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Coexpression patterns of *EGFR*, *HER2*, *HER3* and *HER4* in non-melanoma skin cancer

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Abstract

The receptor tyrosine kinases (RTKs) epidermal growth factor receptor (EGFR), HER2, HER3 and HER4 are involved in the pathogenesis of multiple human malignant neoplasias. However, their role in the carcinogenesis of basal cell carcinomas (BCC) and squamous cell carcinomas (SCC) remains to be elucidated. In order to further define the role of these RTKs, 56 human skin tissue samples of normal skin, BCC and SCC were studied by conventional and differential and quantitative reverse transcriptase–polymerase chain reaction (rtPCR). *EGFR* and *HER3* were predominantly expressed in the BCCs and SCCs, while *HER2* was ubiquitously expressed. *HER4* was not expressed in any sample. Since *in vitro* studies have provided compelling evidence that heterodimer formation of these receptors are associated with different signal transduction processes, coexpression patterns might be decisive for the induction and maintenance of a malignant phenotype. These results confirm this concept: isolated *HER2* expression and *EGFR*/*HER2* were predominantly found in normal skin, while HER2/HER3 and the triple expression of EGFR/HER2/HER3 were seen more frequently in the BCCs and SCCs compared with normal skin (50% and 40% compared with 26%, respectively). The activation of HER3, in addition to EGFR and HER2, might therefore be associated with the malignant phenotype. However, due to the small numbers in this study, further confirmation of the patterns is needed.

Keywords: Receptor tyrosine kinase; erbB; EGFR; HER2; HER3; HER4; Basal cell carcinomas; Squamous cell carcinomas; Skin; rtPCR

1. Introduction

The ErbB family of receptor tyrosine kinases (RTK) includes the epidermal growth factor receptor (EGFR), HER2, HER3, HER4 (also designated ErbB1, ErbB2, ErbB3, ErbB4) and Xmrk, a causative oncogene for melanoma formation in Xiphophorus [1]. Structurally, all RTKs share common features, including a glycosylated extracellular ligand-binding domain, a hydrophobic transmembrane domain and a conserved cytoplasmic catalytic domain [2]. The sequence identity between the subclass I RTKs ranges between 40 and 60% in their extracellular domains and 60–80% in their intracellular domains [3].

Members of the ErbB receptor family have been implicated in the development of a variety of human carcinomas (reviewed in Refs [4,5]). These receptors are thought to contribute to or cause tumorigenesis as a result of their overexpression due to gene amplification or enhanced transcription. EGFR has been found to be amplified and/or overexpressed in human glioblastomas, epidermoid carcinomas, breast cancer, gastrointestinal, urinary and reproductive tract malignancies and malignant melanomas (reviewed in Refs [4–6]), while overexpression of HER2 has been demonstrated, among others, in mammary, ovarian, endometrial and non-small cell lung cancer (NSCLC) [7,8]. HER3 overexpression has not been observed as frequently in human tumours, but it is overexpressed in mammary carcinomas [9–11], head and neck carcinoma cell lines [12] and oral squamous cell carcinomas [13]. Overexpression of HER4 [14,15] has been found predominantly in breast cancer [16–18]. In contrast to the extensive literature concerning these above malignancies, little is known about the expression status of these genes in epithelial cutaneous tumours.

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Expression of these members of the ErbB family in cutaneous non-melanoma skin cancer has mostly been studied immunohistochemically.

Specific binding of EGFR was detected in approximately 50% of basal cell carcinomas (BCC) and 100% of cutaneous squamous cell carcinomas (SCC) [19,20], while another study suggests that this is not always the case [21]. A recent Chinese study found positive reactivity with an EGFR antibody in all tested BCC and weak staining of HER2 in only one-third of BCC. The same study found reactivity of EGFR in 92.3% and of HER2 in 84.6% of cutaneous SCC [22]. An earlier study determined *EGFR* amplification in 19% and overexpression in 53% of SCCs of the head and neck [23]. In benign tumours, such as seborrhoic keratoses or keratoacanthomas, there was an ordered staining at the membrane, whereas in malignant tumours (BCC and SCC), a loss of membrane labelling and accumulation in the cytoplasmic compartment was observed. These authors [24] concluded that dysregulation of EGFR may contribute to the development of cutaneous epithelial tumours. In normal human skin, EGFR was detected immunohistochemically in basal epidermal keratinocytes with decreasing levels towards the corneum layer [25,26], sebocytes, outer root sheath cells in hair follicles, smooth muscle cells of arrector pili muscles and dermal arteries. In benign skin diseases, elevated EGFR levels correlated with proliferating seborrhoic keratoses and skin tags, while viral diseases did not show a distinct pattern [27,28]. Based on these contradictory results, the role of EGFR in the pathogenesis of cutaneous BCC and SCC remains to be determined.

While keratoacanthomas, actinic keratoses and normal epidermis showed little or no HER2 protein immunohistochemically, 20/24 cases of SCCs had elevated expression, with positive staining seen in the cytoplasm. Fewer positive cells and less staining were detected in BCCs compared with SCCs [29]. In oral invasive SCCs, no HER2/neu immunostaining was detectable [30]. In contrast, HER2 oncoprotein expression was associated with poor prognosis in cervical SCC [31].

HER3 has to our knowledge not yet been studied in cutaneous BCCs and SCCs. In SCCs of the head and neck, 9% presented EGFR overexpression, while HER2 and HER3 were not overexpressed in this semiquantitative polymerase chain reaction (PCR) study in 59 cases [32]. Based on the published data, it seems likely that the expression patterns of the ErbB family genes differ with the location, especially between skin and mucosal tissue.

The role of HER4 [15], the most recently identified member of this subfamily of RTKs, has not yet been extensively studied in human cancers [14,15], although its involvement in breast cancer carcinogenesis seems likely [16,18,33]. By immunohistochemistry, positive staining was only detectable in fetal skin, while HER4 expression was not present in adult skin [34].

Regulation of ErbB receptor activation is very complex. A number of different ligands have been described: e.g. epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF) and epiregulin [35,36]. These ligands show different affinities to the ErbB receptors and upon binding induce heterodimerisations of the receptors. Thus, by binding to specific receptors and by forming specific heterodimers, differentiated signal transduction processes can be initiated. The knowledge of co-expression patterns of this ErbB family in benign and malignant tumours will help in the understanding of malignant transformation and might have prognostic or therapeutic significance.

The aim of this study was to determine the differential mRNA expression pattern of *EGFR*, *HER2*, *HER3* and *HER4* in BCCs and cutaneous SCCs in comparison with normal skin. Special emphasis was laid on co-expression which may determine the potency and diversity of intracellular signals leading to malignant transformation and proliferation of malignant tissue.

2. Materials and methods

4–6 mm punch biopsies were taken from 33 non-melanoma skin cancer tissues immediately after excision from macroscopically altered tissue. The tissues of the excised tumours were diagnosed by two independent dermatopathologists on haematoxylin and eosin (H&E) stains. Although special care was taken to study only malignantly transformed tissue, it cannot entirely be ruled out that a small amount of dermal stroma was included. N_2-frozen tissue specimens taken from 16 BCCs, 5 cutaneous SCCs and 16 samples of normal skin were analysed by conventional reverse transcriptase (rt) PCR for *EGFR*, *HER2*, *HER3* and *HER4* expression. A 9-year-old girl with a squamous cell carcinoma suffered from *Xeroderma pigmentosum*. Furthermore, an additional 4 samples of normal skin, 9 BCCs, 6 cutaneous SCCs were studied by differential rtPCR for *HER2* expression and by quantitative rtPCR for *EGFR* and *HER3* expression.

2.1. RNA extraction and rtPCR

Tissue samples were shock frozen in liquid nitrogen immediately after excision. The tissue was minced, total mRNA were isolated from homogenates using the RNA-Clean System (Angewandte Gentechnologie Systeme GmbH, Germany). Using oligo-(dt) primers, the extracted mRNA was transcribed with the Reverse Transcriptase System (Promega, Madison, WI, USA) to cDNA. The cDNAs were then phenol–chloroform extracted, precipitated by ethanol extraction and

redissolved in double distilled (dd) H_2O. The cDNA concentration was calculated from the absorbance measurement at 260 nm. In subsequent PCR reactions (25 μl) 100 ng cDNA template from above was used to investigate the expression of the following genes:

2.1.1. EGFR

5′-ACT AGC CAG GAA GTA CTT CC-3′ and 5′-GGC CTT CTT GGA TCT TTA GT-3′, predicted product size: 398 bp, cycling parameters: an initial denaturation step (94°C 4 min), followed by 93°C 35 s, 60°C 35 s, 72°C 35 s, 35 cycles and one final extension step (68°C 10 min).

2.1.2. HER2

5′-CGG GAG ATC CCT GAC CTG CTG GAA3′ and 5′-CTG CTG GGG TAC CAG ATA CTC CTC-3′, predicted product size: 300 bp, cycling parameters: an initial denaturation step (94°C 4 min), followed by 93°C 35 s, 60°C 35 s, 72°C 35 s, 35 cycles and one final extension step (68°C 10 min).

2.1.3. HER3

5′-CUA CUA CUA CUA GAG GCT GAG CTC CAG GAG AA-3′ and 5′-CAU CAU CAU CAU CTG GGA CCT GGG AGA GAG AG-3′, predicted product size: 814 bp, cycling parameters: an initial denaturation step (94°C 4 min), followed by 93°C 35 s, 56°C 35 s, 72°C 35 s, 35 cycles and one final extension step (68°C 10 min).

2.1.4. HER4 [37]

5′-CCT CTC CTT CCT GCG GTC TGT-3′ and 5′-AAG TCT GGC AAT GAT TTT CTG TGG G-3′, predicted product size: 404 bp, cycling parameters: an initial denaturation step (94°C 4 min), followed by 93°C 35 s, 55°C 35 s, 72°C 35 s, 35 cycles and one final extension step (68°C 10 min).

The PCR mixtures contained 100 ng of template cDNA, 1.5 mM $MgCl_2$, 1 μM sense and antisense primer, 0.2 mM deoxy nucleotide triphosphates (dNTPs) (Promega) and Taq Polymerase 1 U (Boehringer Mannheim, Germany). To circumvent false-negative results, we used primers detecting the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (Stratagene; sense 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ antisense 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′) yielding a 598 bp amplicon. Thermocycling was performed for 29 cycles at 94°C for 45 s, 60°C for 45 s and 72°C for 90 s.

Genomic contamination was ruled out by the use of *HER2* and *HER4* primers with different product sizes for cDNA and genomic DNA. The DNA molecular weight standard (PhiX 174RF/*HaeIII*) was purchased from Boehringer Mannheim. As positive controls, RNA isolated from an amelanotic melanoma cell line C32 obtained by the American Type Culture Collection (ATCC) and an epidermoid carcinoma cell line A431 were amplified. The cell lines were cultured as recommended by ATCC. In the case of *HER4* expression, a *HER4*-positive male mammary carcinoma sample was used as a positive control. Water instead of cDNA was used as a negative control. In parallel, genomic DNA was amplified. Following PCR, identical aliquots (5 μl) were loaded on 2% agarose gels and the products were visualised by ethidium bromide staining (see Fig. 1). The optical density of bands was analysed by a computerised densitometric analysis device (Image master VDS, Pharmacia). In each PCR, a parallel assay with genomic DNA was performed. The optical density of

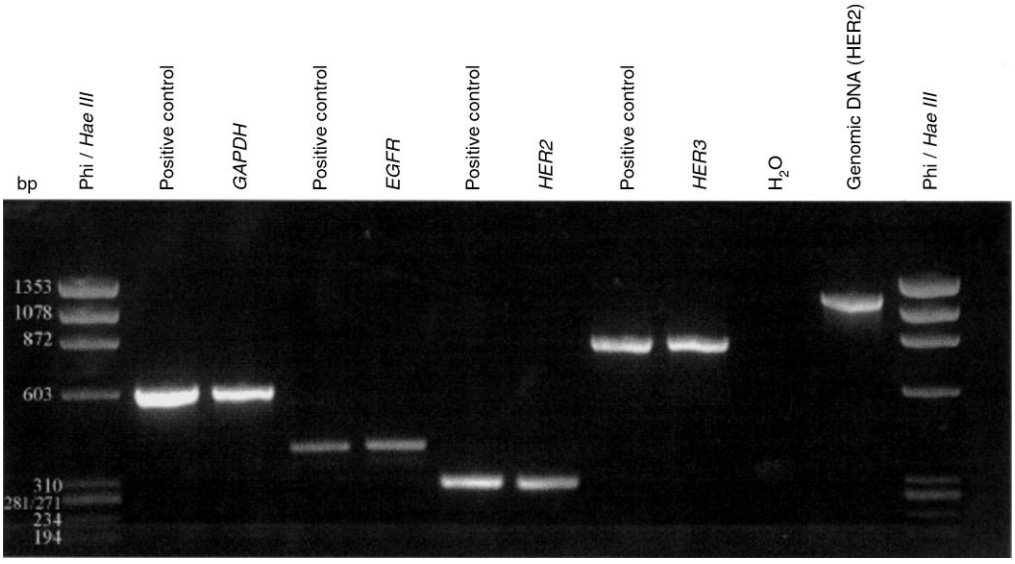

Fig. 1. Representative rtPCR agarose gel. Phi/HAeIII, DNA molecular marker; genomic DNA (HERZ), genomic DNA control.

bands of this genomic DNA was taken as a level for determining weak-positive or strong-positive expression. An optical density measurement lower than the genomic DNA band was regarded as "weak" and similar or higher was regarded as "strong".

2.2. Differential rtPCR

In order to determine the differing expression levels of *HER2,* a differential PCR approach as described in Refs [38–41] was taken. The *HER2* and the *GAPDH* primers, one for the target gene and one for the control gene were present simultaneously in the reaction tube. Cycling parameters were applied as above. The PCR mixtures contained 100 ng of template cDNA, 2 mM $MgCl_2$, 1 μM sense and antisense primer, 0.2 mM dNTPs (Promega) and 1 U Taq Polymerase (Boehringer Mannheim). The DNA molecular weight standard (PhiX 174RF/*HaeIII*) was purchased from Boehringer Mannheim, Germany. As a positive control, cDNA from an amelanotic melanoma cell line C32, obtained from ATCC and cultured according to the protocol recommended by ATCC was used. Water was applied as a negative control. Identical aliquots (5 μl) of PCR products were loaded on 2% agarose gels and visualised by ethidium bromide staining. The optical density of the *GAPDH* and *HER2* bands were analysed by a computerised densitometric analysis device (Image master VDS, Pharmacia) and the *HER2*/*GAPDH* ratios in per cent were determined.

2.3. Quantitative rtPCR

PCR was performed in a GeneAmp 9700 thermocycler (Applied Biosystems, Germany). The final PCR mixture contained 100 ng of targeted cDNA, 2.5 mM $MgCl_2$, 10 mM Tris, 50 mM KCl, 0.5 μM of each primer, 200 μM each of dATP, dTTP, dGTP and dCTP and 25 mU/μl Taq polymerase (Boehringer Mannheim, Germany). The downstream-primer was 5′-labelled with digoxigenin. Each PCR tube contained the same amount of internal controls (=mimics), consisting of the identical primer sequences as the target, but with a different intermediate sequence.

Amplification parameters for the individual genes were as described above.

2.3.1. Construction and stabilisation of internal controls (mimics)

DNA mimics were constructed using the PCR MIMIC Construction Kit (Clontech, USA) following the manufacturer's protocol. These mimic fragments were stabilised by cloning them into a plasmid using the T/A Cloning Kit from Invitrogen (San Diego, CA, USA) following the manufacturer's protocol. Plasmid DNA was extracted using the High Pure Plasmid Isolation Kit (Boehringer Mannheim) and quantified by reading the optical density (OD) at 260 nm. The molar concentration was calculated, and the mimics were appropriately diluted.

2.3.2. Quantitation by PCR enzyme-linked immunosorbent assay (ELISA)

This assay was performed using a commercially available system (PCR ELISA, Boehringer) following the manufacturer's protocol. In brief, part of the respective PCR product was denatured with the same amount of an alkaline solution for 10 min at room temperature. 5-fold dilutions of the denatured PCR-product of each sample were hybridised with 1.5 pmol of a 5′-biotinylated specific oligonucleotide probe in rows A–D of a streptavidin-coated microtitre plate, corresponding to 10.0, 2.0, 0.4 and 0.08 μl of the denatured PCR product. Rows E–H contained 10.0, 2.0, 0.4 and 0.08 μl of the denatured PCR product hybridised with the mimic-specific biotinylated oligonucleotide probe. The total reaction volume was 200 μl. The plate was incubated at 55°C for 1 h, washed 5 times (using the washing buffer included in the kit), incubated with 200 μl of a peroxidase-conjugated anti-digoxigenin antibody at 37°C for 30 min, washed 5 times and incubated with 200 μl of the substrate for peroxidase, 3,3′–5,5′-tetramethylbenzidine (TMB), at 37°C for 15 min. The reaction was stopped by adding 75 μl of 2 M hydrochloric acid, and the OD was measured at a wavelength of 450 nm. By subtraction of the OD of the negative control net ODs were calculated.

Quantitation was performed using the principle of a commercially available system (HIV Monitor, Roche Diagnostic Systems, Branchburg, NJ, USA). For each sample the lowest net-OD for mimic and target over 0.100 was chosen for calculating the target-copy number using the formula:

$$\text{target copies} = (\text{target-net-OD} \times \text{DF}) : (\text{mimic-net-OD} \times \text{DF}) \times \text{input copy number of mimic}$$

DF = dilution factor.

2.3.3. Detection of PCR-products

Firstly, the tissue samples were screened by internally controlled PCR–ELISA for the presence of *GAPDH*-cDNA as a housekeeping gene and for target gene-cDNA. 10 μl of the target and mimic product were denatured with 20 μl denaturation solution, and the PCR–ELISA was continued as described above. Fragment lengths: 598 bp for *GAPDH* and 604 bp for the *GAPDH* mimic, 398 bp for *EGFR* and 440 bp for the *EGFR* mimic, 813 bp for *HER3* and 596 bp for the *HER3* mimic.

In samples that were positive for the target cDNA, copy numbers of *GAPDH* and the target gene were

Table 1
Expression of receptor tyrosine kinases (RTKs) determined by conventional rtPCR (percentage of positive results)

Tissue	*EGFR* *n* (%)	*HER2* *n* (%)	*HER3* *n* (%)	*HER4* *n* (%)
Normal skin	6 (38%)	16 (100%)	6 (38%)	0 (%)
(*n* = 16)	s 4 (25%)	s 15 (94%)	s 1 (6%)	
	w 2 (13%)	w 1 (6%)	w 5 (31%)	
BCC	9 (57%)	16 (100%)	10 (63%)	0 (0%)
(*n* = 16)	s 6 (38%)	s 14 (88%)	s 8 (50%)	
	w 3 (19%)	w 2 (13%)	w 2 (13%)	
SCC	4 (80%)	16 (100%)	4 (80%)	0 (0%)
(*n* = 5)	s 2 (40%)	s 16 (100%)	s 2 (40%)	
	w 2 (40%)	w 0 (0%)	w 2 (40%)	

rtPCR, reverse transcriptase–polymerase chain reaction; s, strong expression; w, weak expression; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; EGFR, epidermal growth factor receptor.

quantitated as described above. To correct for different amounts of the mRNA used intitially and for differing reverse transcription efficiency, copy numbers for the target gene were expressed in relation to *GAPDH* copy numbers.

3. Results

A total of 56 tissue samples from patients with BCCs and SCCs and normal skin samples were studied. Clinicopathological data concerning the tumour type were obtained. Each sample was investigated for *EGFR*, *HER2*, *HER3*, *HER4* and *GAPDH* expression (Tables 1 and 2).

3.1. Conventional rtPCR results

In each PCR, a parallel assay with genomic DNA was performed. The OD of bands from this genomic DNA was taken as a level for determining 'weak-positive' or 'strong-positive' expression as described earlier. Genomic contamination was ruled out by the use of intron-spanning primers for *HER2*, which was expressed in all samples. 8 of the 16 samples from normal skin had been excised from gluteal skin. Non-ultraviolet (UV)-exposed gluteal skin was chosen in order to avoid alterations of gene expression levels by UV radiation. *EGFR* expression was detectable in 38% of normal skin tissue (6/16), of which 4 were strongly positive (25%). 6/16 (38%) of the BCCs strongly and a further 3/16 (19%) weakly expressed *EGFR*, thus a total of 9 (57%) expressed *EGFR*. In the SCCs ($n = 5$) 1 was negative, 2 were strongly and 2 were weakly positive. The SCC of a *Xeroderma pigmentosum* patient had a strong expression.

HER2 was expressed in all samples regardless of the tissue type. 94% ($n = 15$) of normal skin samples strongly expressed *HER2*, only one sample weakly expressed *HER2*. In the BCCs the expression was weak in only 2/16 of the tested samples whereas the rest (88%) strongly expressed *HER2*. All tested SCCs, including the *Xeroderma pigmentosum* sample strongly expressed *HER2*.

Of the normal skin samples 62% were *HER3*-negative. Of the remaining positive samples, only one sample strongly expressed *HER3*, while 5/16 (31%) weakly expressed *HER3*. This was considerably different in BCC: 8/16 (50%) strongly expressed *HER3* while a further 2 weakly expressed *HER3*. In the SCCs the expression rate was the same as for *EGFR*. None of the tested samples, whether normal skin or cancerous skin, were positive for *HER4*.

Regarding coexpression, only the samples with at least one strong expression were included in the study,

Table 2
Co-expression pattern of receptor tyrosine kinetics (RTKs) determined by conventional rtPCR (percentage of positive results)[a]

Tissue	*HER2* *n* (%)	*EGFR*/*HER2* *n* (%)	*HER2*/*HER3* *n* (%)	*EGFR*/*HER2*/*HER3* *n* (%)
Normal skin *n* = 15	7 (47%)	4 (27%)	2 (13%)	2 (13%)
BCC *n* = 14	5 (36%)	2 (14%)	3 (21%)	4 (29%)
SCC *n* = 5	2 (40%)	1 (20%)	1 (20%)	1 (20%)

EGFR, epidermal growth factor receptor; rtPCR, reverse transcriptase–polymerase chain reaction; BCC, basal cell carcinoma; SCC, squamous cell carcinoma.

[a] Numbers vary from Table 1 as only the samples that showed at least one 'strong' expression for *HER2*, *HER3* or *EGFR* were included.

thus 15 normal skin samples, 14 BCC and all 5 SCC remained for evaluation. As demonstrated by the *in vitro* data, we could not detect isolated *HER3* or *EGFR* expression. In all samples with isolated *HER2* expression, the expression level was similar or higher when compared with genomic DNA i.e. 'strong'. The expression pattern in normal skin was characterised by either isolated *HER2* expression (7/15) or *EGFR*/*HER2* expression (4/15). In 2 samples, *HER2*/*HER3* expression was observed and 2 triple expressing samples were also detected. However, in these 4 samples *HER3* expression was weak.

Table 3
Results of quantitative rtPCR, RTK/*GAPDH* ratio

	Normal	BCC	SCC
EGFR (*n*)			
1	0.3	0.6	0.1
2	0.5	2.1	0.7
3	0.4	0.6	0.5
4	0.08	0.2	0.2
5		0.2	0.4
6		0.1	0.2
7		0.3	
8		0.1	
9		0.3	
Mean	0.3	0.5	0.4
% of $\geqslant 0.5$ = strongly positive	25%	33%	33%
HER2 (*n*)			
1	29.4 s	5.2 w	70.2 s
2	24.5 s	41.1 s	40.0 s
3	72.4 s	31.0 s	32.6 s
4	65.5 s	29.3 s	27.3 s
5		16.3 s	37.9 s
6		28.8 s	40.2 s
7		21.7 s	
8		63.0 s	
9		47.9 s	
Mean	48.0 s	31.6 s	41.4 s
HER3 (*n*)			
1	0.9	1.2	0.1
2	0.4	5.1	0.3
3	0.5	3.1	0.3
4	0.2	0.3	0.2
5		0.2	0.2
6		0.2	0.6
7		0.5	
8		0.2	
9		0.5	
Mean	0.5	1.26	0.3
% of $\geqslant 0.5$ = strongly positive	50%	56%	17%

HER2 semiquantitative rtPCR, simultaneous amplification of *HER2* and *GAPDH*, ratio of *HER2*/*GAPDH* given in % conventional rtPCR: s, strongly positive; w, weakly positive. Ethidium bromide stained agarose gel with PCR results determined by conventional rtPCR. rtPCR, reverse transcriptase–polymerase chain reaction; RTK, receptor tyrosine kinase; BCC, basal cell carcinoma; SCC, squamous cell carcinoma.

Isolated expression of *HER2* was also found in 5/14 BCCs and 2/5 SCCs. The *EGFR*/*HER2* pattern was detected in 2/14 BCC samples and 1/5 SCCs. *HER2*/*HER3* was found in 3/14 BCCs and 1 SCC sample. The triple expression of *EGFR*/*HER2*/*HER3* was detectable in 4/14 BCCs and 1 SCC.

3.2. Results of differential and quantitative rtPCR

With these methods *EGFR*, *HER2*, *HER3* were detectable in all samples, even at low levels (Table 3). Due to the expression in normal skin, the level to determine strong expression was set at a RTK/*GAPDH* copy number ratio of 0.5 for *EGFR* and *HER3*. The mean for *EGFR* expression in normal skin was 0.3, in BCC 0.5 and in SCC 0.4. The highest ratio was found with a 2.1 *EGFR*/*GAPDH* copy number ratio in a BCC. 25% of the normal skin samples, 33% of the BCC samples and 33% of the SCCs revealed strong expression with a *EGFR*/*GAPDH* copy number ratio of $\geqslant 0.5$. The mean expression rate of *HER3* was 0.5 for normal skin, for BCC 1.26 and 0.3 for SCC. The levels of *HER3*/*GAPDH* were up to 5.1 and 3.1 in the BCCs. *HER2* expression was determined by differential rtPCR with the exception of one BCC sample, the ratio in per cent was higher than 10%. This single sample (5.2% in a BCC) revealed weak expression, just as was found in the conventional rtPCR. The mean of *HER2*/*GAPDH* in per cent for normal skin was 48.0, for BCC 31.6% and for SCC 41.4%, thus no differential expression in relation to the type of tissue could be determined.

4. Discussion

Despite the growing interest in signal transduction and the expression of associated genes, little is known about the role of the RTKs of subclass I in non-melanoma skin cancers. This is even more astonishing since UV-induction of gene expression such as *EGFR* [42] has been reported *in vitro*. At the same time, there is compelling evidence that the predominant exogenous factor for skin carcinogenesis is UV radiation. With the plethora of intracellular signals due to specific interaction of ligands with ErbB family members and the resulting heterodimer formation as a reaction to carcinogens, this group of genes certainly warrants expression studies in skin cancer.

In this study, we could obtain similar results with two different rtPCR approaches, conventional rtPCR and differential and quantitative rtPCR. In general, the quantitative assay is the more sensitive assay detecting RNA in all samples, rendering a cut-off level necessary to determine strong expression. Based on the expression ratios in normal skin, we set this level at 0.5 for the ratio of RTK/*GAPDH* copy numbers. We found strong

expression of *EGFR* in 25% of the normal tissues with both methods. The corresponding figures in BCC were 38% and 33% and in SCC, 40% and 33%, in the conventional and differential/quantitative methods, respectively.

Our expression data of *EGFR* in BCC and in SCC are in accordance with data obtained by binding assays [19,20]. The high number of strongly expressing BCCs, in particular, argues for the involvement of EGFR in BCC pathogenesis. Furthermore, the 80% of the SCCs expressing *EGFR* in the conventional rtPCR analysis and previous studies [22,23] seem to support the theory that EGFR may also be involved in SCC carcinogenesis.

Since *HER2* was expressed in all tested tissues, it certainly does not qualify as a marker gene for BCC or SCC. Furthermore, the state of proliferation, dedifferentiation or clinical stage were not reflected in the

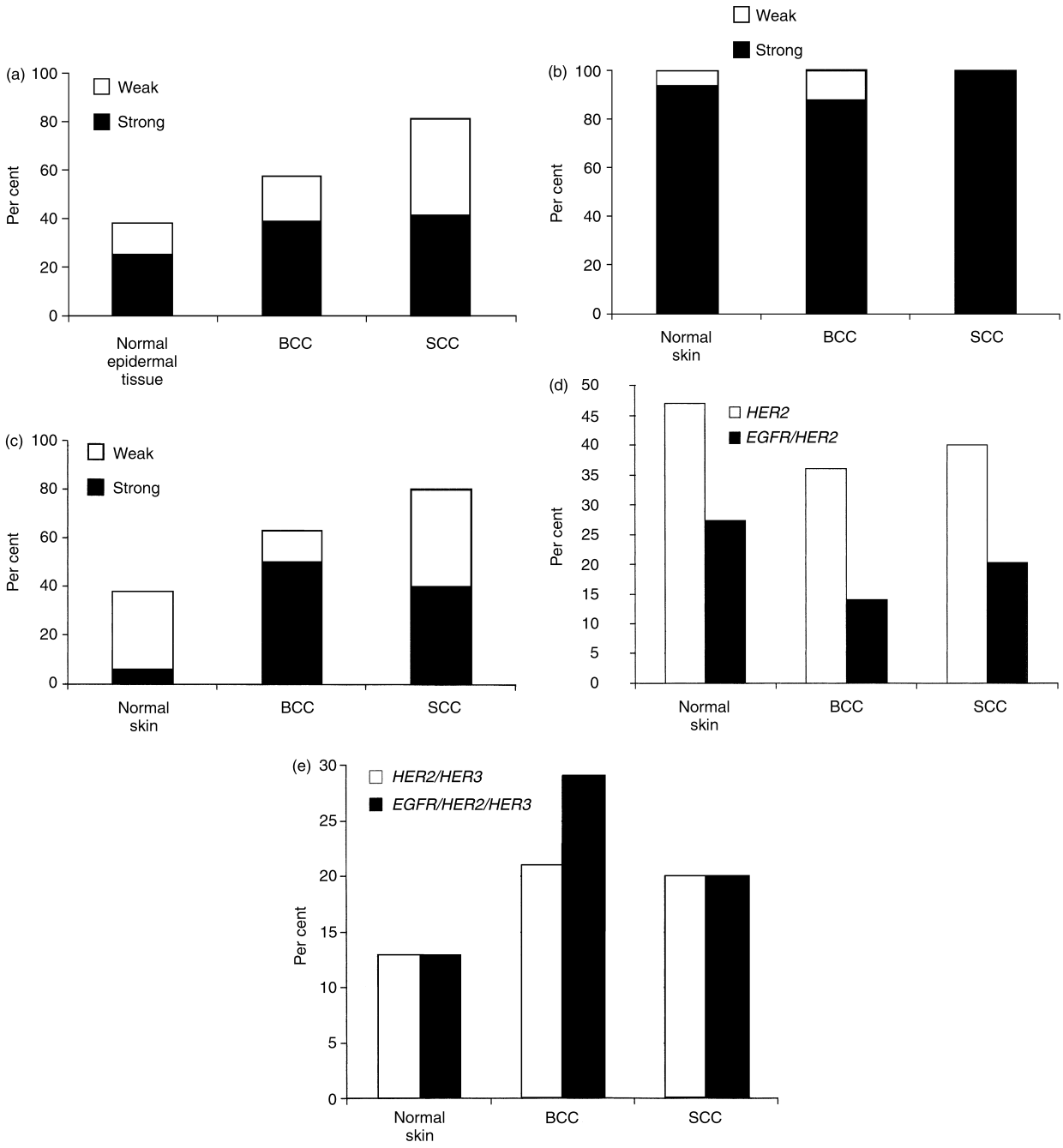

Fig. 2. (a) *EGFR* expression determined by conventional reverse transcriptase–polymerase chain reaction. (b) *HER2* expression determined by conventional rtPCR. (c) *HER3* expression determined by conventional rtPCR. (d) *HER2* and *EGFR*/*HER2* co-expression in normal skin, basal cell carcinoma and squamous cell carcinoma. (e) *HER2*/*HER3* and EGFR/HER2/HER3 co-expression in normal skin, BCC and SCC. BCC, basal cell carcinomas; SCC, squamous cell carcinomas.

expression level of *HER2* in this study. Similar observations have been obtained by us (data not shown) and others [43,44] in melanocytic tumours.

Recent studies have established that the family subclass I of RTKs can associate with each other to form an array of heterodimer complexes [45–50]. Therefore, the activation of one receptor could modulate the activity of the other members of this group of RTKs. Coexpression could extend the repertoire of downstream signalling events. RTK–RTK and ligand–RTK interactions in the EGFR family might enable cells to specifically react to exogenous stimuli.

HER2 is presumably the preferred partner for heterodimerisation of the ErbB family members [51]. The expression of HER2 in all of the tissue samples and the high number of tissues with isolated *HER2* expression seems to support this concept.

In contrast to the ubiquitous expression of HER2, HER3 expression was predominantly found in BCCs and SCCs. Both, the highest mean (1.26) and the highest individual expression (5.1) of the *HER3*/*GAPDH* copy number ratio were found in the BCCs.

As demonstrated by the *in vitro* data, we could not detect isolated *HER3* or *EGFR* expression, arguing for the assumption that these two RTKs are *in vivo* incapable of forming homodimers. This observation is in accordance with the known impaired kinase activity of *HER3*, rendering co-expression of another RTK essential.

Since none of the tested samples revealed the expression of *HER4*, this RTK may not be a causative oncogene in non-melanoma skin cancer. Furthermore, heterodimer formation of HER2 or HER3 with HER4 as described in *in vitro* studies [48,51–53] did not occur in these cutaneous tissues. Thus, in the samples with isolated *HER2* expression, either homodimerisation [54,55] might induce signal transduction or as yet unidentified members of the RTK subfamily I are involved in these cases.

Concerning co-expression patterns, either isolated *HER2* or *EGFR*/*HER2* expression were most commonly observed in normal skin. We found a similar pattern in benign melanocytic nevi (data not shown). In contrast to these benign tissues, *HER2*/*HER3* and *EGFR*/*HER2*/*HER3* expression patterns were mostly found in the BCCs and SCCs. The addition of HER3, rendering new RTK–heterodimer formations possible, may open pathways leading to the induction and/or the maintenance of the malignant phenotype. The distinct expression of HER3 — seen in the elevated rate of positivity (Table 1), might help in the future to distinguish benign and malignant skin lesions.

Thus, knowledge of these RTK expression patterns might bring us a step nearer to understanding the signal transduction processes ocurring in malignant as opposed to 'normal' tissues and might ultimately help to create new therapeutic reagents (Fig. 2).

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