cellular pleomorphism, mitosis rate, character of margins, stromal pattern and existance heterologeous stromal elements. The collection comprised 8 malignant, 10 borderline and 40 benign tumors. Tumor specimens were used for investigation after informed consent. The use of tumor tissue was also approved by the local ethical committee.

Clinical follow-up data could be gathered for 15 patients with phyllodes tumors, 3 of which featured malignant disease. The mean follow-up period was 5.1 years, ranging from 1 to 10 years.

Tissue microarray

A tissue microarray of 58 phyllodes tumors of the breast was constructed according to standard protocols using a dedicated TMA instrument (Beecher Instruments, Silver Spring, Maryland, USA) [15,16]. Six cores of 0.6mm in diameter were punched out of the donor block and placed at a distance of 0.2mm in the recipient block. By using 6 needle cores we even surpassed the recommendations of Hoos and collegues who demonstrated the sufficiency and representativity of at least three cores in TMAs for the investigation of mesenchymal tumors [17]. For the localization of representative tumor areas including epithelial and stromal component, haematoxylin and eosin stained sections were prepared from each original tumor block. A second control tissue array composed of 167 fibroadenomas was constructed in a similar manner.

Immunohistochemistry

For immunohistochemical detection of EGFR, extensive testing of several antibodies was conducted. Thereby we could show considerable differences in EGFR expression frequency exemplarily for soft tissue sarcomas (manuscript accepted for publication). The best suited antibody was then chosen for EGFR staining in this study. Table 1 shows the sources of the antibodies, dilutions, and antigen retrieval methods applied. For all antibodies, endogenous peroxidase activity was blocked for 30 minutes in a methanol solution containing 0.3% hydrogen peroxide after deparaffination and rehydration. After antigen retrieval, a cooling off period of 30 minutes preceded the incubation (overnight at 4°C) with the primary antibody. Before the slides were mounted all sections were dehydrated in alcohol and xylene.

For EGFR, p16, p21, p27, Cyclin A, Cyclin D1 and Cyclin E, primary antibodies were detected using a biotinylated rabbit anti-mouse antibody (DAKO). The signal was amplified by avidin-biotin complex formation and developed with diaminobenzidine followed by haematoxylin counter staining. For EGFR, slides with an EGFR overexpressing cell line (MDA-MB-486) and one without EGFR expression (SKBR-3) were used as positive and negative controls, respectively.

Table 1. Antibodies used for staining of phyllodes tumors.

Antibody	Source	Clone	Dilution	Pretreatment
EGFR	Monosan	EGFR.113	1:20	Steamer, citrate buffer, 35 min
c-kit	DAKO	polyclonal	1:100	Steamer, citrate buffer, 35 min
Ki-67	DAKO	Mib-1	1:1000	Steamer, citrate buffer, 35 min
p53	DAKO	DO-7	1:2000	Steamer, citrate buffer, 35 min
c-myc	Santa Cruz	9E10	1:150	Steamer, citrate buffer, 35 min
$p16^{INK4a}$	Neomarkers	16P07	1:160	EDTA buffer, 20 min, 97°C
p21	Oncogene	WAF1	1:10	Citrate buffer, 20 min, 97°C
$p27^{Kip1}$	BD Pharmingen	57	1:1000	Citrate buffer, 20 min, 97°C
Cyclin A	Monosan	6e6	1:100	Citrate buffer, 20 min, 97°C
Cyclin D1	Novocastra	DCS-6	1:20	EDTA buffer, 20 min, 97°C
Cyclin E	Monosan	13A3	1:200	Citrate buffer, 20 min, 97°C

For c-kit, p53 and Ki67, incubation with the primary antibody was performed for 25 minutes at room temperature using a DAKO Autostainer instrument. The primary antibodies were detected using the LSAB/AP method (DAKO). The signal was then developed with DAKO Red, followed by haematoxylin counter staining.

For c-myc, the slides were incubated with the primary antibody for 16 hours at 4°C. After treatment with a rabbit anti mouse bridge antibody, the signal was detected using the APAAP method. The slides were developed with new fuchsin (DAKO), followed by haematoxylin counter staining.

For evaluation of EGFR expression, membranous and cytoplasmic staining of tumor cells was scored from 0 to 3 (1 weak: at least 10% of tumor cells with a faint staining intensity, 2 moderate: at least 10% with a moderate staining intensity, 3 strong: at least 10% with a strong staining intensity). c-kit expression was graded as previously described [11]. For c-kit and EGFR, cases with a score >1 were regarded as immunoreactive. For p16, p27, Cyclin A, Cyclin E, Ki67 and c-myc, cases with more than 10% of positively staining nuclei were regarded as immunoreactive, for p21 and Cyclin D1 cases with more than 5%. For testing purposes, Ki67 staining scores were grouped from 0 to 3. The different grading systems did not alter the significance of correlations between Ki67 and tumor grade and gene dosage PCR. p53 nuclear staining intensity was scored from 0 to 3, whereas cases >1 were regarded as immunoreactive.

Fluorescence in-situ Hybridization (FISH)

The probe for *EGFR* detection was derived from homo sapiens PAC clone containing the whole *EGFR* gene (GenBank accession no. AC006977). Nick translation was performed following standard protocols for labeling of DNA with digoxigenin-11-dUTP. For denaturation the probe was applied for 5 min at 70°C to 70% formamid-0.6 x SSC. Slides underwent pretreatment with Proteinase K for 45

minutes at 45°C. Hybridization to TMA sections of 4 µm thickness was carried out overnight at 37°C in a 50% formamid-1x SSC-10% dextran sulfate solution in the presence of Cot-1-DNA (Life Technologies, Inc., Carlsbad, USA) and HPL-DNA (Sigma, St. Louis, USA). Post hybridization washes were performed at 45°C in 50% formamide-2x SSC and 0.1x SSC at 60° followed by blocking with 3% BSA in 4x SSC at 37°C. Probes were detected using mouse-anti-digoxigenin (Sigma, St. Louis, USA) and Cy3-labeled goat-antimouse antibodies (Dianova GmbH, Hamburg, Germany) for 45 min each at 37°C.

For each core 20 nuclei were selected for scoring according to morphological criteria using DAPI counterstaining. Only non-overlapping, intact nuclei were scored. Clearly distinguishable non-tumor cells were disregarded. The cutoff for considering a case as amplified was set at 4 signals per nucleus. Scoring was performed as previously published [18,19].

Gene dosage PCR

DNA was extracted from 5 whole paraffin sections of tumor tissue with a thickness of 10µm each according to standard protocols. Furthermore, 10 samples of normal appearing breast tissue surrounding the phyllodes tumors were analyzed.

For detection of amplifications of the first CA repeat in the first intron of the *EGFR* gene, a quantitative realtime PCR was performed targeting the repeat, and two known single-copy genes [20], superoxide dismutase (*sod*) and hemoglobin beta (*hbb*) genes, as reference.

Specific primers for sequences flanking the first CA repeat in the first intron of the *EGFR* gene were designed (CAIfor: 5'-tgaagaatttgagccaaccaaa-3' and CAIrev: 5'-cacttgaaccagggacagca-3') using Primer Express software (Applera). They were chosen since previous studies demonstrated that this primer combination defines amplifications of the whole gene, amplifications restricted to the CA-SSR I repeat and mutations involving this polymorphic sequence. Also a universal, VIC labeled probe consisting of 15 CA repeats (minor groove binder probe: 9 repeats) was constructed. The primers were checked by BLAST search (Internet address: **http://www.ncbi.nlm.nih.gov/Sitemap/index.html#BLAST**) and represented specific sequences for *EGFR*. Primers and probes were also designed for the single-copy genes *sod2* (chromosome 6q25, GenBank accession no. 65965, forward primer: 5'-GGAGAAGCTGACGGCTGC-3', reverse primer: 5'-CCTTATTGAAACCAAGCCAACC-3', VIC-labeled probe: 5'-CAACCTGAGCCTTGGACACCAACAGA-3') and *hbb* (chromosome 11p, GenBank accession No. V00499, forward primer: 5'-GTGAAGGCTCATGGCAAGAAAG-3', reverse primer: 5'-CAGCTCACTCAGTGTGGCAAAG-3', VIC-labeled probe: ATGGCCTGGCTCACCTGGACAACC). The amplicon length was minimized (68–97 bp) for all three of the genes, to allow for the most efficient PCR amplification. PCR analysis was performed using TaqMan Universal Mix (Applera) and detection was

performed on an ABI Prism 7900HT Sequence Detection System (Applera). Cycling conditions were as follows: denaturation: 95°C, 15 s, annealing and extension 60°C, 1 min, using 40 cycles. All PCR reactions were performed in triplicate and in at least two independent reactions. Serial dilutions of DNA were used to ensure accuracy of gene dosage quantification. The copy number of the *EGFR* gene was measured in the breast cancer-derived cell line MDA-MB-468 in comparison to normal leukocytes. MDA-MB-468 DNA reportedly displays a 30–50-fold amplification of the *EGFR* gene and was used as a positive control. DNA concentrations were normalized to both *sod2* and *hbb.*

Statistical analysis

Statistical analysis and tests were performed with SPSS Version 11.5.1. Correlations between EGFR expression, amplification and clinicopathological features were tested with cross tables applying chi-square, and correlation analysis was performed according to Kendall (Tau b).

Figure 1. Photomicrographs of a phyllodes tumor without (a) and with EGFR immunopositivity (b). Noteworthy, the epithelial cell compartment lacks EGFR immunoreactivity (a 10x, b 20x). For color plate see page 187.

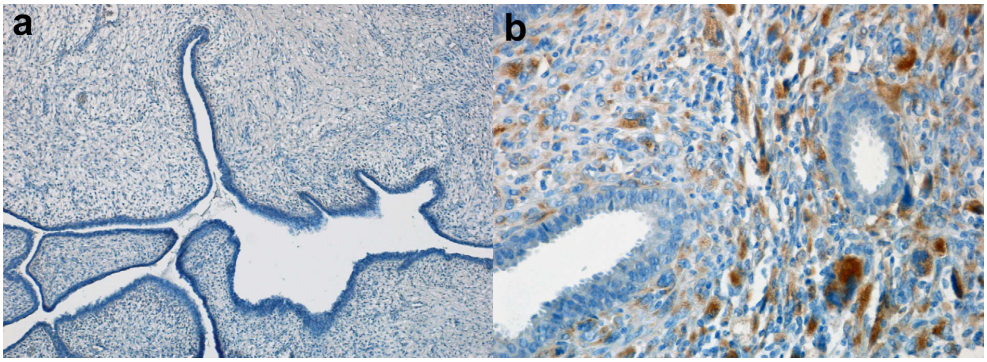


Table 2. EGFR overexpression and *EGFR* amplification in breast phyllodes tumors of different grade.

	Benign		Borderline		Malignant	
	-	+	-	+	-	+
EGFR amplification						
Whole gene	36	3	9	1	6	2
Intron 1 CA repeat	23	15	7	2	2	6
EGFR overexpression						
Stromal	35	5	9	1	3	5
Epithelial	31	2	8	0	3	0

Results

Of the 58 phyllodes tumors, 57 could be analyzed by FISH, 55 by gene dosage PCR, 57 by immunohistochemistry for c-kit, 54 for Cyclin D1 and 52 for p21. For all other markers, all cases could be analyzed. Significant differences could be seen in the reaction patterns between stroma and epithelium. The epithelial component mostly displayed a reaction pattern as seen in the normal breast (data not shown here). Therefore only data on the stromal compartment are presented.

EGFR expression

As shown in Table 2, EGFR overexpression was detected in 19% of all tumors: in 12.5% of benign, in 10% of borderline and 63% of all malignant phyllodes tumors ($p=0.001$). EGFR overexpression was almost exclusively restricted to stromal cells (Fig 1). Epithelial cells stained weakly positive in only two cases of benign phyllodes tumors. Stromal cells usually featured a combined staining of membranes and cytoplasm. Expression of EGFR was often observed in myoepithelial cells but not constantly. Overexpression of EGFR correlated significantly positively with the expression of p53 ($p=0.001$), p16 ($p<0.05$), c-kit ($p<0.01$), Ki67($p<0.05$), Cyclin A ($p<0.01$) and Cyclin E ($p<0.05$) (Table 3). A trend towards statistical significance could be seen for p21 and c-myc.

Amplifications of a regulatory sequence within intron 1 of EGFR

Amplifications of the CA repeat within intron 1 were seen in 23/55 (41.8%) of all cases and were associated with tumor grade ($p<0.05$) (Table 2). This included 15 /38 benign, 2/9 borderline and 6/8 malignant phyllodes tumors. The average gene copy number was 5.2, the maximum observed copy number was 22.9 in one sample. Intron 1 CA repeat amplifications correlated significantly positively with expression of EGFR ($p<0.01$), p53 ($p<0.001$), Cyclin A ($p<0.05$), p16 ($p<0.05$) and p21 ($p<0.01$) (Table 3).

Normal appearing breast tissue surrounding phyllodes tumors showed intron 1 amplifications in 2 of 10 cases.

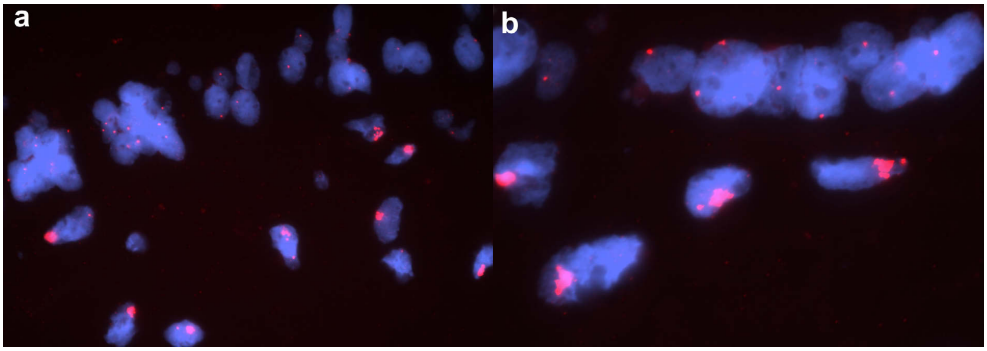
FISH analysis

EGFR whole gene amplifications detected by FISH were present in only 15.8% of all cases (Table 2, 3): in 3/39 benign, 1/10 borderline and 2/8 malignant tumors. Whole gene amplifications occurred exclusively in stromal cells (Figure 2), two of which were high level (>10 copies per nucleus), while the other 4 were low level amplifications. When amplification was observed, the majority of cells were affected. Whole gene amplifications did not correlate with tumor grade (Table 2). A positive correlation could be observed with expression of EGFR ($p<0.001$), p53 ($p<0.01$) and Cyclin E ($p<0.05$), though (Table 3).

Table 3. Comparison between 2 types of *EGFR* amplification, EGFR expression and several biomarkers in breast phyllodes tumors.

		Type of *EGFR* detection					
		Whole gene		Intron 1 repeat		Expression	
Marker		-	+	-	+	-	+
p16	-	13	2	4	11	10	6
	+	38	4	28	12	37	5
p21	-	18	0	14	2	18	1
	+	28	5	16	17	24	9
p27	-	4	0	4	0	4	0
	+	47	6	28	23	43	11
p53	-	46	3	32	15	44	6
	+	5	3	0	8	3	5
Cyclin A	-	48	5	32	19	46	8
	+	3	1	0	4	1	3
Cyclin D1	-	37	6	25	16	35	9
	+	10	0	5	5	8	2
Cyclin E	-	43	3	27	17	41	6
	+	8	3	5	6	6	5
c-kit	-	45	4	30	17	43	7
	+	5	2	2	5	3	4
c-myc	-	34	2	19	16	32	5
	+	16	4	13	7	14	6
Ki-67	-	41	3	27	15	39	6
	+	10	3	5	8	8	5

Figure 2. Fluorescence images of two cases of malignant phyllodes tumor showing stromal tumor cells with *EGFR* whole gene amplifications below a line of epithelial cells with regular number of gene copies (a 20x, b 40x). For color plate see page 186.



Comparison between FISH and gene dosage PCR

Whole gene amplification and intron 1 CA repeat amplification of *EGFR* were significantly correlated with each other ($p<0.01$) and with EGFR overexpression ($p<0.01$ for the intron 1 CA repeat amplifications; $p<0.001$ for whole gene amplifications), but observed frequencies differed clearly. Although all tumors with *EGFR* whole gene amplifications in FISH displayed amplifications in gene dosage PCR, only 9 out of 23 tumors with CA repeat amplification in gene dosage PCR also featured whole gene amplifications in FISH (Table 4).

		Whole gene amplification		Total
		-	+	
Intron 1 amplification	-	31	0	31
	+	17	6	23
Total		48	6	54

Table 4. Comparison between 2 types of *EGFR* amplification in breast phyllodes tumors.

Clinico-pathological correlations

EGFR overexpression ($p=0.001$) and also Cyclin A ($p<0.05$), Cyclin E ($p<0.05$), Ki67 ($p<0.01$), c-kit ($p=0.001$) and p53 ($p<0.001$) immunoreactivity significantly correlated with grade of phyllodes tumors. None of the patients with positive staining for the above markers for which clinical follow-up data was available showed recurrence or metastases (12 benign, 3 malignant).

Fibroadenomas

All 167 fibroadenomas lacked immunopositivity for EGFR. Furthermore, no case with increase of *EGFR* whole gene copy number detectable by FISH could be found. Only one (of 43 tested) revealed an amplification of the CA repeat in intron 1 of *EGFR* by means of gene dosage PCR (2.3%).

Discussion

The pathogenesis of breast phyllodes tumors is poorly understood – especially literature concerning molecular changes within these tumors is sparse. Up to now, only correlations between expression of p53, Ki67, c-kit, PDGF, VEGF and CD10 with tumor grade [9,21-24] have been described. The aim of this study was to expand on these data, especially with regard to other cell cycle regulators and the putative role of *EGFR* amplification by FISH and gene dosage PCR and immunohistochemistry on TMAs. As we were able to confirm the data by Chan et al. [21] and Chen et al. [22] on p53, Ki67 and c-kit, it is rather unlikely that the use of TMAs has biased our results. Unfortunately, clinical data was obtainable in only 15 of 58 cases, so our follow-up information can only be regarded as a small sample.

Our findings show for the first time whole gene and intron 1 CA repeat *EGFR* amplifications and overexpression of EGFR in the stromal component of breast phyllodes tumors. In view of the strong correlations between amplification of the regulatory repeat in intron 1 of *EGFR* and EGFR overexpression on the one hand and tumor grade on the other, these changes are likely to be involved in the pathogenesis and progression of the stromal component of phyllodes tumors. This is supported by our findings in normal breast tissue surrounding the tumors, revealing low level amplifications of the intron 1 CA repeat in 20% of the cases. An association between EGFR overexpression and tumor progression has been suggested earlier [25]. However, we are now able to show that amplifications of genetic sequences covering important regulatory sequences of *EGFR* and to a lesser extent of the whole *EGFR* gene may underlie the overexpression of EGFR in the stromal component of phyllodes tumors. Unfortunately as of today we were not able to perform a complete sequence analysis of the intron 1 CA repeat region, although considerable efforts have been made. It seems, that the nearby fragile site and the length of the repeat itself including its influence on secondary DNA structure [12] contribute to the difficulties encountered.

The correlation between EGFR overexpression and small amplifications in breast cancer has been described earlier by our group [13]. However, the relationship between both features is significantly higher in phyllodes tumors compared to invasive cancer of the breast. Whereas only 25% of EGFR overexpressing invasive breast cancer cases were associated with any kind of *EGFR* amplifications [19], 82% of EGFR-overexpressing phyllodes tumors displayed these amplifications. It further seems that involvement of EGFR on the DNA and protein level is a rather specific finding for phyllodes tumors and not a general event in biphasic tumors of the breast: only one of 43 fibroadenomas featured intron 1 repeat amplifications (and no whole gene amplification in 163 fibroadenomas), but a considerable percentage of benign and an even higher percentage of malignant phyllodes tumors did. This might give further rise to the hypothesis that EGFR is part of a mechanism involved in transition from fibroadenoma towards phyllodes tumors as proposed earlier [1]. It seems more obvious on the other hand, that the *EGFR* pathway is often switched on *after* successful progression to phyllodes tumor and is one important factor that is able to promote malignant transformation besides several others.

Our results further stress the importance of genetic instability in the pathogenesis of these tumors. Phyllodes tumors of the breast are frequently found in patients with Li-Fraumeni syndrome caused by p53 germline mutations and consequently cytogenetic instability [26]. A DNA “fragile site” has been described nearby the *EGFR* locus [13] which might be a possible explanation for the rather high frequency of *EGFR* amplifications in sporadic phyllodes tumors. Consequently the association of p53 and p21 with increased *EGFR* gene dosage could be regarded as

downstream events due to DNA double-strand breaks and insufficient repair mechanisms [27]. However, it is obvious that these mechanisms are rather weak and are directly counterbalanced by an increased proliferation rate as indicated by the correlation of Ki67, Cyclin A and E with EGFR overexpression. In conclusion, the protein expression patterns in phyllodes tumors due to *EGFR* mutations can generally be divided into factors associated with EGFR overexpression and proliferation (Ki67, Cyclin A, Cyclin E, p16) and factors associated with counterbalancing, nevertheless finally insufficient cellular proapoptotic mechanisms due to genetic instability per se (p21).

Unfortunately our results give only limited insight into the relationship between different types of *EGFR* amplifications. We examined two types of *EGFR* amplifications: more common amplifications of a regulatory CA short sequence repeat in intron 1 by gene dosage PCR and rarer amplifications of the whole gene, detectable by FISH-analysis [28]. The frequency of CA repeat amplifications (41%) and whole gene amplifications (15%) point towards a sequence of genetic instability either way. Nevertheless, a dynamic process in the development of *EGFR* amplifications has not been demonstrated so far and it remains to be elucidated if different kinds of *EGFR* amplifications occur in a step-wise manner.

However, it has to be stated that less than half of all phyllodes tumors revealed *EGFR* amplifications and even less displayed an EGFR overexpression. So, EGFR obviously is not the only pathway that promotes growth and progression in phyllodes tumors. Recent studies focussed on the Wnt pathway and its possible interaction with c-myc in the pathogenesis of phyllodes tumors. However, the underlying mechanisms for c-myc deregulation in this tumor entity remained unclear, since no activating mutations could be found for the majority of tumors with overexpression of c-myc. Published data so far point towards a direct relationship between EGFR overexpression and c-myc expression. Nevertheless our results revealed only a statistical trend to support these experimental findings and may give only a limited explanation for c-myc overexpression [11].

In summary, we show for the first time that amplifications of *EGFR* are common and correlate with EGFR overexpression as well as tumor grade, implicating *EGFR* in progression in phyllodes tumors of the breast. As new additional markers for malignancy in phyllodes tumors they may therefore also aid in establishment of the correct diagnosis in questionable cases. Because only one whole gene amplification of *EGFR* was evident in our series of fibroadenomas (2.3%) without EGFR overexpression, EGFR overexpression and amplifications allow for a quite clear distinction of both entities in difficult cases.

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10

Chapter 10

Gene expression signatures of breast phyllodes tumor and fibroadenoma

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Manuscript

Abstract

Phyllodes tumor (PT) and fibroadenoma share morphological similarities but display different clinical behavior. In addition, fibroadenoma may progress to PT by monoclonal stromal expansion. We compared transcriptome-wide levels of gene expression of both tumors and normal breast tissue in order to gain insight into the molecular mechanisms driving development and progression of fibroepithelial tumors.

We used the Affymetrix GeneChip HG-U133A 2.0, which contains more than 22000 probe-sets, to examine gene expression profiles of fibroadenomas (n=5) and phyllodes tumors (n=8). Profiles were compared to signatures of normal breast as well. Immunohistochemical validation of microarray data was performed for two genes, *c-kit* and *CXCR4*, on tissue microarrays (TMA) composed of 58 PTs and 167 fibroadenomas.

We detected many novel genes with a possible role in the pathogenesis of fibroepithelial tumors. *IGF-2* showed strong upregulation in fibroadenoma when compared to normal tissue. Among the genes displaying strongest downregulation in fibroadenoma were several genes involved in steroid binding and metabolism (*SCGB1D2, SCGB2A2, SCGB2A1, SULT2B1*) and galactose metabolism (*LALBA*). In comparison to the normal breast, PT showed downregulation of DNA repair genes (*POLL, RECQL, TP73L*), cell adhesion genes (*PKP1, CX3CL1, FAT2, DSC1*), transcription factors (*ETV4, TP73L, SOX10, KLF5, BCL11B*) and growth factors (*TGFA, EGF, PDGFC*). As compared to fibroadenoma, PT displayed upregulation of 26 genes and downregulation of 52 genes. Downregulation was found of genes involved in transcription (*EGR1, ELF5, LZTS1*), cell adhesion (*JAM2, PCDH11X, CX3CL1*), apoptosis (*TNFSF8, PHLDA1*) and Wnt signaling (*FRZB, SFRP1*). Overexpression was found of factors involved in transcription (*RUNX1, PTN, LHX2, PKNOX2, HOXC13*), cellular integrity (*SGCD, palladin*) and extracellular matrix degradation (*MMP11*). Downregulation of *c-kit* most likely resulted from the relatively smaller amount of epithelium in PT. By immunohistochemistry on TMAs we confirmed downregulation of *CXCR4* in PT and localized it to the epithelial component.

In conclusion, this is the first study comparing gene expression signatures of PT and fibroadenomas. We have identified many novel genes which may contribute to development and progression of fibroepithelial tumors. In addition, several genes may be of value in the differential diagnosis of biphasic breast tumors.

Introduction

Carcinogenesis and cancer progression is driven by a panoply of genetic and epigenetic changes that result in alterations in expression of the involved genes. The recently introduced array technology has made it possible to interrogate transcriptome-wide levels of gene expression in a single experiment. In breast cancer, analysis of expression data derived from the primary tumor identifies different prognostic subgroups [1,2] or predicts response to chemotherapy [3,4]. Transcriptional profiling may even be more powerful than classical grading of breast cancer [1]. Several mesenchymal tumors have been studied by gene expression profiling as well. Some tumors, such as synovial sarcomas, neural tumors and gastrointestinal stromal tumors, seem to have distinct expression profiles, while others, like malignant fibrous histocytoma and liposarcoma, showed considerable overlap in their expression profiles [5]. Still, Linn et al could well differentiate between dermatofibrosarcoma protuberans and other soft tissue tumors based on expression arrays [6]. Similarly, expression profiling distinguished synovial sarcomas and other sarcomas and detected gene expression differences between biphasic and monophasic synovial sarcomas [7].

Fibroadenoma and phyllodes tumor (PT) are biphasic breast tumors harboring both a mesenchymal and an epithelial component. PT is graded as benign, borderline or malignant based on morphology and mitotic index. Malignant PT may resemble soft tissue sarcoma with pronounced stromal overgrowth, nuclear atypia and increased mitotic figures. Differentiating between fibroadenoma and PT can be problematic. In particular, benign PT and fiboadenoma may share morphological similarities [8]. Moreover, fibroadenoma may show stromal progression to PT [9]. The distinction between both tumors is important, though. Although carcinoma *in situ* may occur in the epithelium of fibroadenoma [8], it is basically considered to be a benign tumor that does not recur or metastasize, whereas PT recurs frequently and may even metastasize in up to 25% of malignant PTs [10].

The molecular mechanisms underlying fibroadenoma and PT genesis are largely unclear. Cell cycle deregulation, in particular *TP53* mutation and accumulation, may contribute to PT progression [11]. Further, EGFR overexpression was found in PTs but not in fibroadenoma [12]. Expression profiling of fibroepithelial tumors would be a good instrument to identify further involved genes, but has so far not been performed. A recent study found that solitary fibrous tumor (SFT) and desmoid-type fibromatosis, both soft tissue tumors, had different expression profiles [13]. When evaluating some of these genes by immunohistochemistry on tissue microarrays it was found that fibroadenomas, which functioned as controls, expressed genes in a pattern similar to SFT. All these studies show that gene expression profiling successfully identifies discriminating and corresponding genes between morphologically similar tumors.

In this study, we therefore compared gene expression profiles of normal breast, fibroadenoma and PT to detect genes involved in tumorigenesis. Since fibroadenoma and PT show different clinical behavior, molecular profiling may reveal prognostic genes in addition to markers which could prove to be a diagnostic aid.

Materials and Methods

Tissue samples

Fresh frozen tissue samples were retrieved from the tissue banks of our hospitals. In total, 5 fibroadenomas and 8 PTs were studied as well as 2 samples of normal premenopausal breast (each a pool of two different individuals) to serve as controls. Two 4µm sandwich H&E cryosections were examined to identify and discard any possible normal tissue in the fibroadenomas and PTs, and for grading of PTs according to the criteria of Moffat et al [10]. In brief, based on the degree of stromal overgrowth, margin infiltration, stromal cellularity, stromal atypia and number of mitosis, tumors were graded as benign, borderline or malignant. Mitotic figures were counted using established criteria in ten consecutive fields at 400x magnification [14].

RNA extraction and array hybridization

Sample preparation and processing procedures were performed as described in detail in the Affymetrix GeneChip Expression analysis Manual (Affymetrix, Santa Clara, CA, USA). In brief, snap-frozen tissue was crushed and total RNA was extracted from the crushed tissue with TRIzol reagent (Invitrogen Life Technologies, Rockwell, MD, USA) and cleaned by RNeasy affinity columns (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized from total RNA with use of the T7-(dT24)-oligomer. In vitro transcription was performed by the T7 MEGAScript Labeling Kit (Ambion, Austin, TX, USA) in the presence of biotinylated nucleotides. Double-stranded cDNA and complementary RNA were purified and fragmented with the GeneChip Sample Cleanup Module (Affymetrix). Biotinylated cRNA was hybridized to the Affymetrix GeneChip HG-U133A 2.0 at 45˚C for 16 hours. The HG-U133A 2.0 array contains more than 22000 probe-sets, including 13500 well-characterized human genes. The arrays were washed using Affymetrix fluidics station. Staining was performed with streptavidin-phycoerythrin conjugates. The readout of the probe signals was performed with the GS3000 microarray scanner (Affymetrix, Santa Clara, CA, USA). Expression values for each gene were calculated by using Affymetrix GeneChip analysis software GCOS version 1.3.

Tissue microarray and immunohistochemistry

Two tissue microarrays (TMAs) of respectively 58 PTs and 167 fibroadenomas were constructed according to standard protocols with a dedicated TMA instrument

(Beecher Instruments, Silver Spring, MD, USA) [15,16]. Each case was represented by six cores of 0.6mm in diameter. Representative tumor areas containing both epithelium and stroma were identified on haematoxylin and eosin stained sections of the original tumor blocks. Two differentially expressed genes were validated by immunohistochemical staining of TMAs.

Staining were performed as described previously [12,17]. Endogenous peroxidase activity was blocked for 30 minutes in a methanol solution containing 0.3% hydrogen peroxide after deparaffination and rehydration. Subsequently, antigen retrieval was performed in a pressure cooker for 35 minutes (C-kit) or for 15 minutes in a microwave (CXCR4) in citrate buffer. A cooling off period of 30 minutes preceded the incubation with the primary antibody. For C-kit (polyclonal Ab, 1:100 dilution; DAKO, Glostrup, Denmark), incubation with the primary antibody was performed for 25 minutes at room temperature using a DAKO Autostainer instrument. The primary antibody was detected using the LSAB/AP method (DAKO). For CXCR4 the slides were incubated with the primary antibody for 45 min at room temperature (MAB-171, 1:50; R&D Systems, MN, USA). After treatment with a rabbit anti mouse bridge antibody (30 min, 1:30), the signal was detected using the APAAP method (60 min / 1:100). The slides were developed with new fuchsin (25 min). After counterstaining with haematoxylin, slides were mounted. c-kit and CXCR4 stainings were scored as described previously [18,19].

Table 1A. Upregulated (known) genes in fibroadenomas as compared to normal breast. Functions indicated for the genes were derived from Genecards [66].

Gene symbol	Gene name	Fold change	Function gene product
IGF-2	insulin-like growth factor 2	8.54	growth factor
OLFML3	olfactomedin-like 3	4.04	unknown
ITM2A	Intergral membrane protein 2A	2.99	unknown
COL6A1	collagen, type VI, alpha 1	2.57	extracellular matrix, cell-adhesion
ISLR	immunoglobulin superfamily containing leucine-rich repeat	2.12	protein binding
MAGED1	melanoma antigen family D, 1	2.07	apoptotis pathway
SIGLEC6	sialic acid binding Ig-like lectin 6	1.97	cell adhesion
MAP2K5	mitogen-activated protein kinase kinase 5	1.96	MAPK signalling
RIS	Ras family member Ris	1.90	GTP binding
MAPK8	mitogen-activated protein kinase 8	1.81	MAPK signalling
NGFRAP1	nerve growth factor receptor (TNFRSF16) associated protein 1	1.77	unknown
TIX1	Triple homeobox 1	1.73	transcription factor
MST1	macrophage stimulating 1	1.71	unknown

Statistical analysis

Reasoning from our previously proposed model where fibroadenoma arises from normal breast and PT from either normal breast or through clonal expansion of the stromal component of fibroadenoma [9], we compared expression profiles between fibroadenoma and normal breast, between PT and normal breast, and between PT and fibroadenoma. Each pair was compared with an in-house developed algorithm based on the mathematical software platform SPlus version 6.2. The t test criteria were p value<0.01 to 0.05 depending on the test. Additional filter criteria were applied; fold change>1.5, difference of the normalized signals>10 and F test p value<0.05 [20].

Table 1B. Downregulated (known) genes in fibroadenomas as compared to normal breast. Functions indicated for the genes were derived from Genecards [66].

Gene symbol	Gene name	Fold change	Function gene product
LALBA	lactalbumin, alpha-	0.06	galactose metabolism
SCGB1D2	secretoglobin, family 1D, member 2	0.07	steroid binding
SCGB2A2	secretoglobin, family 2A, member 2	0.10	steroid binding
CLCA4	chloride channel, calcium activated, family member 4	0.12	ion transport
CCL2	chemokine (C-C motif) ligand 22	0.13	chemokine
DF	D component of complement	0.16	complement pathway
SCGB2A1	secretoglobin, family 2A, member 2	0.16	steroid binding
PROL1	Proline rich 1	0.17	unknown
VGLL1	vestigial like 1 (Drosophila)	0.19	transcription regulation
RRP22	RAS-related on chromosome 22	0.20	GTP binding
C7	complement component 7	0.23	complement pathway
KLF5	Kruppel-like factor 5	0.28	transcription factor
MARK2	MAP/microtubule affinity-regulating kinase 2	0.31	cell differentiation
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1	0.34	cell adhesion, anti-angiogenesis
SULT2B1	sulfotransferase family, cytosolic, 2B, member 1	0.34	steroid metabolism
HDAC7A	histone deacetylase 7A	0.35	histone deacetylation , transcription
PDGFC	platelet derived growth factor C	0.40	growth factor
MAZ	MYC-associated zinc finger protein	0.51	transcription factor
STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	0.67	signal transduction, transcription factor
CSK	c-src tyrosine kinase	0.67	protein-tyrosine kinase, cell cycle
SET	SET translocation (myeloid leukemia-associated)	0.67	Multitasking gene

Results

Fibroadenoma vs normal breast

A total of 96 transcripts were found to be differentially expressed between fibroadenoma and normal breast. Among these transcripts we identified 19

upregulated well-known genes and 70 downregulated genes. A selection of genes with altered expression in fibroadenoma is listed in Tables 1A and 1B. Genes downregulated in fibroadenoma are involved in cell adhesion (*CEACAM1, FAT2*), transcription (*KLF5, SET, STAT6, MAZ*), steroid binding and metabolism (*SCGB1D2, SCGB2A2, SCGB2A1, SULT2B1*), galactose metabolism (*LALBA*) and the complement pathway (*DF, C7*). Upregulated genes in fibroadenomas influence processes such as growth factor signaling (*IGF-2*) transcription (*TIX1, MAP2K5, MAPK8*), apoptosis (*MAGED1*), cytoskeleton formation *(CAPZB*) and cell adhesion (*COL6A1, SIGLEC6*).

Table 2. Downregulated (known) genes in phyllodes tumors as compared to normal breast. Functions indicated for the genes were derived from Genecards [66].

Gene symbol	Gene name	Fold change	Function gene product
CSN1S1	casein alpha s1	0.02	transporter activity
RRP22	RAS-related on chromosome 22	0.14	GTP binding protein
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	0.15	tyrosine kinase receptor
SOX10	Sex determining region Y)-box 10	0.15	transcription factor
EGF	Epidermal growth factor	0.17	growth factor
KCNN4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	0.18	ion transport
FOLR1	folate receptor 1 (adult)	0.18	folate metabolism
PADI2	peptidyl arginine deiminase, type II	0.18	protein modification
CX3CL1	chemokine (C-X3-C motif) ligand 1	0.19	chemokine, cell adhesion
PVALB	Parvalbumin	0.19	ion transport
KLF5	Kruppel-like factor 5	0.22	transcription factor
HMGA1	high mobility group AT-hook 1	0.22	DNA binding
TGFA	transforming growth factor, alpha	0.25	protein-tyrosine kinase, growth
CXCL5	chemokine (C-X-C motif) ligand 5	0.25	chemokine
SFRP1	secreted frizzled-related protein 1	0.27	Wnt signalling
PKP1	plakophilin 1	0.27	cell adhesion
BCL11B	B-cell CLL/lymphoma 11B	0.30	transcription regulation
BIRC3	baculoviral IAP repeat-containing 3	0.31	anti-apoptotis
ETV4	ets variant gene 4	0.31	transcription factor
POLL	polymerase (DNA directed), lambda	0.32	DNA repair
PDGFC	platelet derived growth factor C	0.32	growth factor
HIST3H2A	histone 3, H2a	0.33	nucleosome assembly
FAT2	FAT tumor suppressor homolog 2 (Drosophila)	0.33	cell adhesion
TP73L	tumor protein p73-like	0.34	transcription factor
ST14	suppression of tumorigenicity 14	0.35	extracellular matrix degradation
FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	0.35	protein modification
RECQL	RecQ protein-like (DNA helicase Q1-like)	0.46	DNA repair
JAG2	jagged 2	0.57	Notch signaling

PT vs normal breast

A total of 106 transcripts showed differential expression when PT was compared to normal breast. Surprisingly, only 3 transcripts showed overexpression, whereas the remaining 103 transcripts were underrepresented in PT. These transcripts represent 2 overexpressed and 85 downregulated well-known genes. The overexpressed genes concerned *MPPE1*, which is involved in DNA repair, and *SDHB,* an electron transporter. Genes downregulated in PT are involved in cell adhesion (*PKP1, CX3CL1, FAT2, DSC1*), DNA repair (*RECQL, POLL*), transcription (*ETV4, TP73L, SOX10, KLF5, BCL11B*), ion transport (*KCNN4, SCL13A2, PVALB*) and growth factor signaling (*TGFA, EGF, PDGFC*). Table 2 displays a selection of genes downregulated in PT.

Table 3A. Selected genes upregulated in breast phyllodes tumors as compared to fibroadenomas. Functions indicated for the genes were derived from Genecards [66].

Gene symbol	Gene name	Fold change	Function gene product
CTAG1	Cancer/testis antigen 1	47.17	unknown function
CTAG2	Cancer/testis antigen 2	14.05	unknown function
PRAME	Preferentially expressed antigen melenoma	11.87	retinoic acid signalling
HOXC13	Homeobox C13	4.68	transcription factor
CILP	Cartilage intermediate layer protein	4.61	extracellular matrix
ITIH4	Inter alpha inhibitor H4	4.45	acute phase response
LHX2	LIM homeobox 2	3.90	transcription factor
MMP11	Matrix metalloproteinase 11	3.44	extracellular matrix degredation
CLCN4	Chloride channel 4	3.26	ion transport
PKNOX2	PBX/knotted 1 homeobox 2	2.90	transcription factor
NPAS1	Neuronal PAS protein domain 1	2.72	transcription factor
HR	Hairless homolog	2.69	transcription factor
BF	B-factor	2.67	complement system
SGCD	Sarcoglycan delta	2.58	cytoskeleton protein
RUNX1	Runt-related transcription factor	2.39	transcription factor
AMH	Anti Mullerian hormone	2.12	growth factor, sex diffenrentiation
PTN	Pleiotrophin	2.04	cytokine, growth factor activity

PT vs fibroadenoma

A total of 95 transcripts were found to be differentially expressed between PT and fibroadenomas. Thirty-one transcripts were overrepresented in PT, whereas 61 transcripts were underexpressed, representing 26 and 52 well-known genes, respectively. Overexpression was found of factors involved in transcription (*RUNX1, PTN, LHX2, PKNOX2* and *HOXC13*), metabolism (*MTHFD2, P4HA2*), cellular integrity (*SGCD, palladin*), extracellular matrix degradation (*MMP11*) and osmotic regulation (*CLCN4*). Table 3A summarizes several overexpressed genes in PT. Genes underexpressed in PT are involved in apoptosis (*TNFSF8, PHLDA1*), growth

factor signaling (*FGF 7 and 12*), transcription (*EGR1, ELF5*), cell adhesion (*JAM2, PCDH11X, CLDN3*) and cell growth (*FRZB, LZTS1, SFRP1*). The most important downregulated transcripts in phyllodes tumors are shown in Table 3B. Figure 1 depicts the most important implicated genes in our model of pathogenesis of fibroepithelial breast tumors.

Table 3B. Selected genes downregulated in phyllodes tumors as compared to fibroadenoma. Functions indicated for the genes were derived from Genecards [66].

Gene symbol	Gene symbol/Gene name	Fold change	Function gene product
ELF5	E74-like factor 5 (ets domain transcription factor)	0.23	transcription factor
FABP7	Fatty acid binding protein 7, brain	0.27	fatty acid metabolism
BDKRB2	Bradykinin receptor B2	0.29	bradykinin receptor
UGT8	UDP glycosyltransferase 8	0.29	lipid synthesis
PHLDA1	Pleckstrin homology-like domain, family A, member 1	0.31	cell growth, anti-apoptosis
PTGER1	prostaglandin E receptor 1 (subtype EP1)	0.31	prostaglandin receptor
PADI2	Peptidyl arginine deiminase, type II	0.32	protein modification
PLXDC1	Plexin domain containing 1	0.32	unknown
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	0.33	tyrosine kinase receptor
PIGR	Polymeric immunoglobulin receptor	0.35	immunoglobulin transport
PCDH11X	Protocadherin 11 X-linked	0.35	cell adhesion
SLC13A3	Solute carrier family 13 (sodium-dependent dicarboxylate transporter)member 3	0.36	ion transporter
GNAS	GNAS complex locus	0.36	calcium signalling pathway
MS4A1	Membrane-spanning 4-domains, subfamily A, member 1	0.37	b-cell maturation
HIST1H2AG	Histone 1, H2ag	0.39	DNA binding
CX3CL1	Chemokine (C-X3-C motif) ligand 1	0.39	cell adhesion, chemokine
FGF12	Fibroblast growth factor 12	0.40	growth factor
JAM2	Junctional adhesion molecule 2	0.41	cell adhesion
NK4	Natural killer cells protein 4	0.42	cytokine
FGF7	Fibroblast growth factor 7	0.43	growth factor
EGR1	Early growth response 1	0.45	transcription factor
FRZB	Frizzled-related protein	0.46	Wnt signalling
TMPRSS2	Transmembrane protease, serine 2	0.48	serine protease
CXCR4	Chemokine (C-X-C motif) receptor 4	0.50	chemokine receptor
ADAMTS8	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 8	0.51	anti-angiogenesis
MAP2K6	Mitogen-activated protein kinase kinase 6	0.51	MAPK signaling
LZTS1	Leucine zipper, putative tumor suppressor 1	0.51	cell growth, cell cycle regulation
SFRP1	Secreted frizzled-related protein 1	0.60	Wnt signalling

Immunohistochemical staining of TMAs

When staining for c-kit on TMAs, we found a significant positive relation between stromal c-kit expression and tumor grade in PT ($p=0.001$). The stromal component of fibroadenoma stained negative for c-kit. The epithelial component of both tumors showed diffuse weak positive staining for c-kit comparable to that of normal breast tissue. The stromal component of both tumors stained negative for CXCR4. CXCR4 immunoreactivity was practically absent in the epithelium of PTs (3/58 tumors, 5.2%), whereas the epithelium of fibroadenomas more frequently showed weak positive staining (25/138 tumors, 18%; $p<0.05$).

Discussion

PT and fibroadenoma share morphological similarities but display different clinical behavior. The molecular mechanisms underlying development and progression of both tumors are largely unclear. In the present work we compared gene expression profiles of PTs and fibroadenomas with normal breast tissue serving as control. By this approach we have identified genes involved in development of fibroadenoma and PT from normal tissue. We have previously shown that PT may also derive though monoclonal stromal expansion of fibroadenoma [9]. By comparing molecular profiles of both tumors we identify candidate genes which may contribute to this transition.

Our study may have been hampered by small sample size. It has to be realized, however, that PT is a rare tumor with an incidence of only 2.1 per 1 million women per year [21]. Composing a study group of sufficient size was complicated even further by the fact that expression analysis requires fresh frozen tissue. This selection bias may in part explain why some genes that have been detected in PTs by immunohistochemistry did not turn up as differentially expressed in the present microarray study. Further, post-translational modification of proteins may cause some of these discrepancies as well.

An additional problem in our study is the fact that fibroadenoma and phyllodes tumors are biphasic tumors. Changes in gene expression may originate from either compartment. Based on array data alone one cannot localize the expression changes to stroma or epithelium. Staining of altered genes on TMAs meets this problem since the histological context is maintained in the tissue cores. Still, most PTs contain a smaller amount of epithelium and this may elicit apparent loss of genes expressed preferentially in the epithelial component. In fact, this represents an absolute decrease of mRNA in proportion with the reduced epithelial fraction in PT. Therefore, the apparent loss of gene expression in these cases is caused by loss of epithelial cell mass.

Comparing expression signatures of PT and fibroadenomas revealed several genes which may be involved in clonal progression of fibroadenoma to PT or which

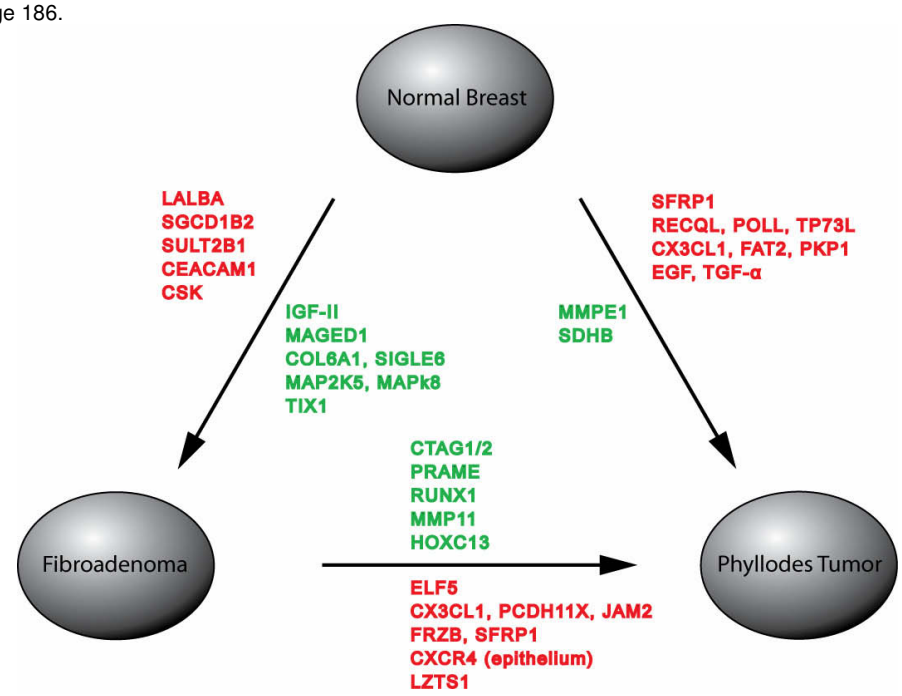
may prove to be a diagnostic aid. The gene showing strongest upregulation in PT was *CTAG1*. It codes for a protein with unknown function. *CTAG1* is normally expressed only in testis but is overexpressed in a wide variety of malignancies including breast cancer [22] and mono- and biphasic synovial sarcoma [23]. Because of its pronounced expression in malignant tissue it is currently under investigation as an immunotherapy target. In fibroadenomas, ~70% showed CTAG1 mRNA expression but CTAG1 immunostaining was negative [22]. It would be interesting to evaluate CTAG1 immunohistochemical staining in PT as it may prove to be a useful marker in the differential diagnosis of fibroepithelial tumors.

RUNX family members are DNA-binding transcription factors that regulate the expression of genes involved in cellular differentiation and cell cycle progression. *RUNX1* expression levels were elevated 2.4 fold in PT when compared to fibroadenoma. In leukemia, *RUNX1 (*also known as *AML-1,* acute myeloid leukemia 1*)* is the most frequent target for chromosomal translocation resulting in oncogenic fusion proteins. Moreover, the *RUNX1* gene is often amplified or overexpressed in cases of acute leukemia. Evidence is emerging that the role of *RUNX1/AML-1* is not limited to hematologic malignancies. Recently, Planaguma et al described its upregulation in endometroid carcinoma [24]. In gastric cancer, on the other hand, *RUNX1* seems to be downregulated [25]. Besides oncogenic overexpression, we also found downregulation of the tumor-suppressor gene *LZTS1* in PT. Downregulation of *LZTS1* has been described in several types of epithelial cancer [26,27]. *LZTS1* probably regulates cell cycle progression by interacting with Cdc2 [28]. Strangely, Linn et al found overexpression of LZTS1 in dermatofibrosarcoma protuberans [6]. No additional data is available on both genes in breast tumors or soft tissue tumors is available in literature. The study of both *RUNX1* and *LZTS1* in fibroepithelial tumors may provide further insight in their pathogenesis.

Metastases are found in up to 25% of malignant PT [10]. In order to acquire a metastatic phenotype cells must be able to escape cellular cohesion and breakdown the surrounding matrix. Matrix metalloproteinases constitute one of the major extracellular matrix degrading enzymic families. We found overexpression of *MMP11* in PT as compared to fibroadenomas. In breast cancer, increased expression of MMP11 has been associated with adverse prognosis [29]. Other genes which downregulation may stimulate dissemination, include cell adhesion genes *JAM2, PCDH11X and CLDN3*. Downregulation of cellular adhesion proteins and overexpression of extracellular proteases in PTs most likely reflects a developing potential to dissemenate.

Comparing PT and normal tissue profiles revealed additional downregulated genes involved in tissue cohesion (*PKP1*) and matrix degradation (*ST14*). In this light, downregulation of *FOLH1* in PT when compared to normal tissue is interesting as well. FOLH1 expression has been demonstrated in normal and hyperplastic prostate tissues, in prostatic intraepithelial neoplasia and in invasive carcinomas [30].

Figure 1. Most important genes with altered expression in proposed model of fibroepithelial tumor genesis. Downregulated genes are shown in red, upregulated genes in green. For color plate see page 186.



Recently, it has been shown that downregulation of *FOLH1* results in an increased invasive potential in prostate cancer cell lines [31]. Although the mechanism is unclear, loss of FOLH1 in PT may contribute to acquisition of a metastatic phenotype. Further, altered expression of genes involved in cellular cohesion and matrix remodeling seem important in development of PT from fibroadenoma and from normal tissue.

Development of genomic instability is, like loss of tissue cohesion and invasion, a critical event in the genesis of human neoplasms [32,33]. Genomic integrity is lost by deregulation of genes involved in DNA damage surveillance and repair. Genomic instabilty at the chromosome level has been demonstrated in PT previously [34,35,36]. Results for fibroadenoma are conflicting [36,37,38]. When comparing PT with normal breast, we found that *RECQL* and *POLL,* both DNA repair genes, were downregulated in PT. *RECQL* is a DNA helicase and has a mismatch repair function [39]. *POLL* is a DNA polymerase involved in base excision repair [40]. Although instability at the DNA level seems the major effect, altered function of these genes may in part explain the chromosomal abnormalities observed in PT. On the other hand, comparing PT to normal tissue revealed overexpression of *MPPE1*, which is a DNA repair gene as well. *MPPE1* functions as a DNA double-strand break repair gene [41]. The effects of overexpression of a DNA repair gene is unknown.

Paradoxically, overexpression of a DNA repair gene can have adverse effects on chromosome stability. Recently, it was demonstrated that overexpression of *POLL* initiates DNA breaks and stimulates DNA exchanges and aneuploidy [42]. When comparing PT to normal tissue another contributing factor emerged. TP73L was downregulated in PT. It was shown that *TP73L*, a p53-family member, is involved DNA damage induced apoptosis [43]. Its loss results in an inability to undergo apoptosis in response to genomic damage. The net-effect of changes in expression of these genes is uncertain, but altered functioning of DNA repair and surveillance genes may be an initiating step in PT genesis.

Recently, the Wnt pathway has been implicated in PT development and progession [44]. Activation of the canonical Wnt signaling pathway leads to stabilization and nuclear accumulation of β-catenin where it directs transcription of a variety of genes. Nuclear accumulation of β-catenin in stroma of PT has been described [44]. When comparing PT to normal tissue, we detected downregulation of *SFRP1*, which is an antagonist of Wnt signaling [45]. In breast cancer, loss of SFRP1 is an early event in breast cancer progression and an adverse prognosticator [46]. Downregulation of SFRP1 by promotor hypermethylation has been described in mesothelioma and colorectal cancer [47,48]. SFRP1 was downregulated in PT when compared to fibroadenoma as well. In addition, another SFRP-family member was downregulated as well, namely *FRZB* (also known as *SRFP3*). Like SRFP1, FRZB is a Wnt signaling antagonist [49]. Since *APC* or β-catenin mutations are rare in PT, it has been hypothesized that stromal β-catenin accumulation results from Wnt5a expression in the epithelial component [44]. Not all cases of β-catenin accumulation are explained hereby and downregulation of SFRP1 and FRZB may contribute to activation of the canonical Wnt pathway.

c-kit is a membrane-bound tyrosine kinase receptor. Overexpression of c-kit is characteristic of gastrointestinal stromal tumors [50]. Surprisingly, c-kit was downregulated in PT when compared to fibroadenomas and normal breast tissue in our group. This result is unexpected since earlier work showed that at higher grade, PT stroma shows overexpression of c-kit [18,51]. Indeed, when staining the TMA for c-kit we found that stromal immunoreactivity was correlated with tumor grade ($p=0.001$). When examining the epithelial component we found weak positive c-kit immunoreactivity in PT and fibroadenoma epithelium. Previously, it has been shown that normal breast epithelium displays c-kit immunoreactivity [52]. Malignant progression of breast epithelium has been related to loss of c-kit expression [53]. However, the apparent downregulation of c-kit in PT is most likely caused by a diminished amount of epithelium in PT. Still, approximately 50% of malignant PT show stromal c-kit overexpression [18]. It is possible that our small group lacked PT with stromal c-kit expression. The discrepancy between immunohistochemical data and the current expression profiling results are therefore not contradictory per se.

Our gene expression profiling results showed that *CXCR4* was downregulated in PT as compared to fibroadenoma. By immunohistochemistry on TMAs we confirmed its downregulation in PT, although this was confined mainly to the epithelial component. CXCR4 and its ligand SDF-1 were first identified in the context of trafficking and homeostasis of immune cells. Later, expression of CXCR4 was found on breast cancer cells and *CXCR4/SDF-1* was thought to govern dissemination patterns of breast cancer [54]. In various neoplasms its overexpression was related to adverse prognosis [55,56,57]. Several factors, such as BCR-ABL [58], CHK [59], pVHL [60] and PTEN [61], have been associated with downregulation of *CXCR4*. Downregulation of *CXCR4* leads to a diminished capacity to disseminate. In PT, the stromal component metastasizes, the epithelial component does not. Therefore, reduced epithelial CXCR4 expression may reflect its indolent nature. Still, the epithelial component of fibroadenoma behaves benign as well. Perhaps, epithelial downregulation of CXCR4 is a result of autocrine or paracrine effects by factors secreted in stroma or epithelium.

When comparing fibroadenoma to normal breast tissue several interesting findings emerged. First, a strong upregulation of IGF-2 was found in fibroadenomas. *IGF-2* has mitogenic and antiapoptotic actions and regulates cell proliferation and differentiation and mediates these effects through *IGF-1R*. Previously, Sawyer et al detected overexpression of IGF-2 by *in situ* hybridization in the stromal component of the majority of breast fibroadenomas [44]. The epithelium lacked IGF-2 expression. By placing its expression under the control of sheep beta-lactoglobulin promotor elements, mice overexpressing IGF-2 showed formation of biphasic breast tumors [62]. This implicates *IGF-2* as a critical gene in fibroadenoma development. In breast cancer, IGF-2 overexpression was found in stromal cells adjacent to and surrounding malignant epithelial cells [63]. These observations make IGF-2 a candidate in epithelial-stromal interactions, which play an important role in breast cancer progression. Stromal IGF-2 overexpression may fulfill such a paracrine or autocrine role in fibroepithelial tumors as well. Among the genes displaying strongest downregulation in fibroadenoma were several genes involved in steroid binding and metabolism (*SCGB1D2, SCGB2A2, SCGB2A1, SULT2B1*) and galactose metabolism (*LALBA*). These observations may implicate that altered steroid regulation contributes to development of fibroadenoma or that in its development fibroadenoma loses some features characteristic of normal breast tissue. It has been suggested previously that fibroadenoma genesis is at least in part hormone dependent [64]. In addition, tamoxifen therapy reduces the risk for fibroadenoma [65]. Although, there is evidence that fibroadenoma is a hormone dependent developmental anomaly and that fibroadenoma is a polyclonal lesion [9], the associated relative risk for breast cancer remains poorly understood. Further, altered expression of cancer related genes in fibroadenomas, such as upregulation of *IGF-2* and downregulation of *CSK* and *CEACAM1,* deserve further attention.

In conclusion, this is the first study comparing gene expression signatures of PT and fibroadenomas in comparison to normal breast to identify genes involved in fibroadenoma and PT genesis. PT differed from fibroadenoma by altered expression of several genes involved in cell growth, apoptosis, transcription and cell adhesion. Downregulation of CXCR4 in PT was confirmed by immunohistochemical staining on TMAs. Interesting genes which may be involved in the transition from fibroadenoma to PT and worth further study include *RUNX1, CTAG1, LZTS1* and *MMP11*. The comparison of fibroadenomas to normal breast tissue implicates *IGF-2* as a critical gene in its pathogenesis and the *IGF*-family may play an important role in fibroepithelial tumors genesis. Additional investigations into these genes may even reveal diagnostic value.

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Chapter 11

Summary and conclusions

In this thesis the focus is on fibroadenoma and phyllodes tumor, both fibroepithelial breast tumors. These tumors are biphasic, composed of stroma and epithelium. Fibroadenoma is the most prevalent of the fibroepithelial tumors and is generally regarded as a benign tumor. Phyllodes tumor is rare and of unpredictable clinical behavior making its recognition therefore of importance. Classification criteria for phyllodes tumor and fibroadenoma show considerable overlap. Moreover, it has been suggested that fibroadenoma may progress to phyllodes tumor. Overall, little is known on the pathogenesis of both tumors. The exact role of and the relation between both components of fibroepithelial tumors has been subject to controversy. Some assign the epithelium an active role in tumorigenesis, for example by secretion of growth factors, while others regard this component as merely reactive. Further, the molecular changes driving progression of both components are largely unclear. In this thesis we explore several major phenomena in carcinogenesis for their role in development of these fibroepithelial tumors. Cell cycle deregulation, angiogenesis, genetic instability and growth factor expression (EGFR) were evaluated for their contribution in phyllodes tumor and fibroadenoma pathogenesis. Some molecular markers may differentiate between fibroadenoma and phyllodes tumors and prove a valuable diagnostic aid in problematic cases. Histology of both tumors and clonality of its both compartments were studied and integrated. Finally, we compared gene expression profiles of both tumors. By this approach we attempted to gain insight in pathogenesis and progression of fibroepithelial breast tumors.

In **chapter 2** we review the world literature on fibroepithelial tumors, including sclerosing lobular hyperplasia and hamartoma. We provide extensive information on microscopy, clinical behavior, molecular biology and genetics. A relative paucity of studies addressing the molecular mechanisms underlying the development and progression of both tumors characterizes available literature. This lack of information on several subjects prompted some of the work described in this thesis.

Large epidemiological studies relate the presence of fibroadenoma to an increased relative risk for invasive breast cancer. The mechanism for this association is unknown, but it seems to be related in part to microscopical features of the fibroadenoma. Further, malignant transformation of the epithelial component has been described in a small percentage of tumors. For a highly prevalent tumor like fibroadenoma this may have clinical consequences. Partially due to the lack of comprehensive studies on the histology of fibroadenoma and its surrounding parenchyma, no clear guidelines exist on the management of fibroadenoma. By carefully studying its histopathology in **chapter 3** we attempted to construct a proposal for its management. Fibroadenomas displayed a wide variety of proliferative and non-proliferative histological lesions. We identified several phyllodes tumors which had initially been classified as fibroadenoma, underlining this diagnostic

problem. Further, 3 fibroadenomas with an area of apparent stromal expansion were detected. Possibly these tumors display stromal progression to phyllodes tumor. In 44% of tumors, epithelial proliferative disease was found. Epithelial hyperplasia has been associated with an increased relative risk for invasive breast cancer of up to 5, making a contribution to the risk associated with fibroadenoma not unconceivable. However, hyperplasia in the adjacent tissue was seen less frequently (9%). Therefore, it is unknown if the high prevalence of hyperplasia in fibroadenomas reflects the status of the entire breast and its significance remains to be determined. In 2% of fibroadenomas we found carcinoma in situ (CIS). The youngest of these patients was 40 years old. It is obvious that CIS, a direct precursor of invasive breast cancer, should be excised. When combining our data with that of previous literature, removal of fibroadenomas in women over 35 years of age will identify all in situ and invasive carcinomas. Ideally, this approach should be tested in a prospective trail. This will be difficult, however, since many women choose for excision even after a triple-diagnostic procedure. Regarding its associated increased risk for breast cancer, fibroadenoma may serve as a marker to identify women at high risk. However, for as long as it its unclear if this risk should have therapeutic consequences, such as intensified follow-up or chemoprevention, removal of all fibroadenomas is not warranted.

In **chapter 4** we describe a case report of a women with three fibroadenomas simultaneously giving rise to CIS. One fibroadenoma harbored DCIS, whereas LCIS developed from the other two. Due to a positive family history for breast and ovarian cancer, a suspicion for a BRCA mutation existed. A missense mutation in exon 16 of BRCA1, which is now regarded as a polymorphism, was detected. Although malignant transformation of fibroadenomas is infrequent, this case stresses the need for histological evaluation of all breast masses in women with a strong positive family history for breast and/or ovarian cancer.

Progression of the stromal component of fibroadenoma to phyllodes tumor has been postulated in the literature but direct evidence is lacking. In the work described in chapter 3 we identified several cases of fibroadenoma with an area of apparent stromal progression. In **chapter 5** we subjected these cases and phyllodes tumors of various grades, normal fibroadenomas and fibroadenomas with epithelial hyperplasia and CIS to PCR-based clonality assay. Further, by integrating the results we attempted to construct a model displaying possible progression routes within and between these tumors. Clonality analysis takes advantage of the silencing by methylation of one of both X-chromosomes. In a monoclonal process, thus derived from one progenitor cell, the same X-chromosome is inactivated in all cells. In a polyclonal cell population, in 50% of cells the maternal X-chromosome is inactivated and the paternal X-chromosome in the other half. Inconspicuous fibroadenomas

showed polyclonal stroma and epithelium. Hyperplasia in fibroadenomas was polyclonal as well. We firstly demonstrate that the areas of apparent stromal expansion in fibroadenomas were monoclonal, suggesting stromal progression. As expected, CIS in fibroadenoma was monoclonal. Mostly, stroma of phyllodes tumors was monoclonal, which was expected since this is the component that metastasizes. Surprisingly, stroma of a few benign and borderline phyllodes tumors was polyclonal. Thereby we advance molecular evidence supporting the hypothesis that the phyllodes-like stromal expansion in fibroadenomas may be an early tumor progression event. We are the first to demonstrate that, in addition to epithelial progression to CIS, stroma of fibroadenomas may progress to phyllodes tumor. Further, our results also suggest the existence of clonal evolution in the progression of phyllodes tumors of the breast.

Transition through the cell cycle is regulated by cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors. Disruption of one or more components of the cell cycle machinery may lead to uncontrolled progression through the cell cycle and rapid cell growth. The most extensively studied cell cycle control pathways are the apoptosis controlling p53 pathway and the proliferation controlling cyclin D1/p16/pRb pathway. In **chapter 6** we studied the role and prognostic relevance of several representatives of these two major cell cycle controlling pathways in phyllodes tumors of the breast. Besides studying primary tumors, we compared several paired primary and recurrent tumors. We found an increasing level of cell cycle distortion with higher tumor grade in the stromal component. Stromal overexpression of $p16^{INK4A}$, $p21^{waf1}$, cyclin A, p53, p21 and pRb all were found more frequently at higher tumor grade. In addition, the number of aberrantly expressed cell cycle proteins in the stromal compartment increases with tumor grade as well. Stromal cyclin A expression was the single best separating factor between tumor grades and may therefore aid grading. Cyclin A, p53, Ki67 and pRb expression, number of altered cell cycle proteins, tumor grade and tumor size were predictive of recurrent disease. However, the number of stromal aberrations and stromal p53 overexpression were independent prognosticators for recurrent disease. In concordance with its inconspicuous microscopical appearance, the epithelial component mostly showed no altered expression of cell cycle proteins. No differences were found between primary and recurrent tumors, which confirms that wide local excision is the treatment of choice. However, in our group, margin status was not predictive of recurrence. In accordance with the theory that multiple derailments in one pathway do not add to a greater growth advantage, we found no correlation between altered expression of cell cycle markers from the same pathway. However, correlations existed between stromal overexpression of $p16^{INK4A}$ and $p21^{waf1}$, $p16^{INK4A}$ and p53, and p53 and pRb, hereby disrupting both pathways. These results point an important role of p53 and other cell cycle proteins in phyllodes tumor progression. The progressive

dedifferentiation of the stromal component at higher tumor grade is reflected in the increasing level of cell cycle disruption. Cyclin A expression may be useful for accurately grading of phyllodes tumors. However, as compared to classical prognosticators such as tumor grade, margin status or proliferation makers, stromal p53 expression may prove superior in predicting prognosis. Cell cycle derailment does not seem to play a role in the epithelial component of phyllodes tumors. In stroma, however, it may be major force driving progression of this component.

A tumor can only grow beyond several mm^3 if it is able to form new microvessels in order to sustain a higher proliferation rate over that of apoptosis. Hypoxia inducible factor 1α (HIF-1α) has a central role in this process and activates genes involved in angiogenesis, glycolysis, erythropoiesis and apoptosis. In **chapter 7** we studied the relation between angiogenesis and proliferation in fibroepithelial breast tumors. Expression of HIF-1α, and its downstream targets CAIX and VEGF was investigated by immunohistochemistry in a group of 37 primary phyllodes tumors and 30 fibroadenomas. Expression levels were related to microvessels counts, proliferation and clinicopathological parameters. Fibroadenoma mostly lacked HIF-1α and CAIX reactivity. In phyllodes tumors, however, stromal HIF-1α expression was correlated with tumor grade, proliferation, p53 accumulation and microvessel counts. Necrosis and CAIX expression were rarely found in stroma. All malignant phyllodes tumors showed HIF-1α overexpression. Concerted CAIX and HIF-1α expression was frequently found in morphologically normal epithelium of phyllodes tumors. By measuring the distance of microvessels to epithelium in fibroadenomas and phyllodes tumors we conclude that this is most likely explained by relatively distant microvasculature in phyllodes tumors causing mild hypoxia. This probably reflects a physiological adaptation to microenvironmental disturbance by rapidly proliferating stroma with lagging peri-epithelial angiogenesis and probably has no tumor biological significance in itself. Indeed, in contrast to stromal expression, epithelial HIF-1α expression lacked prognostic power. Still, paracrine stimulation of the stroma by HIF-1α induced epithelial mitogens can not be ruled out completely. Microvessel counts and VEGF expression as such did not differ between fibroadenomas and phyllodes tumors. Stromal HIF-1α overexpression in phyllodes tumors was predictive of disease free survival. In phyllodes tumors, stromal HIF-1α overexpression predicts prognosis and may play an important role in stromal progression. Due to the absence of necrosis and CAIX expression in phyllodes tumors, HIF-1α upregulation seems normoxic and may be best explained by changes in expression of oncogenes, tumor suppressor genes or growth factors. The strong relation between stromal HIF-1α expression and p53 upregulation makes p53 a possible candidate. However, numerous factors interact with HIF-1α and the mechanisms of non-hypoxic HIF-1α upregulation in phyllodes tumors remains unknown. Techniques covering a variety of molecular elements, such as DNA microarrays may help to unravel the complex

mechanisms underlying the presumed non-hypoxic upregulation of HIF-1α in the stroma of phyllodes tumors. Further, any possible downstream effects of epithelial HIF-1α overexpression may reveal themselves by this approach as well. Alternatively, assessing growth factor expression in phyllodes tumors with HIF-1α positive and negative epithelium might demonstrate such an effect as well. In addition, this will address possible epithelial-stromal cross-talk as well, which as a general phenomenon in carcinogenesis, raises increasing interest.

Human neoplasm development is initiated by changes in function of genes that directly regulate cell death and growth. One of the mechanisms leading to aberrant gene expression is DNA copy number change. Recently developed array based CGH allows genome wide screening for copy number changes and direct mapping of alterations to the human genome sequence. In **chapter 8** we used array CGH to study copy number alterations in phyllodes tumors and fibroadenomas. We used arrays composed of 2464 genomic clones, providing a resolution of ~1.4Mb across the genome. No copy number changes were found in fibroadenomas, even single clone alterations were rare. On the other hand genetic instability was found in all but one phyllodes tumor. A mean of 5.3 (range 0-16) chromosomal events was seen per case. A mean of 2.0 gains (range 0-10) and 2.8 losses (range 0-7) was seen per case of PT. Amplifications were rare. We observed recurrent losses on chromosome 1q, 4p, 10, 13q, 15q, 16, 17p, 19 and X. Copy number gains were seen on 1q, 2p, 3q, 7p, 8q, 16q and 20. No relation was found between number of copy number changes and tumor grade. Although some changes were seen only in malignant tumors, no significant differences were found between grades. Some alterations are firstly described here, probably due to the superior resolution of array CGH as compared to chromosome CGH. Several recurrent regions of copy number alterations harbored well-known cancer related genes, such as *TP53* and several *Cadherin* family members. Although not related to grade, it seems that genomic instability plays a role in development of phyllodes tumors. These results should be confirmed in a larger series, although this will be difficult due to the low incidence of phyllodes tumors. In fibroepithelial tumors the epithelium is mostly a two-layered lining making it almost impossible to obtain sufficient DNA. Our results therefore reflect copy number changes in the stroma. With optimalization of genomic amplification techniques it will become feasible to perform array CGH of the epithelial component. Ultimately, the effect of gene dosage on expression determines its effect on tumor progression. In the near future we therefore plan to compare array-based expression data to gene dosage in the same group of tumors.

Overexpression of the epidermal growth factor receptor (EGFR) in invasive breast cancer was recently demonstrated to be related to the length of a polymorphic CA repeat located at the 5'-regulatory sequence in intron 1 of the *EGFR* gene. In

addition, in became clear that allelic imbalances restricted to the CA repeat were based on amplifications of that region. These amplifications are associated with EGFR overexpression. In **chapter 9** we studied the relation between EGFR expression, *EGFR* whole gene amplifications and amplification status of a short CA repeat within intron 1 of *EGFR* in fibroadenomas and phyllodes tumors. These findings were related to expression of several cell cycle markers, which were assessed by immunohistochemistry on tissue micro arrays. Stromal EGFR immunoreactivity was detected in 19% of phyllodes tumors (75% of all malignant tumors). Whole gene amplifications were seen by FISH in 16% (in stromal cells only) and intron 1 repeat amplifications by gene dosage PCR in as much as 42% of all phyllodes tumors. 82% of EGFR overexpressing tumors showed *EGFR* gene amplification. EGFR immunoreactivity or gene amplification was not observed in the epithelial component. Tumor grade was significantly correlated to EGFR overexpression ($p=0.001$) and intron 1 repeat amplifications ($p<0.05$). EGFR overexpression further correlated positively with immunohistochemical staining for p53, p16, cyclin A, cyclin E, Ki67 and c-kit. Intron 1 repeat amplification correlated with p16 ($p<0.01$), p21 ($p=0.009$) and p53 ($p<0.001$) immunoreactivity. EGFR overexpression nor whole gene amplification was observed in 167 fibroadenomas and only one of 43 (2.3%) showed intron 1 amplification. These results show that activating mutations in and overexpression of *EGRF* are associated with the progression in grade of phyllodes tumors of the breast. Like cell cycle deregulation (chapter 6), *EGFR* overexpression and amplification is confined to the stromal component. *EGFR* amplification and overexpression is not seen in fibroadenomas and is therefore not a common feature of biphasic tumors. Thus, EGFR expression may differentiate between phyllodes tumor and fibroadenoma in case of diagnostic difficulties.

Tumorigenesis and cancer progression are driven by a multitude of genetic and epigenetic changes that result in altered expression of involved genes. The Human Genome Project has revealed detailed information about the structure of all human genes. Based on this knowledge, high performance molecular screening techniques have been developed. DNA microarrays allow the simultaneous analysis of the expression of thousands of genes. In **chapter 10** we applied gene expression profiling to fibroadenomas and phyllodes tumors. We found many novel genes which may contribute to genesis or progression of fibroepithelial tumors. *IGF-2* showed strong upregulation in fibroadenoma when compared to normal tissue. Among the genes displaying strongest downregulation in fibroadenoma were several genes involved in steroid binding and metabolism and galactose metabolism. Phyllodes tumor showed altered expression of DNA repair genes, cell adhesion, transcription factors and growth factors when compared to the normal breast. As compared to fibroadenoma, phyllodes tumor displayed upregulation of 26 genes and

downregulation of 52 genes. Downregulation was found of genes involved in transcription, cell adhesion, apoptosis and Wnt signaling. Overexpression was found of factors involved in transcription, cellular integrity and extracellular matrix degradation. *CTAG1*, a gene of unknown function, showed a nearly 50-fold upregulation in phyllodes tumor, which suggests that it may aid diagnostics. *c-kit* and *CXCR4* were downregulated in phyllodes tumor and these genes were immunohistochemically examined on tissue microarrays (TMA). The apparent downregulation of c-kit is probably caused by a relatively smaller epithelial component, whereas CXCR4 seems to be downregulated in the epithelial component. This is the first study comparing gene expression signatures of phyllodes tumors and fibroadenomas. We have identified many novel genes which may contribute to development and progression of fibroepithelial tumors. In addition, several genes may be of value in the differential diagnosis of biphasic breast tumors. Therefore, we plan to further investigate several interesting genes by immunohistochemistry on TMAs.

Concise conclusions

1. Since malignant transformation of the epithelium of fibroadenomas is not seen or is extremely rare under 35 years of age, only fibroadenomas in women above this age should be excised.
2. In women with a family history of breast cancer, all breast masses should be evaluated histologically.
3. Clonality analysis indicates that benign fibroadenoma may progress to unpredictable phyllodes tumor.
4. Cell cycle deregulation drives stromal progression of phyllodes tumor, whereas in fibroadenomas no cell cycle aberrations are apparent.
5. Stratifying by p53 expression may be superior to classic grading in predicting prognosis in phyllodes tumors.
6. In contrast to fibroadenomas, genetic instability is found in most phyllodes tumors.
7. HIF-1α expression predicts prognosis in phyllodes tumors and may play an important role in the adaptive process in growing phyllodes tumors.
8. Activating mutations in and overexpression of *EGFR* are not found in fibroadenomas but are associated with progression of phyllodes tumors.
9. This thesis contains no data that suggests an active role of the epithelium in fibroepithelial tumors pathogenesis.
10. Although morphologically fibroadenoma and phyllodes tumor are alike, on the whole both tumors are different molecular entities.

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Chapter 12

Samenvatting en conclusies

Dit proefschrift richt zich op fibroadenomen en phyllodes tumoren, beide fibroepitheliale tumoren. Deze tumoren worden bifasisch genoemd omdat zij opgebouwd zijn uit twee componenten, een epitheliale en een stromale. Het fibroadenoom is de meest voorkomende fibroepitheliale tumor en wordt gezien als een goedaardige tumor. Phyllodes tumoren zijn zeldzaam, gedragen zich onvoorspelbaar en kunnen zelfs uitzaaien. Het onderscheid tussen beide tumoren is derhalve van belang. De diagnostische criteria vertonen echter een behoorlijke overlap. Tevens is gesuggereerd dat fibroadenomen progressie tot phyllodes tumoren kunnen vertonen. Over de onderliggende pathogenese van beide tumoren is weinig bekend. De precieze rol en de onderlinge relatie van beide componenten is onderwerp van discussie. Sommigen kennen het epitheel een actieve rol in de tumorontwikkeling toe, bijvoorbeeld door secretie van groeifactoren, terwijl anderen deze component slechts als reactief beschouwen. Verder zijn de moleculaire veranderingen die progressie van beide componenten in de hand werken grotendeels onbekend. In dit proefschrift onderzoeken wij verscheidene belangrijke carcinogenetische fenomenen op een mogelijke de rol in de ontwikkeling van fibroepitheliale tumoren. Ontsporing van cel cyclus regulatoren, angiogenese (vaatnieuwvorming), genetische instabiliteit en groeifactor regulatie (EGFR) werden onderzocht op hun bijdrage in de pathogenese van fibroadenomen en phyllodes tumoren. Sommige moleculaire markers die werden onderzocht zouden kunnen differentiëren tussen beide tumoren en dus diagnostische waarde kunnen hebben. Tevens bestudeerden wij de histologie van beide componenten en integreerden deze gegevens met clonaliteitsdata. Als laatste werden genexpressie-profielen van fibroadenomen en phyllodes tumoren vergeleken. Middels deze aanpak trachtten wij inzicht te verkrijgen in de pathogenese (ontwikkeling) en progressie van fibroepitheliale borsttumoren.

Hoofdstuk 2 is een samenvatting van de beschikbare literatuur over fibroepitheliale tumoren, inclusief scleroserende lobulaire hyperplasie en hamartoom. In dit hoofdstuk geven wij uitgebreide informatie over microscopie, klinisch gedrag, moleculaire biologie en genetica. De beschikbare literatuur wordt gekenmerkt door een relatieve schaarste aan studies over de moleculaire mechanismen die ten grondslag liggen aan de ontwikkeling en progressie van fibroepitheliale tumoren. Dit gebrek aan informatie op sommige gebieden was de aanzet tot een deel van het werk in dit proefschrift.

Uit grote epidemiologische studies werd duidelijk dat er een verband bestaat tussen fibroadenomen en een toegenomen risico op borstkanker. Het mechanisme achter deze associatie is onbekend, maar het lijkt deels samen te hangen met de microscopische kenmerken van het fibroadenoom. Ook maligne transformatie van het epitheel van fibroadenomen wordt beschreven in een klein percentage van

tumoren. Deze feiten kunnen voor een veel voorkomende tumor als het fibroadenoom klinische consequenties hebben. Gedeeltelijk door het gebrek aan uitgebreide histologische studies van het fibroadenoom en het omgevende borstweefsel zijn er geen duidelijke richtlijnen voor de behandeling van deze tumor. Door een zorgvuldige studie van de histopathologische kenmerken van het fibroadenoom trachtten wij in **hoofdstuk 3** te komen tot een behandelingsvoorstel. Een scala aan proliferatieve en niet-proliferatieve afwijkingen werd gezien. Verschillende phyllodes tumoren bleken initieel als fibroadenoom te zijn gediagnosticeerd, wat een bekend diagnostische probleem is. Tevens werden drie fibroadenomen met een gebied van schijnbare stromale expansie ontdekt. Mogelijk dat er in deze tumoren sprake is van progressie tot phyllodes tumoren. 44% van tumoren vertoont epitheliale proliferaties. In de “normale” borst wordt epitheliale hyperplasie geassocieerd met een 5 maal verhoogd relatief risico op borstkanker. Dit maakt een bijdrage van hyperplasie in fibroadenomen aan het eerder beschreven verhoogd risico op borstkanker niet ondenkbaar. Toch wordt hyperplasie in het omgevende weefsel veel minder (9%) gezien dan in de tumor zelf. Het blijft daarom nog onduidelijk of de hoge incidentie van hyperplasie in fibroadenomen de status van de overige borst weerspiegelt. De precieze waarde van hyperplasie in fibroadenomen is derhalve nog niet duidelijk. In 2% van de tumoren werd carcinoma in situ (CIS) gevonden. De jongste patiënte was 40 jaar. Het is duidelijk dat CIS, een directe voorloper van invasief carcinoom, verwijderd dient te worden. Als men onze gegevens combineert met die uit eerdere literatuur, dan lijkt het aangewezen fibroadenomen bij vrouwen boven de 35 jaar te verwijderen. Met deze aanpak worden alle in situ en invasieve carcinomen geïdentificeerd. Idealiter zou men deze hypothese moeten testen in een prospectieve klinische trail. Dit is echter moeilijk te realiseren daar het bekend is dat de meeste vrouwen ondanks uitgebreide diagnostiek (lichamelijk onderzoek, beeldvorming en cytologisch- of naald-biopt) kiezen voor excisie. Gezien het verhoogde risico op borstkanker zouden fibroadenomen kunnen dienen als marker om hoog risico vrouwen te identificeren. Echter, zolang het onduidelijk blijft of dit verhoogde risico consequenties heeft, zoals intensieve controle of chemopreventie, is verwijdering van alle fibroadenomen niet aangewezen.

In **hoofdstuk 4** beschrijven we een casus van een patiënte met 3 fibroadenomen van waaruit zich gelijktijdig CIS ontwikkeld. Een tumor bevatte DCIS, de twee andere tumoren LCIS. Gezien de positieve familie anamnese voor borst- en ovarium-kanker bestond er een verdenking op een BRCA mutatie. Een missense mutatie in exon 16 van BRCA1 werd gevonden, maar deze mutatie wordt tegenwoordig als een polymorphisme gezien. Hoewel maligne transformatie van fibroadenomen weinig frequent wordt gezien, benadrukt deze casus de noodzaak tot

histologische evaluatie van alle borstlesies bij vrouwen met een positieve familie anamnese voor borst- en ovarium-kanker.

Over progressie van stroma van fibroadenomen tot phyllodes tumor wordt in de literatuur gespeculeerd maar direct bewijs ontbreekt. In hoofdstuk 3 beschreven wij enkele gevallen van fibroadenomen met een gebied van ogenschijnlijke stromale progressie. In **hoofdstuk 5** onderwierpen wij deze gevallen en phyllodes tumoren van verschillende graad, normale fibroadenomen en fibroadenomen met epitheliale hyperplasie en CIS aan PCR gebaseerde clonaliteitsanalyse. Door deze clonaliteitsresultaten te integreren met de histologie trachtten wij een model te construeren waarin mogelijke progressieroutes in deze tumoren en tussen de tumoren onderling worden weergegeven. Clonaliteitsanalyse maakt gebruik van de inactivering door methylatie van één van beide X-chromosomen. In een monoclonaal proces, dwz ontstaan vanuit één voorlopercel, is in iedere cel hetzelfde X-chromosoom geïnactiveerd. In een polyclonaal proces daarentegen, is in 50% van de cellen het maternale X-chromosoom geïnactiveerd en in de andere helft het paternale. Zowel stroma als epitheel van “gewone” fibroadenomen bleek polyclonaal. Hyperplasie in fibroadenomen was tevens polyclonaal. Als eersten toonden wij aan dat de gebieden van ogenschijnlijke stromale progressie in fibroadenomen monoclonaal zijn. Dit suggereert inderdaad progressie naar phyllodes tumor. Zoals verwacht was CIS monoclonaal. De stromale component van phyllodes tumoren was meestal monoclonaal, wat te verwachten valt aangezien dit de component is die metastaseert. Opvallend was dat het stroma van enkele benigne en borderline phyllodes tumoren polyclonaal bleek te zijn. Er lijkt daarom sprake van clonale evolutie (van polyclonaal naar monoclonaal met hogere graad) in de progressie van phyllodes tumoren. Wij leveren moleculair bewijs voor de hypothese dat phylloid-achtige stromale expansie in fibroadenomen een vroege uiting is van tumorprogressie. Wij tonen daarom voor eerste maal aan dat fibroadenomen naast epitheliale progressie tot CIS ook stromale progressie tot phyllodes tumoren kunnen vertonen.

Voortgang door de celcyclus wordt gereguleerd door cyclinen, cycline-afhankelijke kinasen (CDK) en CDK remmers. Verstoring van één of meer componenten van dit systeem kan leiden tot ongecontroleerde voortgang door de celcyclus en snelle celgroei. De meest uitgebreid bestudeerde celcyclus “pathways” zijn de apoptose controlerende p53-route en de proliferatie controlerende pRb-route. In **hoofdstuk 6** bestudeerden wij de rol en prognostische waarde van verschillende vertegenwoordigers van deze belangrijke celcyclus controle systemen in phyllodes tumoren. Naast primaire tumoren includeerden wij ook enkele bijbehorende recidief tumoren. Een toename van celcyclus deregulatie in de stromale component werd gevonden met hogere tumorgraad. Stromale overexpressie van $p16^{INK4A}$, $p21^{waf1}$,

cycline A, p53, p21 en pRb waren geassocieerd met hogere tumorgraad. Het aantal afwijkende celcyclus eiwitten in de stromale component bleek eveneens toe te nemen met tumorgraad. Cycline A expressie in stroma was de beste onderscheidende factor tussen tumorgraden en zou de conventionele gradering kunnen verbeteren. Cycline A, p53, Ki67 en pRb expressie, aantal afwijkende celcyclus eiwitten, tumorgraad en tumorgrootte bleken allen voorspellend voor recidief. Echter, het aantal afwijkende eiwitten in stroma en p53 overexpressie in stroma waren de enige onafhankelijke voorspellers van recidief. In overstemming met de onopvallende microscopische verschijning, liet het epitheel geen afwijkende expressie van celcyclus eiwitten zien. Als behandeling van eerste keuze van phyllodes tumoren wordt ruime excisie aangeraden. Het feit dat er geen verschillen werden gevonden in de expressie van celcyclus regulatoren tussen primaire en recidief tumoren ondersteunt dit. Echter, de status van de snijvlakken had in onze groep geen prognostische waarde. In overeenkomst met de theorie dat meerdere afwijkingen in één celcyclus controle route geen extra groeivoordeel voor een tumorcel geven, vonden wij geen relatie tussen afwijkende expressie van eiwitten uit dezelfde route. Wel toonden wij significante relaties aan tussen stromale overexpressie van $p16^{INK4A}$ en $p21^{waf1}$, $p16^{INK4A}$ en p53, en p53 en pRb waardoor beide celcyclus controle routes onklaar worden gemaakt. Deze resultaten wijzen op de belangrijke rol van p53 en andere celcyclus regulatoren in progressie van phyllodes tumoren. De progressieve dedifferentiatie van de stromale component met hogere tumorgraad wordt weerspiegeld in de mate van celcyclus verstoring. Cycline A expressie kan een toegevoegde waarde zijn bij nauwkeurige gradering van phyllodes tumoren. Gradering lijkt echter niet de beste manier om prognose te voorspellen. Vergeleken met klassieke voorspellende factoren, zoals tumorgraad, radicaliteit en proliferatiemarkers, lijkt p53 expressie van superieure prognostische waarde. Celcyclus afwijkingen spelen geen rol in de epitheliale component. In het stroma, daarentegen, is het mogelijk een drijvende kracht achter progressie van deze component.

Zonder nieuwe bloedvaten te vormen kan een tumor kan slechts enkele mm^3 groot worden. Deze zijn noodzakelijk om celproliferatie op een hoger niveau te laten verlopen als apoptose (celdood). Hypoxie induceerbare factor 1α (HIF-1α) heeft een centrale rol in dit proces en activeert genen die betrokken zijn bij angiogenese (vaatnieuwvorming), glycolyse (afbraak van glucose) en apoptose. In **hoofdstuk 7** wordt de relatie tussen angiogenese en proliferatie in fibroepitheliale tumoren beschreven. In een groep van 37 primaire phyllodes tumoren en 30 fibroadenomen werd de expressie van HIF-1α en doelgenen CAIX en VEGF onderzocht middels immunohistochemie. De expressie niveaus werden gerelateerd aan aantallen microscopische bloedvaatjes, proliferatie en clinicopathologische parameters. In het merendeel van fibroadenomen werd geen expressie van HIF-1α en CAIX gezien. In

slechts 2/30 fibroadenomen was enige HIF-1α reactiviteit te vinden. HIF-1α expressie in phyllodes tumoren was gecorreleerd met tumorgraad, proliferatie, p53 accumulatie en aantallen bloedvaatjes. Opvallend was dat HIF-1α overexpressie gezien werd in alle maligne phyllodes tumoren. Necrose en CAIX expressie waren zeldzaam in de stromale component van phyllodes tumoren. In morfologisch normaal epitheel van phyllodes tumoren werd frequent gecombineerde CAIX en HIF-1α expressie gezien. Door het meten van de afstand van epitheel tot dichtstbijzijnde bloedvaten werd aannemelijk gemaakt dat er meest waarschijnlijk sprake is van mild zuurstoftekort in het epitheel door achterblijvende peri-epitheliale vaatgroei. Het lijkt daarom een fysiologische aanpassing zonder verdere tumorbiologische betekenis. Epitheliale HIF-1α expressie had inderdaad geen prognostische waarde. Toch kan paracriene stimulatie van de stromale component door HIF-1α geïnduceerde stoffen uit het epitheel niet geheel worden uitgesloten. Er was geen verschil tussen fibroadenomen en phyllodes tumoren wat betreft aantallen bloedvaatjes en VEGF expressie. HIF-1α overexpressie in stroma van phyllodes tumoren bleek voorspellend voor ziekte vrije overleving. HIF-1α heeft prognostische waarde in phyllodes tumoren en speelt mogelijk een belangrijke rol in tumorprogressie. Gezien de afwezigheid van necrose en CAIX expressie is HIF-1α overexpressie in phyllodes tumoren meest waarschijnlijk normoxisch and wordt het wellicht veroorzaakt door veranderde expressie van oncogenen, tumorsuppressor genen of groeifactoren. De significante relatie tussen p53 accumulatie en HIF-1α overexpressie maakt p53 een mogelijke kandidaat hiervoor. Er zijn echter vele factoren die interactie met HIF-1α vertonen en de mechanismen achter de vermoedelijke non-hypoxische HIF-1α overexpressie in phyllodes tumoren blijven vooralsnog onduidelijk. Een benadering met technieken die verschillende elementen op grote schaal inventariseren, zoals expressie microarrays, zou mogelijk de onderliggende complexe mechanismen kunnen blootleggen. Mogelijke paracriene effecten van epitheliale HIF-1α overexpressie op stroma zou men op deze manier tevens kunnen aantonen. Immunohistochemische bepaling van groeifactoren in phyllodes tumoren met HIF-1α negatief en positief epitheel zou een alternatieve benadering kunnen zijn. Op deze manier zou men tevens eventuele epitheliale-stromale interactie kunnen onderzoeken, wat als algemeen verschijnsel in groeiende belangstelling staat.

Tumor ontwikkeling wordt geïnitieerd door veranderingen in de functie van genen die direct betrokken zijn bij celdood en celgroei. Één van de mechanismen die genexpressie beïnvloeden is kopie-aantal verandering van chromosomen en chromosoomgebieden. Een toename van kopie-aantal van een chromosoomgebied noemt men een gain, een verlies van kopie-aantal een deletie. Met de recent ontwikkelde array CGH techniek is het mogelijk om in één experiment het gehele genoom te screenen op chromosoomafwijkingen en de afwijkingen direct te relateren aan de sequentie van het menselijke genoom. In **hoofdstuk 8** gebruiken wij array

CGH om chromosoomveranderingen in fibroadenomen en phyllodes tumoren te bestuderen. Arrays opgebouwd uit 2464 genomische clones met een resolutie van 1.4Mb werden gebruikt. In fibroadenomen werden geen chromosoomafwijkingen gevonden, zelfs afwijkingen van één clone waren zeldzaam. In phyllodes tumoren daarentegen vonden wij genetische instabiliteit in praktisch alle tumoren (91%). Een gemiddelde van 5.3 (bereik 0-16) chromosomale afwijkingen [2.0 gains (0-10) en 2.8 deleties (0-7)] werd gevonden per tumor. Amplificaties waren zeldzaam. Verlies van genomisch materiaal werd gezien op chromosomen 1q, 4p, 10, 13q, 15q, 16, 17p, 19 en X. Toename van DNA kopie aantal werd gezien op 1q, 2p, 3q, 7p, 8q, 16q, 20. Chromosomale veranderingen waren niet gerelateerd aan tumorgraad. Hoewel sommige veranderingen alleen in maligne tumoren werden gezien, was geen enkele afwijking specifiek voor een bepaalde tumorgraad. Door de superieure resolutie van array CGH beschreven wij sommige veranderingen voor de eerste maal. In sommige gebieden van chromosoomafwijkingen zijn bekende kanker gerelateerde genen gelokaliseerd, zoals *TP53* en verschillende leden van de *Cadherine* familie. Hoewel niet geassocieerd met tumorgraad, lijkt het erop dat genomische instabiliteit een rol speelt bij de ontwikkeling van phyllodes tumoren. Bij voorkeur zullen deze resultaten moeten worden bevestigd in een grotere groep. Gezien de lage incidentie van phyllodes tumoren zal dit echter moeilijk zijn. In fibroepitheliale tumoren is de epitheliale component vaak slechts twee cellagen dik. Dit maakt het praktisch onmogelijk om uit deze component voldoende DNA te oogsten. Onze resultaten betreffen derhalve de stromale component. Met verdere optimalisatie van amplificatietechnieken zal het mogelijk worden array CGH van het epitheel te verrichten. Gezien de controverse rondom de mogelijke rol van de epitheliale component zou dit erg interessant zijn. Uiteindelijk bepaalt de invloed van gendosis op genexpressie het effect op tumorprogressie. Het vergelijken van gendosis zoals verkregen met array CGH met array-gebaseerde expressiedata in dezelfde groep tumoren lijkt daarom een zinvolle volgende stap.

Recent werd aangetoond dat overexpressie van de epidermale groei factor receptor (EGFR) in borstkanker geassocieerd is met de lengte van een polymorphe CA repeat welke is gelokaliseerd in de eerste 2000 baseparen van intron 1 van *EGFR*. Tevens werd duidelijk dat amplificatie van de intron 1 CA repeat een mechanisme van EGFR overexpressie is. In **hoofdstuk 9** bestudeerden wij de relatie tussen EGFR expressie, *EGFR* genamplificatie en amplificatie status van de CA repeat in intron 1 in phyllodes tumoren en fibroadenomen. Middels weefsel-microarrays werden expressieniveaus van verschillende celcyclus markers bepaald. EGFR immunoreactiviteit in stroma werd gezien in 19% van phyllodes tumoren (75% van maligne tumoren). Genamplificatie zoals bepaald middels FISH werd aangetoond in 15.8% van stromale cellen en niet in epitheliale cellen. Intron 1 repeat amplificaties werden bepaald met gendosis PCR en konden worden aangetoond in

42% van alle phyllodes tumoren. 82% van de tumoren met EGFR overexpressie lieten amplificatie van het gen zien. EGFR expressie of genamplificatie werd niet gezien in de epitheliale component. Tumorgraad was significant gerelateerd aan EGFR overexpressie ($p=0.001$) en intron 1 repeat amplificatie ($p<0.05$). Verder bleek dat EGFR overexpressie gecorreleerd was met overexpressie van p53, p16, cyclin A, cyclin E, Ki67 en c-kit. Er was een significante relatie tussen intron 1 repeat amplificatie en p16 ($p<0.01$), p21 ($p=0.009$) en p53 ($p<0.001$) immunoreactiviteit. EGFR overexpressie en amplificatie van het hele gen werd niet gezien in 167 fibroadenomen en slechts in één tumor (1/43=2.3%) werd intron 1 repeat amplificatie gevonden. Dit alles toont aan dat activerende mutaties in en overexpressie van *EGFR* betrokken zijn bij de progressie in tumorgraad van phyllodes tumoren. Zoals afwijkende expressie van celcyclus regulatoren (hoofdstuk 6) is overexpressie en amplificatie van *EGFR* een eigenschap van de stromale component van phyllodes tumoren. *EGFR* amplificatie en overexpressie wordt niet gezien in fibroadenomen en is dus geen gemeenschappelijk kenmerk van bifasische tumoren. EGFR expressie zou dus een doorslaggevende factor kunnen zijn bij diagnostische problemen.

Het ontstaan en progressie van tumoren wordt voortgedreven door een grote hoeveelheid genetische en epigenetische veranderingen die resulteren in veranderde genexpressie. Het “Human Genome Project” heeft gedetaileerde informatie over alle menselijke genen opgeleverd. Hierop voortbordurend zijn verschillende moleculaire screenings technieken ontwikkeld die tegelijkertijd een groot aantal elementen kunnen inventariseren. DNA microarrays kunnen gelijktijdig de expressie van duizenden genen bepalen. In **hoofdstuk 10** bepaalden wij de expressieprofielen van fibroadenomen en phyllodes tumoren. Wij vonden vele nieuwe genen die een rol zouden kunnen spelen bij het ontstaan of de progressie van fibroepitheliale tumoren. De expressie van *IGF-2* was sterk verhoogd in fibroadenomen vergeleken met normaal weefsel. Onder de genen wiens expressie het meest was verlaagd in fibroadenomen bevonden zich een aantal genen die betrokken zijn bij de binding en het metabolisme van steroïden en galactose metabolisme. Vergeleken met normaal weefsel, was er in phyllodes tumoren sprake van een veranderde expressie van genen die betrokken zijn bij DNA reparatie, celadhesie, transcriptie en groeifactoren. Uit de directe vergelijking van phyllodes tumoren met fibroadenomen bleek dat 26 genen verhoogd en 52 genen verlaagd tot expressie komen in phyllodes tumoren. Een verlaagde expressie werd gezien van genen die betrokken zijn bij transcriptie, celadhesie, apoptose en de Wnt “pathway”. Verhoogde expressie van verschillende genen op het gebied van transcriptie, celstructuur en stromadesintegratie werd gedetecteerd in phyllodes tumoren. *CTAG1*, een gen met onbekende functie, werd 50-maal zo sterk tot expressie gebracht in phyllodes tumoren in vergelijking met fibroadenomen. Dit grote verschil zou kunnen betekenen dat *CTAG1* een nuttige diagnostische marker zou kunnen zijn. Er werd een verminderde expressie van *c-kit*

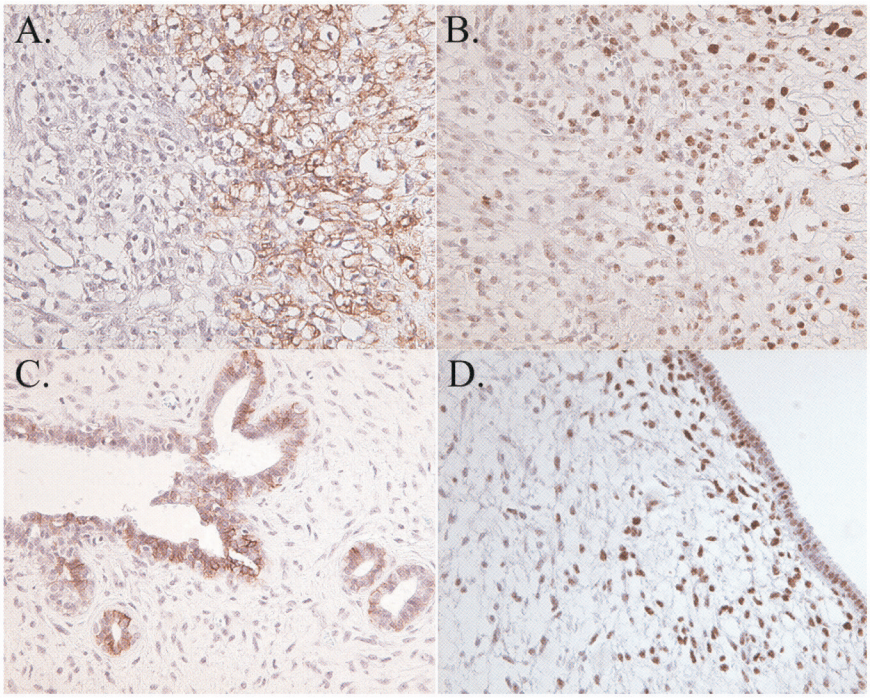
en *CXCR4* gezien in phyllodes tumoren. Expressie van beide genen werd middels immunohistochemie op weefselmicroarrays verder onderzocht. Het lijkt erop dat de verminderde expressie van *c-kit* met name wordt bepaald door de verminderde hoeveelheid epitheel in phyllodes tumoren. *CXCR4* lijkt in het epitheel inderdaad verminderd tot expressie te komen in phyllodes tumoren. Dit is de eerste studie waarin expressieprofielen van phyllodes tumoren en fibroadenomen worden vergeleken. We vonden vele nieuwe genen die mogelijk betrokken zijn bij ontwikkeling en progressie van fibroepitheliale tumoren. Verschillende genen zouden diagnostische waarde kunnen hebben. Een aantal interessante kandidaten worden verder onderzocht middels immunohistochemie op weefselmicroarrays.

Beknopte conclusies

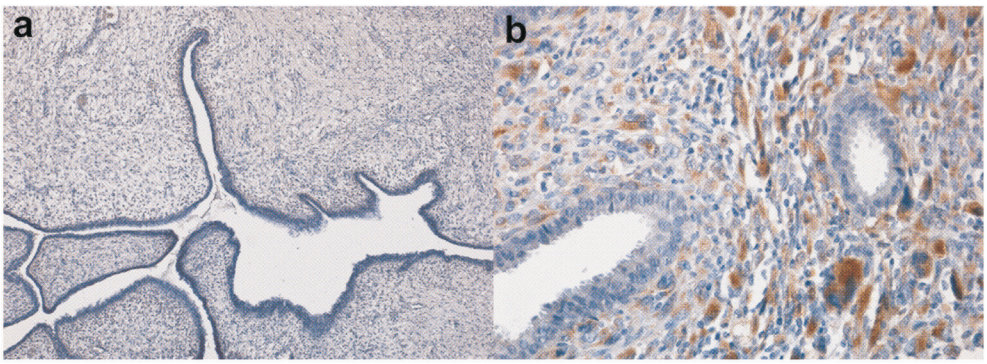
1. Omdat maligne transformatie van het epitheel van fibroadenomen niet wordt gezien of hooguit extreem zeldzaam is bij vrouwen jonger dan 35 jaar, moeten fibroadenomen bij vrouwen boven deze leeftijd worden verwijderd.
2. Alle borstlesies bij vrouwen met een positieve familie anamnese voor borstkanker moeten histologisch worden beoordeeld.
3. Clonaliteitsanalyse toont aan dat goedaardige fibroadenomen progressie tot de onvoorspelbare phyllodes tumor kunnen vertonen.
4. Celcyclus deregulatie is een drijvende kracht achter stromale progressie in phyllodes tumoren. In fibroadenomen daarentegen worden geen afwijkende celcyclus proteïnen gevonden.
5. Stratificatie volgens p53 expressie is mogelijk superieur aan conventionele gradering wat betreft prognostische waarde.
6. In tegenstelling tot fibroadenomen, wordt genetische instabiliteit in het merendeel van phyllodes tumoren gevonden.
7. HIF-1α expressie heeft prognostische waarde in phyllodes tumoren en speelt mogelijk een belangrijke rol in het aanpassingsproces van groeiende tumoren.
8. Activerende mutaties in en overexpressie van *EGFR* zijn geen eigenschap van fibroadenomen maar zijn wel geassocieerd met progressie van phyllodes tumoren.
9. Dit proefschrift bevat geen data die pleiten voor een actieve rol van de epitheliale component in de pathogenese van fibroepitheliale tumoren.
10. Hoewel fibroadenomen en phyllodes tumoren microscopisch op elkaar kunnen lijken, zijn deze tumoren moleculair gezien verschillende entiteiten.

Color plates

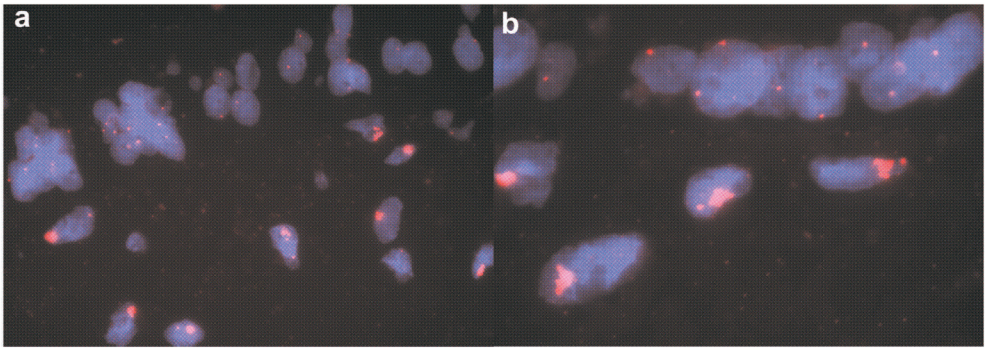
Chapter 7, Figure 1. Examples of immunostaining for HIF-1α and CAIX in breast phyllodes tumors. Malignant phyllodes tumor with stromal CAIX expression (A); Same tumor as A with topographically overlapping HIF-1α overexpression (B); Benign phyllodes tumor with CAIX positive staining epithelium (C); Borderline phyllodes tumor with HIF-1α overexpression in normal appearing epithelium and in subepithelial stroma (D).



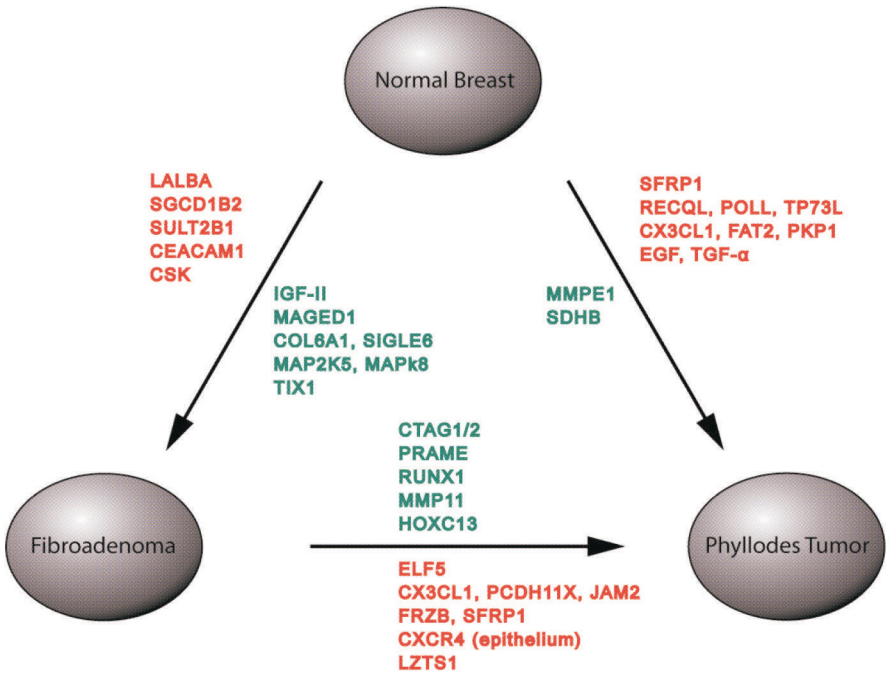
Chapter 9, Figure 1. Photomicrographs of a phyllodes tumor without (a) and with EGFR immunopositivity (b). Noteworthy, the epithelial cell compartment lacks EGFR immunoreactivity (a 10x, b 20x).



Chapter 9, Figure 2. Fluorescence images of two cases of malignant phyllodes tumor showing stromal tumor cells with *EGFR* whole gene amplifications below a line of epithelial cells with regular number of gene copies (a 20x, b 40x).



Chapter 10, Figure 1. Most important genes with altered expression in proposed model of fibroepithelial tumor genesis. Downregulated genes are shown in red, upregulated genes in green.



Curriculum vitae

Arno Kuijper werd geboren op 13 augustus 1975 in Leiden. Hij voltooide het VWO aan het Bisschop Bekkers College in Eindhoven. Na 1 jaar Geneeskunde te hebben gestudeerd aan het Rijks Universitair Centrum Antwerpen, België, startte hij in 1995 met dezelfde studie aan de Vrije Universiteit Amsterdam. Naast zijn studie werkte hij op het MS-MRI centrum van het VUMC, waar hij in het kader van verschillende onderzoeken laesies in het centraal zenuwstelsel van MS patiënten kwantificeerde. Tijdens zijn doctoraal fase begon hij met het hier beschreven onderzoek onder leiding van prof PJ van Diest en prof E van der Wall. Na zijn doctoraalfase, die cum laude werd afgesloten, richtte hij zich ruim een jaar fulltime op dit onderzoek. Ook verrichtte hij hiervoor korte researchstages op de afdelingen Pathologie, Universiteit Münster, Duitsland (hoofd: prof W Boecker) en Laboratory Medicine, University of California, San Francisco, USA (hoofd: prof DG Albertson). Na zijn co-schappen, die eveneens cum laude werden afgesloten, verbleef hij wederom voor een half jaar op het vertrouwde lab. Het toekennen van de Nijbakker-Morra studenten prijs in 2002 stelde hem in staat een groot internationaal congres te bezoeken. In 2003 begon hij met de opleiding tot internist in het St Elisabeth ziekenhuis Tilburg (dr C van der Heul, prof KML Leunissen).

Dankwoord

Aan een buitenstaander is het moeilijk uit te leggen wat promoveren precies inhoudt. Ik hoop dat het inzien (lezen is misschien te veel gevraagd) van mijn proefschrift enige duidelijkheid biedt. Misschien niet op wetenschappelijk-inhoudelijk gebied maar wel op de inspanning die het heeft gevraagd. Hoewel ik (bijna) altijd met plezier aan mijn proefschrift heb gewerkt, is het wel zo dat de blikvernauwing, die vooral in de laatste maanden toesloeg, zijn weerslag heeft gehad op de overige aandachtsgebieden in het leven. Die schade wordt ingehaald in de komende tijd!

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Arno