

Egfr amplification specific gene expression in phyllodes tumours of the breast

Konstantin Agelopoulos^a, Christian Kersting^a, Eberhard Korsching^a, Hartmut Schmidt^a, Arno Kuijper^b, Christian August^a, Pia Wülfing^c, Joke Tio^c, Werner Boecker^a, Paul J. van Diest^b, Burkhard Brandt^d and Horst Buerger^{a,e,*}

^a *Institute of Pathology, University of Muenster, Muenster, Germany*

^b *Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands*

^c *Department of Obstetrics and Gynecology, University of Muenster, Muenster, Germany*

^d *Institute of Tumour Biology, University of Hamburg, Germany*

^e *Institute of Pathology, Paderborn, Germany*

Abstract. *Background:* Recently, we were able to show that amplifications of the epidermal growth factor receptor (*egfr*) gene and the overexpression of EGFR were associated with the initiation and progression of phyllodes tumours. *Methods:* In order to gain further insights into regulation mechanisms associated with *egfr* amplifications and EGFR expression in phyllodes tumours, we performed global gene expression analysis (Affymetrix A133.2) on a series of 10 phyllodes tumours, of these three with and seven without amplifications of an important regulatory repeat in intron 1 of *egfr* (CA-SSR I). The results were verified and extended by means of immunohistochemistry using the tissue microarray method on an extensively characterized series of 58 phyllodes tumours with antibodies against caveolin-1, eps15, EGF, TGF- α , pErk, pAkt and mdm2. *Results:* We were able to show that the presence of *egfr* CA-SSR I amplifications in phyllodes tumours was associated with 230 differentially expressed genes. Caveolin-1 and eps15, involved in EGFR turnover and signalling, were regulated differentially on the RNA and protein level proportionally to *egfr* gene dosage. Further immunohistochemical analysis revealed that the expression of caveolin-1 and eps15 were also significantly correlated with the expression of pAkt ($p < 0.05$), pERK ($p < 0.05$), mdm2 ($p < 0.01$) and EGF ($p < 0.001$ for caveolin-1). Eps15 and pERK were further associated with tumour grade ($p < 0.01$ and $p < 0.001$, respectively). *Conclusion:* Our results show that amplifications within regulatory sequences of *egfr* are associated with the expression of eps15 and caveolin-1, indicating an increased turnover of EGFR. The interplay between EGFR and caveolin-1, eps15, pAkt, mdm2 and pERK therefore seems to present a major molecular pathway in carcinogenesis and progression of breast phyllodes tumours.

Keywords: Phyllodes tumours, *egfr*, caveolin-1, eps15

1. Introduction

Phyllodes tumours (PT) of the breast are rare biphasic tumours which account for less than 1% of all breast tumours. Their pathogenesis and underlying genetic alterations have been poorly understood. Recently, we were able to show that overexpression of the Epidermal Growth Factor Receptor (EGFR) was correlated with the progression of these tumours. Furthermore, EGFR overexpression was strongly associated with whole gene amplifications of *egfr* gene as well as with amplifications within intron 1 of *egfr*, the lat-

ter containing important regulatory sequences for the transcription of the gene (CA-SSR I) [9]. These results were largely in line with previous results from the literature for other proteins and provided for the first time a more comprehensive idea about *egfr* biology of these tumours [2–4]. Since genetic alterations of *egfr*, which are associated with overexpression of its protein, are among the first recurrent molecular alterations described in phyllodes tumours, amplifications of *egfr* may play a role in the initiation and progression of a large subset of phyllodes tumours. Amplifications of CA-SSR I have also repeatedly been described in invasive breast cancer and were similarly correlated with EGFR overexpression [3,31]. Nevertheless, the affected intracellular pathways associated with these mutations could not be defined so far.

*Corresponding author: Horst Buerger, MD, PhD, Professor of Pathology, Institute of Pathology, University of Münster, Albert-Schweitzer-Str. 33, 48149 Münster, Germany. Tel.: +49 5251 8700523; Fax: +49 5251 8700529; E-mail: buerger@histopatho.eu

In this study we aimed to define intracellular relationships and possible pathways downstream of *egfr* by means of global gene chip analysis in a series of PT, characterized for their *egfr* CA-SSR I amplification status. This was followed by the immunohistochemical verification of candidate genes in a tissue microarray approach. Our findings provide more insight into putative regulation pathways associated with *egfr* amplifications in PT of the breast and stress the importance of *egfr* as a driving force in the initiation and progression of these intriguing tumours.

2. Materials and methods

2.1. Materials

Fresh frozen tumour tissue of 10 PT taken from the archives of the Departments of Pathology, Universities of Muenster and Utrecht, were used for gene chip analysis. These consisted of 5 benign, 3 borderline and 2 malignant tumours according to the criteria of Moffat et al. [21]. Scoring of tumour grade as well as immunohistochemical scoring was performed independently by two pathologists (C.K and H.B.). Cases with disagreement were scored jointly using a double-headed microscope.

All 10 tumours were evaluated for the expression of EGFR on fresh frozen tissue, since sufficient paraffin-embedded tumour material was not for all available anymore. All ten tumours showed positive EGFR immunoreactivity within the stromal and the myoepithelium compartments. The glandular epithelium was consistently negative. Paraffin blocks of 58 PT of the female breast were retrieved from the archives 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 use of tumour tissue was also approved by the local ethical committee.

All tumours were characterized for their *egfr* gene dosage status by means of quantitative real-time PCR and FISH analysis. In addition, expression of EGFR, p16, p21, p27, p53, c-myc, Cyclin A, Cyclin D1, Cyclin E, c-kit and Ki-67 was studied by immunohistochemistry as previously described [8,9,35].

2.2. mRNA expression microarray

Total RNA (tRNA) was isolated from each of the samples using the RNeasy Mini Kit (Qiagen GmbH) according to the manufacturer's instructions. Quantity and purity (260/280 nm ratio) was determined using a BioPhotometer (Eppendorf, Germany). Quality control of the isolated tRNA was done on an automated electrophoresis system (BIORAD Experion Bioanalyser, USA) using RNA StdSens Chips. With this LabChip microfluidic system, the 28S/18S ratio was calculated and only tRNA without degraded amounts of tRNA was used in this study.

1 µg of RNA per sample was introduced in the process. If the concentration of the RNA was lower than 1 µg/8 µl an enrichment step was applied (Microcon filter, Millipore). All further steps were strictly based on the Affymetrix protocol and the Affymetrix kits. The cDNA synthesis was done with the 'One-Cycle' process. cDNA clean up, *in vitro* transcription and cRNA clean up were performed with the respective kits/procedures provided by Affymetrix. The quantity of the biotin-labeled cRNA was checked by measuring the 260/320 nm ratio. Hybridization and scanning were performed individually for each of the samples on the basis of the oligonucleotide microarray HG U133Av2 (22277 probe sets, Affymetrix) according to the manufacturer's protocols.

2.3. Microarray analysis

GeneChip® Operating Software (GCOS, Affymetrix) version 1.4 was used to evaluate the raw data (for GCOS algorithms see Affymetrix: Statistical Algorithms Description Document – [sadd_whitepaper.pdf](#)). Signal values were exported from GCOS and imported into the statistics software package S-Plus 6.2. In this environment the different experiments were scaled to the same interquartile range and median. The resulting new experimental reference parameters were strictly comparable concerning the median and the spread of the data. The significant gene expression differences between the defined experimental groups were evaluated using *t*-test (two-sample, two-sided, variance equal, $\alpha < 0.05$). The additional filter criteria to call a gene "differentially expressed" were an *F* test *p*-value < 0.05 , a fold change value > 1.5 and a difference of the group means > 10 .

2.4. Tissue microarray

A tissue microarray of 58 phyllodes tumours of the breast with six cores of 0.6 mm in diameter were punched out of the donor block according to standard protocols using a dedicated TMA instrument (Beecher Instruments, Silver Spring, Maryland, USA) [13,23] and transferred to a recipient block as previously described [32].

2.5. Immunohistochemistry

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. Only the immunohistochemical results of the stromal part (dominating phyllodes tumours) were used for detailed analysis. Staining intensities were graded semiquantitatively from 0 (weak) to 3 (strong) using positive controls recommended by the antibody-supplier.

2.6. 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).

3. Results

3.1. Gene chip analysis

From all tumours sufficient high quality RNA could be isolated. All tumours presented unaltered copy numbers for the *egfr* gene by means of FISH analyses. Three tumours (1 malignant; 2 benign) displayed amplifications within intron 1 of *egfr*, seven tumours (1 malignant; 3 borderline; 3 benign) revealed a normal gene dosage for this specific locus using quantitative real time PCR. 213 genes were upregulated and 17 genes were downregulated in PT with CA-SSR I amplifications at a significance level of 0.05 with multiple *t*-testing (Table 2). The fold change value was set to a moderate level of greater 1.5 fold. The resulting number of differentially regulated genes is in accordance with the applied constraints. Numerous genes were found involved either in nucleic acid metabolism ($n = 42$) or signal transduction ($n = 40$). Further, twelve genes were found to be directly involved in receptor tyrosine kinase signalling pathways as shown in Table 3.

3.2. Immunohistochemical verification of differentially regulated genes

Due to lack of sufficient paraffin embedded material the samples used for expression profiling were not included in the tissue microarray. In the used cases amplifications of CA-SSR I were found in 15/38 benign, 2/9 borderline and 6/8 malignant phyllodes tumours.

Differential regulation of genes, all known to be associated with EGFR signalling turnover and regulation, were verified on the protein level: caveolin-1, eps15, pAkt, mdm2, pERK, EGF and TGF- α . Representative images are shown in Fig. 1. Overviews about all significant correlations and about staining frequencies are given in Tables 4 and 5, respectively.

Table 1
Overview of all antibodies used in this study, including source, pretreatment, dilution and detection systems

Antibody	Source	Clone	Pretreatment	Dilution
Caveolin-1	BD Biosciences	2297	30' steamer citrate buffer pH 6.0	1:200
eps15	Santa Cruz	rabbitt polyclonal	–	1:200
pAkt	Cell Signaling Technology	rabbitt polyclonal	30' steamer citrate buffer pH 6.0	1:200
pERK	Cell Signaling Technology	E 10	30' steamer citrate buffer pH 6.0	1:100
EGF	Acris	rabbit polyclonal	30' steamer TRS pH 9.0	1:20
TGF- α	Zyomed	MF9 +TG86	30' steamer citrate buffer pH 6.0	1:15
mdm2	Zyomed	IF 2	30' steamer TRS pH 9.0	1:50

Table 2

List of candidate genes with upregulation and downregulation (italics) in phyllodes tumours with amplifications including sequences of intron 1 of the *egfr* gene

Amino acid metabolism

GLUL; TDO2; MGC15523

Apoptosis

BNIP3; CLU; KIAA0367; CASP4; GADD45A

Carbohydrate metabolism

FLJ21865; PPP1R3C; ENO2; ME1; A4GALT; MAN2C1; SLC2A5

Cell adhesion

ITGB1BP1; LRRC15; PPFIBP1

Cell cycle

11-Sep; GAS2; RECK; TBCD

CDC2; CETN2

Cell structure, proliferation and differentiation

AHI1; ANK2; BST2; CDC42BPA; COL13A1; COL16A1; COL6A2; IFI44; NEF3; NEFL; PTDSR; SGCD

ACTB

Developmental processes

DVL3; IFRD1; LPIN1; MATN2; MBNL1; MEF2A; PKD2

ANXA2P2

Immunity and defense

C1QTNF3; CFH; CFH /// CFHL1; NCF2; ORM1; PACS2; TNFAIP2

Intracellular protein traffic

EPS15; EXOC7; GDI1; ITSN2; LRP1; MRC2; MYO1D; PHLDB1; PPIG; TGOLN2

Lipid, fatty acid and steroid metabolism

APOL1; LSS; PIK4CA /// LOC220

PSAP

Nucleoside, nucleotide and nucleic acid metabolism

ADARB1; ADNP; AKAP8L; ARHGEF10; BCL6; CNOT4; DICER1; DPYD; ERCC3; GATAD1; GUK1; HIST1H2BD; IRF3; KIAA0476; KIAA1466; LRRFIP1; MBD4; PLAG1; PTBP2; PTOV1; RBM6; RBPMS; SAFB2; SF1; SFRS11; SHOX2; SIN3B; SMARCC2; SMARCD3; SOX13; SRRM1; SYNE1; THRAP2; THRAP5; TRA2A; TWIST1; VGLL4; ZBTB1; ZIC1; ZNF160; ZNF202; ZNF500; ZNF516

SET; MGMT; RINGT; CPSF5

Oncogenesis

EPB41L1; MAGEA9; TPD52L2

Protein metabolism and modification

AAK1; CSNK1G2; CTSB; FASTK; HSPB7; IMP-3; LONP; MAST2; MKNK1; MMP14; NPEPL1; PAM; PTK2B; SERPINE1; SERPINI1; TAOK3; TTC15; TYK2; UBE2D2; USP53; WNK1

MRPS16; RPL5; YME1L1; OAZ2

Protein targeting and localization

AKAP10; GOLGA2; PDE4DIP; PPFIBP1 /// LOC44

Signal transduction

ADCY7; APBB3; C5R1; CAV1; CELSR1; CLEC7A; DDEF1; DDEF2; DDEF1L1; DLC1; DMPK; EPOR; FGFR1; FN1; GNA11; GPR153; IL6R; LAMA2; LAT; LEFTY2; LRP5; MAP2K3; MAPK8IP3; MERTK; MTSS1; PIK3R1; PKD1; PPAP2A; PRELP; RASAL2; RFNG; RGS1; ROCK2; SH2B; SHC1; SHC2; SN; STK24; TNFRSF25; TSPAN8

Transport

ABCF2; ATP2B4; KCNAB1; MSCP; SCN4A; SFXN3; SLC1A3

Table 2
(Continued)**Biological process unclassified**

ABI3BP; BGN /// SDCCAG33; CHCHD7; DKFZP434C171; DYM; EHP1L1; EHD2 /// RAB42; FAM13A1; FLJ10534; FLJ12788; FLJ13769; FLJ20507; FLJ43663; GOLPH4; IDS; KIAA0100; KIAA0265; KIAA1078; KIAA1536; KIAA1659; LOC399491; LOC51326; MUM1; PLEKHA5; RCK; REC14; RSAD1; SLC26A10; TAZ; TMEM45A; ZCWPW1; ZGPAT; ZNF638

C21orf33; FLJ10156; HBXIP; SPFH1

For all genes either a known or an inferred biological function was identified from Panther (www.pantherdb.org/genes), NETAFFX (www.affymetrix.com/analysis/netaffx/index.affx), OMIM (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM) and GO (www.geneontology.org) nomenclature.

Table 3

List of differentially expressed candidate genes involved in signal transduction with association to the EGFR pathway

Gene symbol	Chromosomal localization	Biological function
LAT	16p12.1	Ras protein signal transduction
RASAL2	12q23–q24	
C5R1	19q13.3–q13.4	MAPK cascade
MAP2K3	17q11.2	
FGFR1	8p11.2–p11.1	MAPKKK cascade
IL6R	1q21	
SH2B	16p12.1	
SHC1	1q21	
SHC2	1q21	
TNFRSF25	6p21.1–12.2	
CAV1	7q31.1	Receptor mediated endocytosis
EPS15	1p32	

All shown genes were upregulated in phyllodes tumours with amplifications including sequences of intron 1 of the *egfr* gene. For all genes either a known or an inferred biological function was identified from Panther, NETAFFX, OMIM and GO nomenclature.

Caveolin-1 was expressed in the cytoplasm of 9/53 (17%) PT. Staining was observed in both stromal and epithelial components. Caveolin-1 expression was positively correlated with the CA-SSR I gene dosage ($p < 0.05$), pAkt ($p < 0.01$), EGF ($p < 0.001$), mdm2 ($p < 0.01$), pERK ($p < 0.05$), eps15 ($p < 0.05$), c-kit ($p < 0.001$), p53 ($p < 0.01$), Ki-67 ($p < 0.01$) and Cyclin A ($p < 0.05$).

eps15 showed a positive cytoplasmic immunoreactivity in 10/51 (20%) PT. Staining was observed in both stromal and epithelial components. Eps15 was significantly correlated with pAkt ($p < 0.05$), mdm2 ($p < 0.01$), pERK ($p < 0.01$), c-kit ($p < 0.01$), p53 ($p < 0.001$), Cyclin A ($p < 0.05$) and tumour grade. A borderline statistical significance could be shown for the association between eps 15 and CA-SSR I gene dosage ($p = 0.05$).

pAkt revealed a positive immunostaining in the cytoplasm (5/55; 9%) of stromal and epithelial cells. Cytoplasmic staining of pAkt was positively associated with caveolin-1 and eps15 for EGF ($p < 0.01$), TGF- α

($p < 0.01$), and pERK ($p < 0.01$). Nuclear staining correlated significantly with EGFR expression ($p < 0.01$). A trend towards statistical significance could be seen for the correlation with mdm2 ($p = 0.05$).

pERK expression was sparse and could be seen in epithelium and stroma of 4 out of 56 cases (7%). Myoepithelial cells were mostly positive. Besides the above mentioned correlations, pERK expression was associated with TGF- α ($p < 0.01$), c-kit ($p < 0.05$), p53 ($p < 0.001$), Ki-67 ($p < 0.01$) and tumour grade ($p < 0.001$).

EGF was expressed in stromal cells of 10 out of 53 PT cases (19%), but was observed predominantly in the epithelial cell component. A significant positive correlation could be demonstrated for pAkt ($p < 0.05$), TGF- α ($p < 0.01$), c-kit ($p < 0.05$), Caveolin ($p = 0.001$) and Cyclin E ($p < 0.05$).

TGF- α expression was observed predominantly in epithelial tumour cells. Cytoplasmic staining in stromal cells was seen in 11 out of 54 cases (20%). TGF-

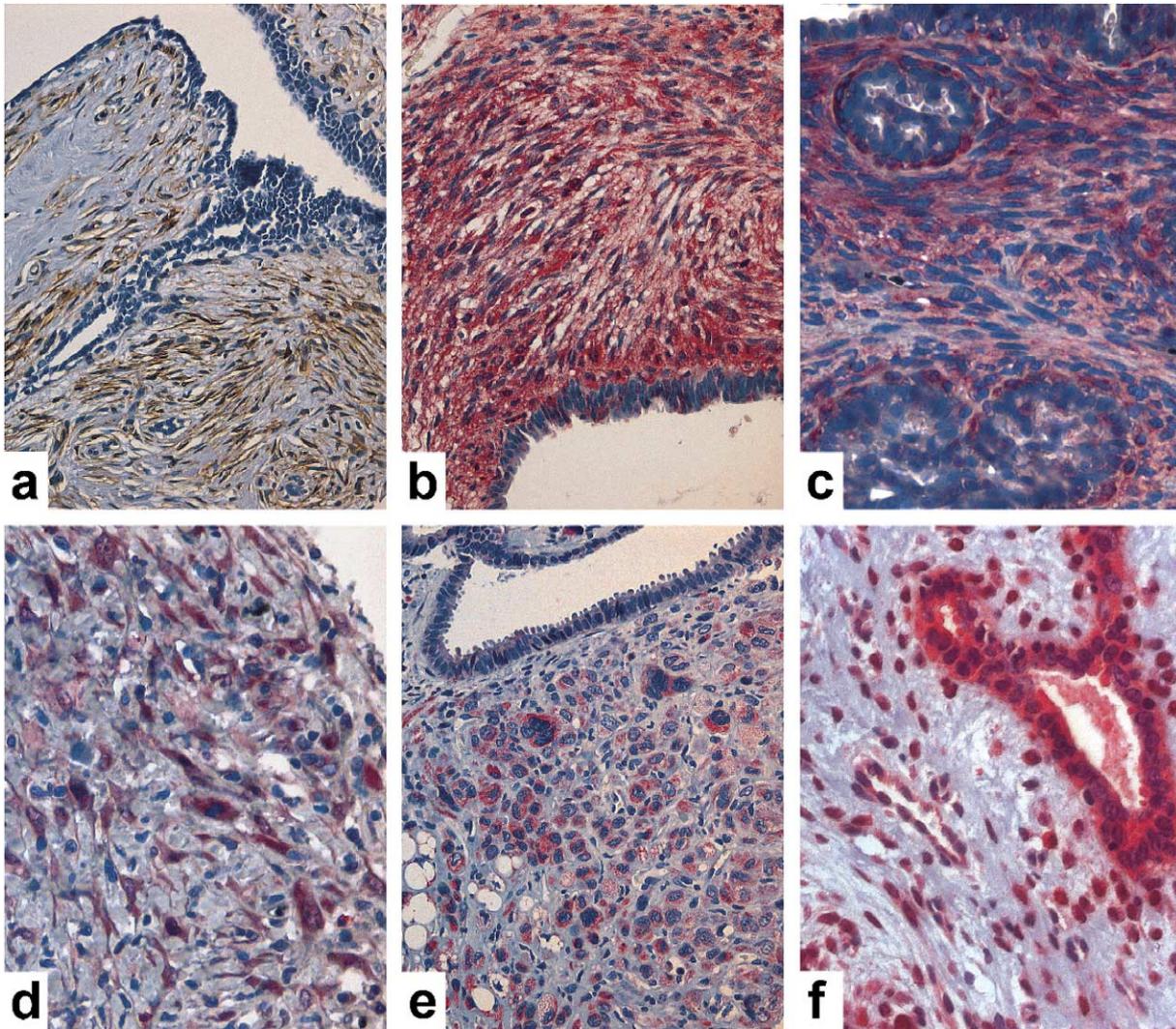


Fig. 1. Example of immunohistochemical staining patterns of caveolin-1 (a), eps15 (b), pERK (c), pAkt (d), EGF (e) and mdm2 (f) in phyllodes tumours of the breast.

α expression was further significantly associated with tumour grade ($p < 0.001$).

Nuclear **mdm2** expression was seen in 11 of 54 cases (20%), predominantly in the stromal component. **mdm2** expression in stromal cells was associated with expression of EGFR ($p < 0.05$), c-kit ($p < 0.01$), p53 ($p < 0.01$), Mib-1 ($p < 0.01$), p21 ($p < 0.001$), and Cyclin E ($p < 0.01$).

4. Discussion

Our own recently published study showed correlations between amplifications of *egfr* and EGFR over-

expression in breast PT [9]. However, these results, in context with other reports, raised important questions and urged the need for an improved understanding of receptor tyrosine kinase regulated pathways in these tumours [26,27]. Unfortunately, at the present state and to the best of our knowledge, no cellular models like PT cell lines are available for detailed functional research. In order to circumvent these problems and to improve the knowledge about these tumours, we performed global gene expression analysis on a series of 10 phyllodes tumours with known status of *egfr* CA-SSR I amplifications. We are aware that the number of tumours might be too small to draw definite conclusions, but one has to take into account the low in-

Table 4

Overview about the significance of found immunohistochemical correlations

	pAKT	Cav-1	eps15	pERK	MDM2	TGF- α	EGF
pAKT		0.002	0.020	0.001	0.001	0.001	0.009
Cav-1	0.002		0.001	0.048	0.003		0.001
eps15	0.020	0.001		0.002	0.009		
pERK	0.001	0.048	0.002			0.003	
MDM2	0.001	0.003	0.009				
TGF- α	0.001			0.003			0.006
EGF	0.009	0.001				0.006	
p53		0.003	0.000	0.000	0.010		
c-kit		0.000	0.003	0.010	0.004		0.044
Cyclin E					0.001		0.012
Cyclin A		0.019	0.036				
p21					0.006		
EGFR	0.001				0.011		
Ki-67		0.006		0.006	0.001		
CA-SSR I		0.022	0.050				
tumour grade			0.002	0.000		0.000	

Table 5

Overview about the frequencies of immunohistochemical staining of the investigated molecular markers

	Location of expression	Frequency of marker expression in stromal cells	
		Cytoplasm	Nucleus
Caveolin	stroma & epithelium	17% (9/53)	–
Eps15 (H896)	stroma & epithelium	19.6% (10/51)	–
pAKT	stroma & epithelium	8.6% (5/55)	10.9% (6/55)
pERK	stroma & epithelium	7.1% (4/56)	–
EGF	mostly epithelium	18.9% (10/53)	–
TGF- α	mostly epithelium	20.4% (11/54)	–
mdm2	mostly stromal cells	–	20.4% (11/54)

idence of these tumours [21] precluding large series with frozen tissue available. However, since the results were verified by means of immunohistochemistry on a tissue microarray containing a significantly larger series of PT, we think that the disadvantage of the relatively small number of tumours could be overcome. Our investigations revealed 213 upregulated and 17 downregulated genes in phyllodes tumours with am-

plifications within CA-SSR I of *egfr*. Most of them ($n = 47$) were found to be involved in ‘nucleosid, nucleotid and nucleic acid transport’, followed by ‘signal transduction’ ($n = 40$) and ‘protein metabolism and modification’ ($n = 25$). Candidate genes found in these as well as the other categories not mentioned here will be investigated by qRT-PCR as well as immunohistochemically. Of special interest for this study were twelve genes found to be associated with receptor tyrosine kinase pathways. Out of these, two genes were directly involved in EGFR signalling and turnover. Therefore we focussed the subsequent immunohistochemical analyses on molecules involved directly in the EGFR pathway. A higher expression of caveolin-1 and eps15 on the RNA level was seen predominantly in PT with amplifications in CA-SSR I. This association between *egfr* intron 1 gene dosage and caveolin-1 and eps15 expression could also be verified on the protein level. Caveolin-1, as a major structural protein of caveolae, is involved in the storage of EGFR molecules and the mediation of specific EGFR-signals in mesenchymal cells [33]. After EGF stimulation the content of EGFR in caveolae rapidly decreases [20]. This is followed in fibroblasts by an internalization of the receptor, mainly mediated by eps15 [4,29,30]. The transfer of these findings on the functional level therefore points to an increased activity and consequently turnover of EGFR in *egfr* amplified tumours. It is noteworthy that this especially holds true in PT with a similar EGFR expression. This is further supported by the significant correlation between EGF and TGF- α , as major ligands of EGFR, caveolin-1 as seen before [12], and a differential expression of 10 more genes (Table 3), all described to be involved in EGFR signalling at various stages of internalization and signalling. It is important to note that all previous studies on EGFR expression in PT, but also in other mesenchymal tumours [10], accented predominantly a cytoplasmic EGFR staining pattern.

Within recent years, the importance of caveolin-1 in tumour progression, especially in relation to *egfr* amplification and overexpression, has become obvious [12]. Caveolin-1 has been described as a major regulator of phosphorylated Akt (pAkt) through inhibition of distinct phosphatases [17]. Our tissue microarray results support these findings. pAkt expression was significantly associated with eps15, caveolin-1, EGF and TGF- α expression in this study. Since this change could not be observed in gene chip analysis it is likely that an increase of pAkt half-time, as previously reported, is responsible for this correlation. However, the

survival advantage mediated by pAkt might be seen on various stages. The pro-proliferate effect might be reflected in the positive correlation with pErk ($p < 0.01$), but anti-apoptotic effects have also been associated with pAkt [18]. Therefore, the interplay between EGFR, caveolin-1 and pAkt represents a reasonable and logical explanation for essential tumour biological features of a subset of PT. However, this model is furthermore able to integrate other previous findings on the overexpression of receptor tyrosine kinases in PT [7,34]. For example IGF-R and c-kit have also been shown to be involved in the progression of PT [26,27]. Especially for IGF-R, an interplay with caveolin-1 and Akt has been described in detail in other cellular models [24,25,28]. Therefore, one might speculate that the caveolin-1/pAkt – axis activated by a number of receptor tyrosine kinases might represent the central key element in the progression of PT. Even though metastases in PT are rare, the inhibition of this putative molecular “bottle neck” might be a therapeutic option for these clinically advanced tumours in the future [11]. Since a functional connection has also been described between pAkt and β -catenin [22], an increase of pAkt might further give an explanation for the association of β -catenin with the progression of PT [26].

In order to find additional support for our hypothesis, we investigated the expression status of mdm2 in PT. This was based on reports that mdm2 expression is mediated by growth factor stimulation and/or genetic alterations such as genetic amplifications [5,16] via Akt [2]. Our immunohistochemical results support this interpretation since we could show a significant correlation between nuclear mdm2 and pAkt ($p < 0.01$). The transfer of mdm2 into the nucleus is mainly due to phosphorylation by pAkt [19]. In addition, mdm2 expression was also associated with expression of EGFR, caveolin-1, eps15, p53 and p21. Taking into account the previous observation of a significant correlation between EGFR and p53 expression, the presumed pro-apoptotic effect of p53, due to the genetic instability reflected in *egfr* amplifications, may be strongly counterbalanced by mdm2 as negative regulator of p53 [6]. These results are in contrast with previous reports on mdm2 expression in PT [14]. However, from a biological point of view the expression of mdm2 in these tumours could further logically explain the expression of Hif-1 α in phyllodes tumours [1,15].

In summary, amplifications of *egfr* are associated with an increased turnover of EGFR and consequently an increase of pAkt, mdm2 pERK and p53 via caveolin-1 in breast phyllodes tumours. Since this path-

way has also been described for other growth factor receptors, it might be that this pathway represents a central, dominating pathway in phyllodes tumours and allows a unifying concept for the pathogenesis of phyllodes tumours.

Acknowledgements

We thank Ulrike Neubert and Petra Meier for help with the immunohistochemical staining and FISH analysis and Inka Buchroth for performing the gene chip analysis. We appreciated the contribution of tumour material from Christian Gustmann, Institute of Pathology, Limburg, Jens Packeisen, Institute of Pathology, Osnabrück and Bernd Hinrichs, Institute of Pathology, Köln-Rodenkirchen for the construction of the tissue microarray.

This work was supported by grants from the Peter- and-Traudl-Engelhornstiftung (K.A.), the IMF Münster (KE220507), the National Cancer Center, New York (H.B.) and the First Aegon International Scholarship in Oncology (A.K.).

References

- [1] J.I. Bardos and M. Ashcroft, Negative and positive regulation of HIF-1: a complex network, *Biochim. Biophys. Acta* **1755** (2005), 107.
- [2] J.I. Bardos, N.M. Chau and M. Ashcroft, Growth factor-mediated induction of HDM2 positively regulates hypoxia-inducible factor 1 α expression, *Mol. Cell Biol.* **24** (2004), 2905.
- [3] H. Buerger, F. Gebhardt, H. Schmidt, K. Huttmacher, A. Beckmann, R. Simon, W. Boecker and B. Brandt, Length and loss of heterozygosity of an intron 1 polymorphic sequence of *egfr* is related to cytogenetic alterations and EGFR-expression, *Cancer Res.* **60** (2000), 854.
- [4] R. Carbone, S. Fre, G. Iannolo, F. Belleudi, P. Mancini, P.G. Pelicci, M.R. Torrisi and P.P. Di Fiore, eps15 and eps15R are essential components of the endocytic pathway, *Cancer Res.* **57** (1997), 5498.
- [5] R. Fukuda, K. Hirota, F. Fan, Y.D. Jung, L.M. Ellis and G.L. Semenza, Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells, *J. Biol. Chem.* **277** (2002), 38205.
- [6] S.L. Harris and A.J. Levine, The p53 pathway: positive and negative feedback loops, *Oncogene* **24** (2005), 2899.
- [7] T. Hasebe, S. Imoto, S. Sasaki, Y. Tsubono and K. Mukai, Proliferative activity and tumor angiogenesis is closely correlated to stromal cellularity of fibroadenoma: proposal fibroadenoma, cellular variant, *Pathol. Int.* **49** (1999), 435.
- [8] A. Husdal, G. Bukholm and I.R. Bukholm, The prognostic value and overexpression of cyclin A is correlated with gene

- amplification of both cyclin A and cyclin E in breast cancer patient, *Cell. Oncol.* **28** (2006), 107.
- [9] C. Kersting, A. Kuijper, H. Schmidt, J. Packeisen, C. Liedtke, N. Tidow, C. Gustmann, B. Hinrichs, P. Wulfing, J. Tio, W. Boecker, P. Van Diest, B. Brandt and H. Buerger, Amplifications of the epidermal growth factor receptor gene (egfr) are common in phyllodes tumors of the breast and are associated with tumor progression, *Lab. Invest.* **86** (2006), 54.
- [10] C. Kersting, J. Packeisen, B. Leidinger, B. Brandt, R. von Wasielewski, W. Winkelmann, P.J. van Diest, G. Gosheger and H. Burger, Pitfalls in immunohistochemical assessment of EGFR expression in soft tissue sarcomas, *J. Clin. Pathol.* **59** (2006), 585.
- [11] D. Kim, H.C. Dan, S. Park, L. Yang, Q. Liu, S. Kaneko, J. Ning, L. He, H. Yang, M. Sun, S.V. Nicosia and J.Q. Cheng, AKT/PKB signaling mechanisms in cancer and chemoresistance, *Front Biosci.* **10** (2005), 975.
- [12] Y.N. Kim, P. Dam and P.J. Bertics, Caveolin-1 phosphorylation in human squamous and epidermoid carcinoma cells: dependence on ErbB1 expression and Src activation, *Exp. Cell. Res.* **280** (2002), 134.
- [13] J. Kononen, L. Bubendorf, A. Kallioniemi, M. Barlund, P. Schraml, S. Leighton, J. Torhorst, M.J. Mihatsch, G. Sauter and O.P. Kallioniemi, Tissue microarrays for high-throughput molecular profiling of tumor specimens [see comments], *Nat. Med.* **4** (1998), 844.
- [14] V. Kuenen-Boumeester, S.C. Henzen-Logmans, M.M. Timmermans, I.L. van Staveren, A. van Geel, H.J. Peeterse, J. Bonnema and E.M. Berns, Altered expression of p53 and its regulated proteins in phyllodes tumours of the breast, *J. Pathol.* **189** (1999), 169.
- [15] A. Kuijper, G.P. van der, W.E. Van Der and P.J. van Diest, Expression of hypoxia-inducible factor 1 alpha and its downstream targets in fibroepithelial tumors of the breast, *Breast Cancer Res.* **7** (2005), R808.
- [16] E. Laughner, P. Taghavi, K. Chiles, P.C. Mahon and G.L. Semenza, HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression, *Mol. Cell. Biol.* **21** (2001), 3995.
- [17] L. Li, C.H. Ren, S.A. Tahir, C. Ren and T.C. Thompson, Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PPI and PP2A, *Mol. Cell. Biol.* **23** (2003), 9389.
- [18] P.K. Majumder and W.R. Sellers, Akt-regulated pathways in prostate cancer, *Oncogene* **24** (2005), 7465.
- [19] L.D. Mayo and D.B. Donner, The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network, *Trends Biochem. Sci.* **27** (2002), 462.
- [20] C. Mineo, G.L. James, E.J. Smart and R.G. Anderson, Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane, *J. Biol. Chem.* **271** (1996), 11930.
- [21] C.J. Moffat, S.E. Pinder, A.R. Dixon, C.W. Elston, R.W. Blamey and I.O. Ellis, Phyllodes tumours of the breast: a clinicopathological review of thirty-two cases, *Histopathology* **27** (1995), 205.
- [22] P.M. Navolanic, L.S. Steelman and J.A. McCubrey, EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (Review), *Int. J. Oncol.* **22** (2003), 237.
- [23] J. Packeisen, E. Korsching, H. Herbst, W. Boecker and H. Buerger, Demystified... tissue microarray technology, *Mol. Pathol.* **56** (2003), 198.
- [24] D. Ravid, S. Maor, H. Werner and M. Liscovitch, Caveolin-1 inhibits cell detachment-induced p53 activation and anoikis by upregulation of insulin-like growth factor-I receptors and signaling, *Oncogene* **24** (2005), 1338.
- [25] S. Repetto, B. Salani, D. Maggi and R. Cordera, Insulin and IGF-I phosphorylate eNOS in HUVECs by a caveolin-1 dependent mechanism, *Biochem. Biophys. Res. Commun.* **337** (2005), 849.
- [26] E.J. Sawyer, A.M. Hanby, R. Poulson, R. Jeffery, C.E. Gillett, I.O. Ellis, P. Ellis and I.P. Tomlinson, Beta-catenin abnormalities and associated insulin-like growth factor overexpression are important in phyllodes tumours and fibroadenomas of the breast, *J. Pathol.* **200** (2003), 627.
- [27] E.J. Sawyer, R. Poulson, F.T. Hunt, R. Jeffery, G. Elia, I.O. Ellis, P. Ellis, I.P. Tomlinson and A.M. Hanby, Malignant phyllodes tumours show stromal overexpression of c-myc and c-kit, *J. Pathol.* **200** (2003), 59.
- [28] D.G. Sedding and R.C. Braun-Dullaeus, Caveolin-1: dual role for proliferation of vascular smooth muscle cells, *Trends Cardiovasc. Med.* **16** (2006), 50.
- [29] T. Sorkina, A. Bild, F. Tebar and A. Sorkin, Clathrin, adaptors and eps15 in endosomes containing activated epidermal growth factor receptors, *J. Cell. Sci.* **112** (1999), 317.
- [30] F. Tebar, T. Sorkina, A. Sorkin, M. Ericsson and T. Kirchhausen, Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits, *J. Biol. Chem.* **271** (1996), 28727.
- [31] N. Tidow, A. Boecker, H. Schmidt, K. Agelopoulos, W. Boecker, H. Buerger and B. Brandt, Distinct Amplification of an Untranslated Regulatory Sequence in the egfr Gene Contributes to Early Steps in Breast Cancer Development, *Cancer Res.* **63** (2003), 1172.
- [32] M.M. Vleugel, R. Bos, H. Buerger, P. van der Groep, O.R. Saramäki, T. Visakorpi, E. van der Wall and P.J. van Diest, No amplifications of hypoxia-inducible factor-1alpha gene in invasive breast cancer: a tissue microarray study, *Cell. Oncol.* **26** (2004), 347.
- [33] H. Waterman and Y. Yarden, Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases, *FEBS Lett.* **490** (2001), 142.
- [34] P.V. Woolley, S.M. Gollin, W. Riskalla, S. Finkelstein, D.F. Stefanik, L. Riskalla, W.P. Swaney, L. Weisenthal and R.J. McKenna, Jr., Cytogenetics, immunostaining for fibroblast growth factors, p53 sequencing, and clinical features of two cases of cystosarcoma phyllodes, *Mol. Diagn.* **5** (2000), 179.
- [35] K.R. Zalata, W.A. Nasif, S.C. Ming, M. Lotfy, N.A. Nada, N. Gad El-Hak and S.H. Leech, p53, Bcl-2 and C-Myc expressions in colorectal carcinoma associated with schistosomiasis in Egypt, *Cell. Oncol.* **27** (2005), 245.



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