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.

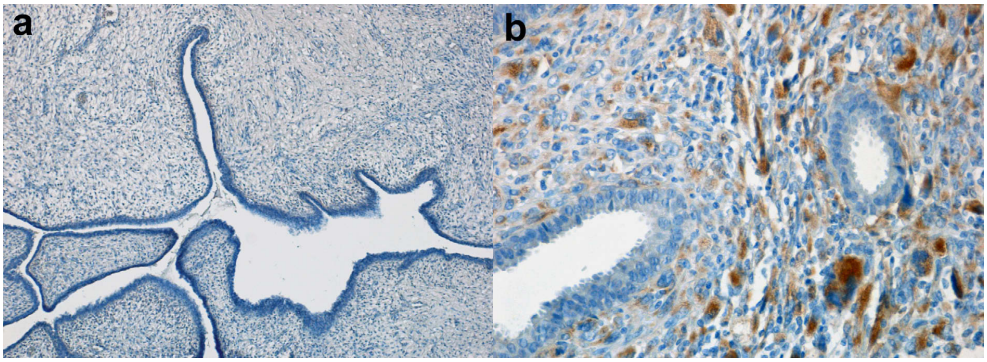


Table 2. EGFR overexpression and *EGFR* amplification in breast phyllodes tumors of different grade.

	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.

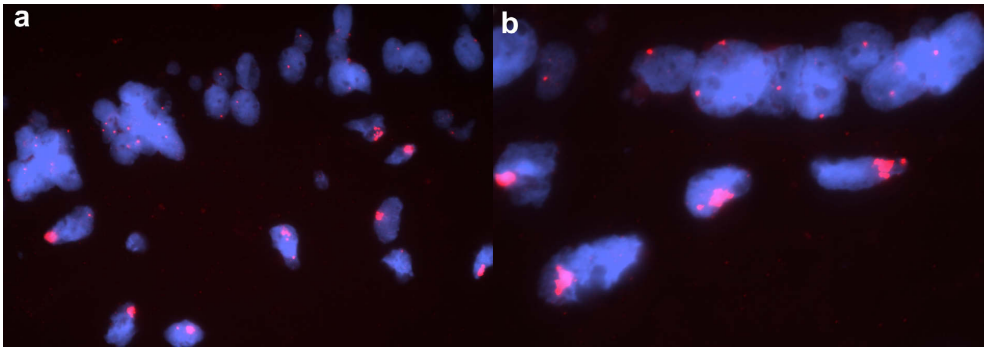
FISH analysis

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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