Virchows Archiv (1995) 426:107–115

REVIEW ARTICLE

Christian R. De Potter · Anne-Marie Schelfhout

The *neu*-protein and breast cancer

Received: 1 August 1994 / Accepted: 3 October 1994

Abstract The *neu*-protein is overexpressed in about 20% of invasive duct cell carcinomas of the breast. The only reliable sign for *neu*-overexpression by immunohistochemistry is membrane staining. Its overexpression is correlated with decreased overall survival and disease free survival due to increased metastatic activity of *neu*-overexpressing tumour cells. This increased metastatic potential is a consequence of the motility enhancing activity of the *neu*-protein, which is exclusively expressed on pseudopodia, and to a lesser extent of its growth stimulating effect. From a clinical point of view, the assessment of *neu*-overexpression in breast cancer might become a useful tool in the future treatment of patients by chemotherapy, since patients whose tumour shows *neu*-overexpression benefit from higher doses of chemotherapy. The molecule plays a key role in the pathogenesis of Paget's disease of the breast. A chemotactic factor which is secreted by epidermal keratinocytes attracts the Paget cells to spread into the epidermis and acts via the *neu*-protein. In ductal carcinoma in situ, the combination of *neu*-overexpression and large cell type is highly correlated with extent of disease and therefore *neu*-overexpression might be a predictive marker for recurrence of disease after tumour resection.

Key words *neu* · c-*erb*B2 · Oncogene · Breast cancer · Paget's disease

Introduction

In recent years, the expression of the *neu*-protein has gained increasing importance for the biology and prognosis of breast cancer. This review focusses on the different biological and clinical aspects of the protein. From the biological point of view, its function as a motility factor receptor is stressed. Clinical applications of this motility function are found in the pathogenesis of Paget's disease, in the predictive value of *neu*-overexpression in the assessment of the extent of disease in ductal carcinoma in situ (DCIS) and in the aggressive metastatic behaviour of *neu*-overexpressing tumours in the first years after diagnosis. The ongoing debate on the correlation of *neu*-overexpression with prognosis and the search for a specific ligand are discussed.

C.R. De Potter (✉) · A.-M. Schelfhout
N. Goormaghtigh Institute of Pathology, University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium

The *neu*-oncogene: structure and function

The *neu*-oncogene is a protooncogene which codes for a membranous protein: the *neu*-protein. The *neu*-protein belongs to the *erb*B protooncogene family or type I family of tyrosine kinase receptors. This *erb*B family of tyrosine kinase receptor molecules is constituted by epidermal growth factor receptor (EGF-R), *neu*, c-*erb*B3/HER3 and c-*erb*B4/HER4. Their molecular weight is determined as 170 kDa, 185 kDa, 160 kDa and 180 kDa, respectively [38, 55]. All of these proteins share considerable homology with EGF-R, the first detected member of this oncogene family [4, 13, 62, 63, 66, 84]. All these transmembrane proteins function by phosphorylation of tyrosine for instance after triggering the intracellular tyrosine kinase domain by a ligand-receptor interaction. This phosphorylation presumably leads to signal transduction by phosphorylating second messenger proteins. Phosphorylation of a particular oncoprotein, triggered by a specific binding with its ligand, may lead to cross-phosphorylation of a related oncoprotein. So, the *neu*-protein can be cross-phosphorylated after phosphorylation of EGF-R by its ligands, EGF or transforming growth factor-α (TGF-α), which are not specific ligands for the *neu*-protein [11].

The *neu*-protein is composed of a cell-external ligand binding domain, a transmembranous component, which keeps the molecule in the lipid bilayer of the cell membrane and a cell-internal domain with tyrosine-kinase activity, involved in signal transduction [13]. The *neu*-oncogene was first described in rat neuro/glioblastoma cells, induced by treatment with a carcinogen [62, 63].

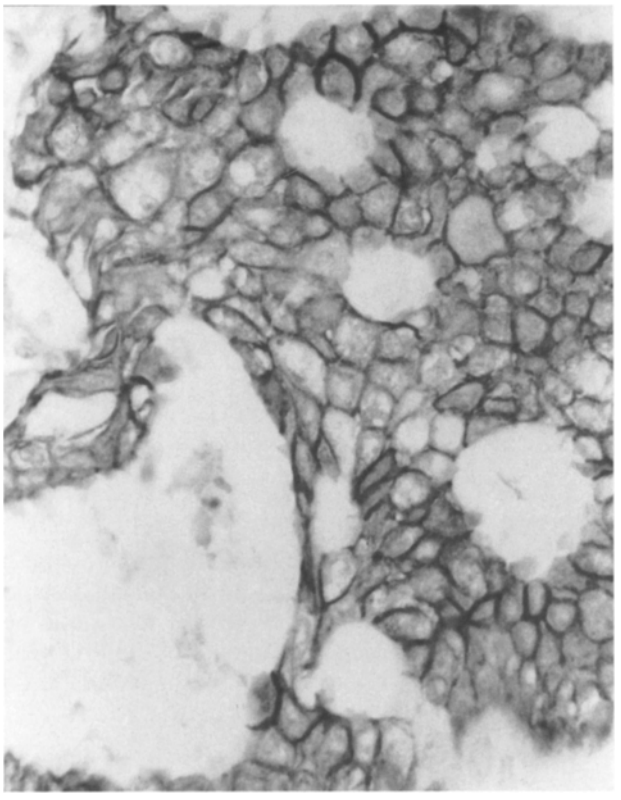

Fig. 1 Invasive ductal carcinoma immunostained for the *neu*-protein. All tumour cells show distinct expression at the plasma membrane. (×440)

The human equivalent of the rat *neu*-gene, which was independently cloned from a cDNA library was called HER-2 [13]. After cloning from genomic DNA it was designated as c-*erb*B-2 and after cloning from a breast carcinoma cell line as an *erb*B-related gene [61, 66]. The gene is localized on the long arm of chromosome 17 [26]. So far, no viral oncogenic counterpart has been identified.

The oncogenic potential of the *neu*-protein was initially demonstrated after transfection experiments of the gene in NIH/3T3 cells. The gene lacked transforming activity after transfection despite expression of detectable levels of its protein, but a further five- to tenfold increase in its expression was associated with activation of the gene as a potent oncogene. These findings suggest that overexpression alone may convert the gene protein from a normal receptor into an oncogene protein [22].

Cellular localization of the *neu*-protein

Initially, overexpression of the *neu*-protein was assessed by Southern blot analysis of its gene. Overexpression of the protein can also be evaluated in a reliable way by immunohistochemistry and correlates with gene amplification [74]. Amplification of the gene has been reported in 10–40% of the breast carcinomas [74, 75]. Detected by immunohistochemistry, the *neu*-protein is overexpressed

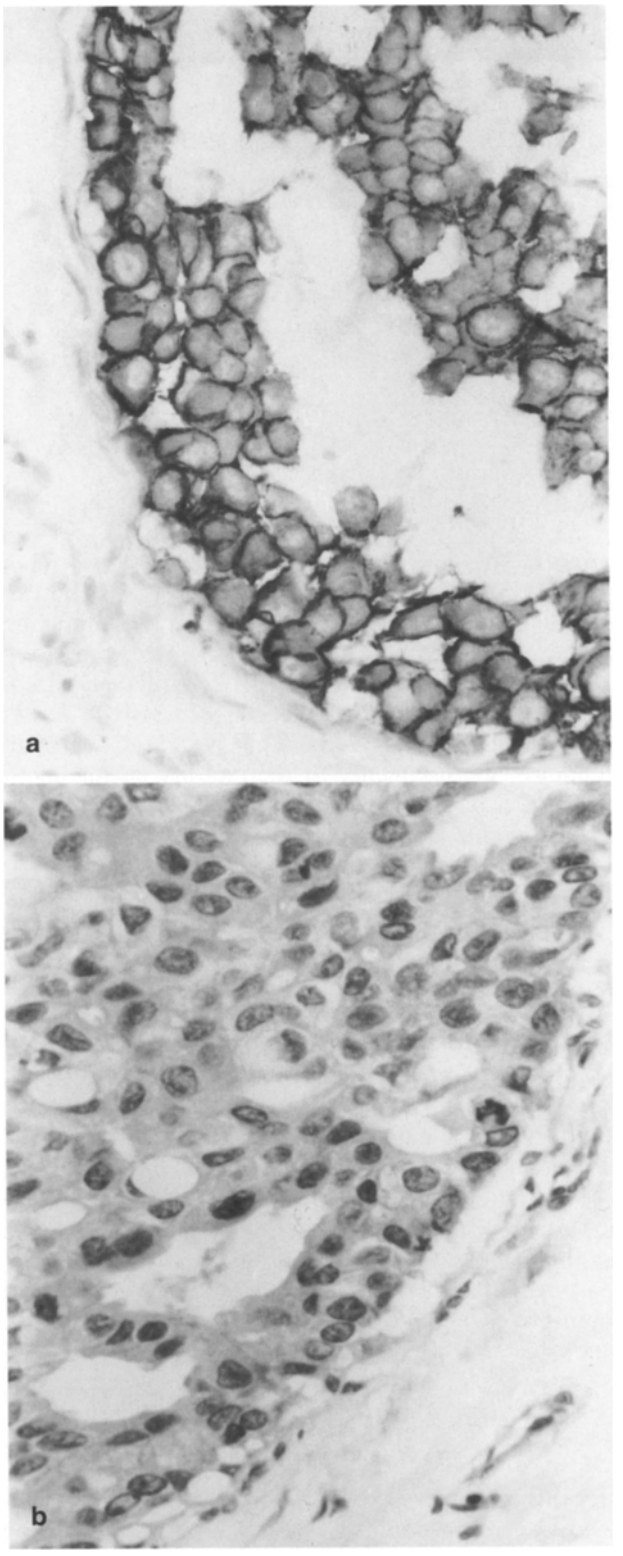

Fig. 2a Ductal carcinoma in situ immunostained for the *neu*-protein. All tumour cells show distinct expression at the plasma membrane. **b** These in situ tumours are typically composed of large cells with abundant cytoplasm. Usually the nuclei are pleomorphic. (Haematoxylin and eosin ×440)

in about 20% of all breast cancers and in 25% of invasive duct cell carcinomas [17]. No overexpression has been described in the invasive lobular carcinoma nor in the in situ lobular carcinoma [31, 59).

In order to investigate overexpression by immunohistochemistry, it should be kept in mind that only membrane staining can be considered as a reliable sign of *neu*-overexpression (Figs. 1, 2). Several authors have observed a cytoplasmic reactivity by light microscopy, although the *neu*-protein is expected to be a plasma membrane protein. This cytoplasmic distribution was demonstrated in kidney cells, oral mucosa, urothelium [29], normal breast tissue [17, 77] and breast carcinoma cells [7]. A cytoplasmic localization of a membrane bound receptor could be explained by internalization of the receptor, but it is difficult to understand why a substantial immunoreactivity can be detected in cells with a barely detectable *neu*-mRNA level as was described in some fetal tissues [37]. The detection of a mitochondrial protein, different from the *neu*-protein but cross-reacting with antibodies directed against the intracellular domain of the *neu*-protein may lead to a false positive granular cytoplasmic staining, which has nothing to do with *neu*-overexpression [18, 68] (Fig. 3). The inconsistent interpretation of *neu*-positivity elucidates several discrepant findings on prognosis in the literature.

At the ultrastructural level, the distribution of the *neu*-protein is not diffuse, but resides on specific cell organelles. In the proximal tubule cells of the kidney, the normal expression was observed on the cell membrane of the microvilli of the brush border [18]. In *neu*-overexpressing breast carcinoma cells obtained from breast biopsy material, the protein was accumulated at plasma membrane protrusions [18]. Since plasma membrane protrusions such as microvilli, filopodia, lamellipodia and lobopodia, collectively designated as pseudopodia are obligatory for functions such as cell spreading, locomotion, cell motility and phagocytosis, it seems reasonable to suggest that the *neu*-protein is involved in these cellular functions.

Subsequently similar results on the distribution of the protein on the cell membrane were obtained from experimental data. In unstimulated *neu*-overexpressing SK-BR-3 human breast cancer cells, the *neu*-protein is similarly localized on small microvilli and plasma membrane protrusions throughout the cell surface as in breast biopsies and normal human glandular tissues. After stimulation of motility and chemotaxis, the small microvilli increase considerably in size. During this period, pseudopodia and long thin plasma membrane extensions are formed and aggregation of the *neu*-protein takes place on these enlarged membrane extensions [16].

The *neu*-protein and cell growth

From the earliest papers linking the *neu*-protein with poor prognosis and due to its similarity to EGF-R and its structural organization, it was postulated that the *neu*-protein should be a receptor for a growth factor. The introduction of the *neu*-protein into the genome by transfecting 3T3 cells not only led to an altered genotype with a malignant phenotype, but also resulted in an increased growth potential [22]. The production of a hybrid protein, consisting of the EGF-R extracellular, transmembrane and protein kinase C substrate domains linked to the intracellular tyrosine kinase and carboxyl terminal domain of the rat *neu* cDNA, has evoked a similar result. Upon transfection with this construct and after stimulation of this membrane molecule by EGF and TGFα, tyrosine phosphorylation of the chimeric receptor protein and stimulation of DNA synthesis, resulting in mitogenic and transforming properties of the transfected cells were obtained [40]. Furthermore, anti-*neu* antibodies, regardless of isotype exerted a selective cytostatic effect on the growth of *neu*-transformed cells [23]. In vivo treatment with anti-*neu* antibodies was able to significantly inhibit the tumorigenic growth of *neu*-transformed NIH/3T3 cells implanted into nude mice. The antibody treatment was also able to inhibit the growth of the rat neuroblastoma cells from which the *neu*-oncogene was initially isolated [24]. However, experiments with several putative ligands have led to other conclusions. These putative ligands induced differentiation and led to complete cessation of cell growth (see below).

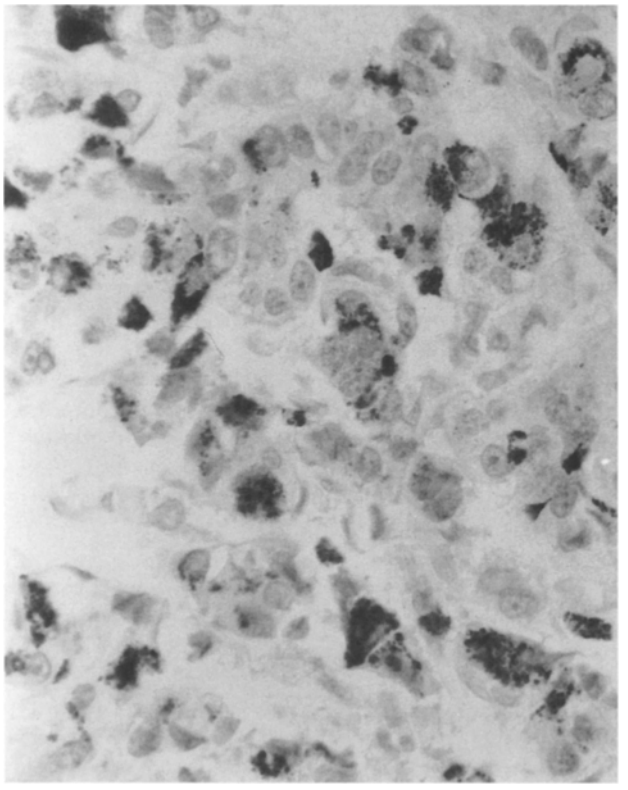

Fig. 3 Invasive lobular carcinoma, showing a granular cytoplasmic reaction after immunostaining for the *neu*-protein. This staining pattern should be considered as nonspecific and has nothing to do with *neu*-overexpression. It is frequently observed after immunohistochemistry with polyclonal and monoclonal antibodies directed against the intracellular domain of the *neu*-protein. (×440)

The *neu*-protein and cell motility

The precise morphological localization of the molecule on the cell membrane was helpful in elucidating its function in motility. The data obtained from experiments carried out with SK-BR-3 cells confirmed the initial morphological observations that the *neu*-protein exclusively resides on microvilli and pseudopodia [16]. The involvement of the *neu*-protein in cell motility is further supported by the detection of a 50 kDa *neu*-protein binding motility factor, which was purified from conditioned medium of malignant keratinocytes. This motility factor causes fast spreading, plasma membrane ruffling, cell differentiation, translocation, stimulation of chemotaxis and at high concentration growth arrest of SK-BR-3 cells. Motility and chemotaxis induced by the 50 kDa motility factor can be inhibited by antibodies directed against the extracellular domain of the *neu*-protein. The inhibition of the provoked motility again proves the function of the *neu*-protein in cell motility. The motility factor is considered as a *neu*-ligand, different from other, previously described putative ligands [15]. The association of the *neu*-protein with microfilaments via a large glycoprotein complex in mammary carcinoma microvilli is consistent with these findings [9].

Although it was generally accepted that the *neu*-protein would be a growth factor receptor, most experiments with putative ligands led to growth inhibition instead of growth stimulation. This finding seems to contrast with the oncogenic activity of the molecule. However, recently reported functions in cell motility are at least as important as a role in cell growth with regard to the oncogenic activity of the molecule. Cancer cells can only exhibit their invasive activity and the destruction of normal tissues by the action of motility factors and motility factor receptors. Both types of molecules render the tumour cell capable of penetrating the normal tissue and of spreading into the body, which may lead to the formation of metastases. The tissue specific routes of spread of *neu*-overexpressing tumour cells, namely, to the liver and not to the lymph nodes, can be explained by the action of specific motility factors secreted by particular organs and attracting the target cells which exhibit the particular receptor.

These statements are further supported by experiments carried out on mouse 3T3 cells, which were transfected with the mutation-activated rat *neu*-oncogene. The 3T3 cells exhibited mutatic properties both in vivo and in vitro, while parental 3T3 cells did not [87]. Monoclonal antibodies capable of inducing down regulation of the *neu*-protein as well as the adenovirus 5 E1A gene product can suppress the *neu*-induced transformation of 3T3 cells. Both abrogated the metastatic properties of the *neu*-transformed 3T3 cells [88].

The ligand

Several groups of researchers have dealt with one or more putative ligands for the *neu*-protein. Initially, by employing a series of biochemical assays and particularly by phosphorylation assays, conditioned media from *ras*-transformed fibroblasts were identified as sources of a possible ligand, which affected receptor down-regulation and tyrosine autophosphorylation of the *neu*-protein [86]. From *ras*-transformed fibroblast conditioned medium a 44 kDa *neu* stimulatory factor (NDF) was purified [53]. NDF is considered to be a differentiation factor, which induces altered morphology of breast cancer cells and synthesis of milk components [53]. Although NDF was considered as a specific ligand for the *neu*-protein after cross-linking experiments with radiolabelled NDF resulting in labelling of the *neu*-protein, the molecule is not able to affect tyrosine phosphorylation of *neu*-overexpressing ovarian cancer cells. Neither does NDF bind with high affinity to the ovarian cells, as would be expected from a ligand [54]. From these conflicting results it was concluded that NDF would require an unknown second cellular component in order to affect the *neu*-protein [54].

Almost simultaneously with the reports on NDF, heregulin was identified as a 45 kDa protein, purified from the conditioned medium of MDA-MB-231 human breast cancer cells [35]. Heregulin specifically induces tyrosine phosphorylation of the *neu*-protein. Heregulin is considered as the human equivalent of NDF. Both molecules behave similarly in bioassays. Heregulin has been identified as a ligand for the c-*erb*B-3 gene product [10]. It also activates tyrosine phosphorylation of HER4 and is considered as a specific ligand for HER4. It fails to induce phosphorylation of the *neu*-protein in the absence of HER4 [56, 57]. The second cellular component, necessary for binding NDF or heregulin onto the *neu*-protein seems to be the c-*erb*B-3 oncogene product. Coexpression of the *neu*-protein and the c-*erb*B-3 molecule reconstitutes a high affinity receptor for heregulin [71]. It has been shown that different NDF isoforms are generated by alternative splicing and perform distinct tissue-specific functions, such as differentiation of breast cells and growth of Schwann cells [79].

Several other candidate ligands for the *neu*-protein have been reported. One of these is the 50 kDa motility factor, mentioned above [15]. A 30 kDa factor secreted from MDA-MB-231 breast cancer cells was shown to be a candidate ligand for *neu* [42). At low concentration, the molecule stimulates growth of *neu*-overexpressing cells while it inhibits growth at higher concentration. Similar results on growth stimulation and inhibition were obtained with the 50 kDa motility factor [15]. A 25 kDa polypeptide purified from bovine kidney was also identified as a ligand for the *neu*-protein. This factor, designated NEL-GF was mutagenic for *neu*-overexpressing cells and stimulates autophosphorylation of the molecule [36]. Its relation with another 25 kDa peptide considered to be a *neu*-ligand and secreted by activated macrophages is unknown [73]. A *neu*-protein-specific activating factor (NAF) was partially purified from medium conditioned by the transformed human T-cell line ATL-2. NAF was able to stimulate tyrosine-specific kinase activity, induce

Table 1 Positive (+) or no (−) correlation of various prognostic factors with *neu*-oncogene amplification or *neu*-protein overexpression (*ER* oestrogen receptor status, *PR* progesteron receptor status, *LN* lymph node status, *OST* overall survival time)

First author of reference	Age	Premeno-pause	ER[a]	PR[a]	Grade	S-phase	LN	Aneu-ploidy	DFS	OST	Tumour size
Allred [1]	+	+	+	+	+						
Anbazaghan [2]			+	+	+	+	+	+	+	+	
Barnes [5]			+	+	+		−		−	−	
Berger [7]			+		+		+				
Borg [8]			+		+		+		+		−
De Potter [19]			+	+			−		+		
Guérin[b] [28]							+				
Gusterson [31]							−				
Heatley [34]			+				+				
Lovekin [41]			+		+		−			+	
Marks [43]									+	+	
McCann [45]	−	−	−		+		−		+	+	−
Mizukami [47]			−	−			−	−	−		
Paterson[b] [52]									+		
Poller [58]			+								
Richter King[b] [61]							−				
Schroeter [65]	−		+		+		−		+	+	+
Seshadri [67]							+				
Slamon[b] [69]	−		−	−					+	+	−
Slamon [70]									+	+	
Van De Vijver [74]							−		−	+	+
Varley[b] [76]									+	+	
Wilbur [80]			+	+	+						
Wright [82]			+		+		−		+	+	−
Zeillinger [89]			+	+							
Zhou[b] [90]			−	−			−		−		−
Zhou[b] [91]	−		−		−		−		+		−

[a] + means inverse correlation between the factor and *neu*-expression
[b] Southern blot

dimerization and internalization, and increase the growth of cells bearing the *neu*-protein [25]. Since none of these molecules has been sequenced and cloned, the structure and relation with NDF or heregulin is unknown. It is still waiting for the full identification of a molecule which acts as an unequivocal ligand for the *neu*-protein.

The *neu*-protein and prognosis of breast cancer

The relationship between the *neu*-protein and prognosis of breast cancer has been examined extensively, with considerable attention to tumour recurrence and patient survival. From the earliest investigations, several authors found that overexpression of the *neu*-protein is an indicator for poor prognosis in breast cancer [7, 69, 76]. In some multivariate analyses *neu*-overexpression retained its prognostic significance, being second to the classical prognostic indicators in breast cancer such as the number of invaded axillary lymph nodes, tumour size, tumour differentiation and receptor status [30, 75). According to some investigators, *neu*-overexpressing tumours seem to behave worse particularly the first years after diagnosis [19, 75], and these patients suffer from a shorter disease free survival time and shorter overall survival time between the 3rd and 4th year after diagnosis [65, 75]. The difference in prognosis seems to vanish from the 5th year after diagnosis. However, other authors failed to find a significant relation between overall survival time, recurrence rate and the *neu*-protein, when examined in a multivariate analysis. The most relevant prognostic factors and their relation with *neu*-overexpression are summarized in Table 1.

A strong negative association between *neu*-overexpression and oestrogen or progesteron receptor status has also been described [41, 65, 89]. The fact that most *neu*-overexpressing tumours lack steroid receptors can be explained on the one hand by the repressed expression of the *neu*-protein during oestrogen-induced proliferation and on the other by its enhanced expression during growth arrest and/or differentiation of mammary cells. Breast tumours constitutively expressing the *neu*-protein may escape oestrogen growth regulation and this condition may be associated with a particular invasive potential [14]. Experiments with human breast cancer cell lines indicate that oestrogen but not progestin decreases *neu*-protein expression [60].

Most authors did not consider any significant relationship between *neu*-overexpression and nodal status [17, 30, 75], but, haematogenous metastases, organ metastases and liver metastases in particular, are more frequently observed in *neu*-overexpressing breast cancer patients [19, 65].

Apart from its role in the assessment of prognosis, evaluation of the *neu*-protein in breast cancer patients from a clinical point of view might become important in

the future treatment of patients by chemotherapy. An analysis of molecular markers in breast cancer suggests that increasing the dose intensity of adjuvant chemotherapy may not result in a similar benefit for all patients with positive nodes. It appears that patients whose tumours overexpress the *neu*-protein may have the greatest benefit from higher dosage of chemotherapy. Among patients who received 6 months of chemotherapy, disease free survival was significantly longer when the tumours did not express *neu*-protein. There is a significant dose-response effect of adjuvant chemotherapy with cyclophosphamide, doxorubicin and fluorouracil in patients with overexpression of the *neu*-protein but not in patients with no *neu*-overexpression. Overexpression of the *neu*-protein may therefore be a useful marker to identify the patients who are most likely to benefit from high doses of adjuvant chemotherapy [50].

The *neu*-protein and DCIS

The expression of the *neu*-protein in DCIS has been investigated by several authors. A correlation with comedo-subtype was reported [6, 75]. A relation between *neu*-overexpression and DCIS, characterized by pleomorphic nuclei and large cell type was also found [6, 21, 49]. Recently, a highly significant relationship between *neu*-overexpression, cell type and extent of disease is described [21]. The extent of disease of the *neu*-positive DCIS is significantly larger than the extent of disease of the *neu*-negative cases. *neu*-positive DCIS always belongs to the large cell group and never to the small cell group [21] (Fig. 2). According to these authors, the combination of large cell type DCIS and *neu*-overexpression particularly, is highly correlated with tumour size and might therefore be of value in predicting recurrence after tumorectomy, due to occult DCIS in the remaining breast tissue.

This last hypothesis remains to be investigated in future prospective studies.

The *neu*-protein and Paget's disease of the breast

In Paget's disease of the breast, the epidermis of the nipple shows invasion by large neoplastic cells with a large nucleus and ample pale-staining cytoplasm. They are devoid of intercellular bridges [3]. It is most widely accepted that the intra-epidermal Paget cells originate from an underlying ductal carcinoma [51]. The cytoplasmic expression of low molecular weight cytokeratin in Paget cells and the absence of high molecular weight cytokeratin correspond with the glandular origin of these malignant cells. Low molecular weight cytokeratins are characteristic of simple and glandular epithelia and of tumour cells derived therefrom, while high molecular weight cytokeratins are cytoskeletal proteins of cornifying keratinocytes and of tumour cells originating from this epithelium [12, 27, 48].

Overexpression of the *neu*-protein was found in an unusually high percentage of cases in Paget's disease. Almost a 100% positivity for the *neu*-protein has been described in Paget's disease of the breast [39, 46, 81]. The unusually high expression of an oncogene in a particular disease is highly suggestive for its causative role in the pathogenesis of this disease. Therefore, it was hypothesized that the *neu*-protein migh play an important role in the pathogenesis of Paget's disease and that it might induce the spreading of the Paget cells into the epidermis.

Experiments with *neu*-overexpressing SK-BR-3 human breast cancer cells have proven that the Paget breast cancer cells spread into the epidermis because of the activity of a chemotactic factor, which is released by the epidermal cells and acts through the *neu*-protein. The chemotactic motility factor was able to induce motility of the *neu*-overexpressing SK-BR-3 cells. The factor secreted in the conditioned medium by the normal keratinocytes also provoked chemotaxis of SK-BR-3 cells and not of MCF-7 cells which are human breast carcinoma cells lacking *neu*-overexpression. Again, antibodies against the extracellular domain of the *neu*-protein were able to inhibit the chemotactic activity of the motility factor in a specific way. Taken together, these experiments suggest that the chemotactic activity of the epidermal motility factor is mediated through the *neu*-protein. The action of the epidermal motility factor through the *neu*-protein explains the pathogenesis of Paget's disease of the breast [20].

The *neu*-protein and other malignancies

neu-overexpression was found in tumours other than breast tumours. In ovarian adenocarcinomas amplification of the *neu*-gene detected by southern blotting was described in 26% of cases, which correlated with amplification detected by immunohistochemistry. Similarly to the correlation of prognosis and *neu*-overexpression in breast cancer, a statistically significant correlation between gene amplification and survival was found [70]. Again, other authors failed to demonstrate any independently adverse effect [32].

In pancreatic epithelial malignancies, *neu*-overexpression evidenced as membrane staining was only noticed in 2% of cases [33]. However, a 45% *neu*-immunoreactivity was described recently, especially in well differentiated adenocarcinomas of the pancreas [85]; the first group [33] considered cytoplasmic staining to be non-specific, the latter also included cytoplasmic staining as a sign for *neu*-positivity. *neu*-overexpression is not only confined to adenocarcinomas [72]. Membrane staining was also observed in 2–36% of bladder carcinomas and in 1–36% of squamous cell carcinomas of the bronchus [44, 78, 83]. By Southern blot *neu*-expression was shown to be very rare in small cell lung cancer [64].

Conclusion

Immunohistochemical expression of the *neu*-protein on the tumour cell membrane in nearly 20% of breast carci-

nomas is correlated with a decreased overall and disease free survival and a negative oestrogen and progesterone receptor status. *neu*-overexpression may increase the metastatic potential due to a growth enhancing effect and, particularly, to a motility stimulating effect. The *neu*-protein plays a crucial role in the pathogenesis of Paget's disease of the breast and may predict larger extent of disease in DCIS.

References

1. Allred DC, Clark GM, Molina R, Tandon AK, Schnitt SJ, Gilchrist KW, Osborne CK, Tormey DC, McGuire WL (1991) Overexpression of HER-2/*neu* and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. Hum Pathol 23:974–979
2. Anbazhagan R, Gelber RD, Bettelheim R, Goldhirsch A, Gusterson BA (1991) Association of c-*erb*B-2 expression and S-phase fraction in the prognosis of node positive breast cancer. Ann Oncol 2:47–53
3. Azzopardi JG (1979) Special problems in breast pathology. In: Azzopardi JG (ed) Problems in breast pathology. Saunders, London, pp 258–333
4. Bargmann CI, Hung M-C, Weinberg RA (1986) The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature 319:226–230
5. Barnes DM, Lammie GA, Millis RR, Gullick WL, Allen DS, Altman DG (1988) An immunohistochemical evaluation of c-*erb*B-2 expression in human breast carcinoma (1988) Br J Cancer 58:448–452
6. Bartkova J, Barnes DM, Millis RR, Gullick WJ (1990) Immunohistochemical demonstration of c-*erb*B-2 protein in mammary ductal carcinoma in situ. Hum Pathol 21:1164–1167
7. Berger MS, Locher GW, Saurer S, Gullick WJ, Waterfield MD, Groner B, Hynes NE (1988) Correlation of the c-*erb*B-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. Cancer Res 48:1238–1243
8. Borg A, Tandon AK, Sigurdsson H, Clark GM, Ferno M, Fuqua SA, Killander D, McGuire WL (1990) HER-2/*neu* amplification predicts poor survival in node-positive breast cancer. Cancer Res 50:4332–4337
9. Carothers Carraway CA, Carvajal ME, Li Y, Carraway KL (1993) Association of $p185^{neu}$ with microfilaments via a large glycoprotein complex in mammary carcinoma microvilli. J Biol Chem 268:5582–5587
10. Carraway KL, Sliwkowski MX, Akita R, Platko JV, Guy PM, Nuijens A, Diamonti JA, Vandlen RL, Cantley LC, Cerione RA (1994) The *erb*B3 Gene Products Is a Receptor for Heregulin. J Biol Chem 269:14303–14306
11. Connelly PA, Stern DF (1990) The epidermal growth factor receptor and the product of the *neu* protooncogene are members of a receptor tyrosine phosphorylation cascade. Proc Natl Acad Sci USA 87:6054–6067
12. Cooper D, Schermer A, Tung-Tien Sun (1985) Classification of human epithelia and their neoplasms using monoclonal antibodies to keratin: strategies, applications and limitations. Lab Invest 52:243–256
13. Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, Levinson A, Ullrich A (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 230:1132–1139
14. Dati C, Antoniotti S, Taverna D, Perroteau I, De Bortoli M (1990) Inhibition of c-*erb*B-2 oncogene expression by estrogens in human breast cancer cells. Oncogene 5:1001–1006
15. De Corte V, De Potter C, Vandenberghe D, Van Laerebeken, Azam M, Roels H, Mareel M, Vandekerckhove J (1994) A 50-kDa protein present in conditioned medium of COLO-16 cells stimulates cell spreading and motility and activates tyrosine phosphorylation of *neu*/Her-2 in human SK-BR-3 mammary cancer cells. J Cell Sci 107:405–416
16. De Potter CR, Quatacker J (1993) The $p185^{erbB2}$ protein is localized on cell organelles involved in cell motility. Clin Exp Metastasis 11:462–472
17. De Potter CR, Van daele S, Van de Vijver MJ, Pauwels C, Maertens G, De Boever J, Vandekerckhove D, Roels H (1989) The expression of the *neu*-oncogene product in normal fetal and adult human tissues. Histopathology 15:351–362
18. De Potter CR, Quatacker J, Maertens G, Van daele S, Pauwels C, Verhofstede C, Eechaute W, Roels H (1989) The subcellular localization of the *neu* protein in human normal and neoplastic cells. Int J Cancer 44:969–974
19. De Potter CR, Beghin C, Makar AP, Vandekerckhove D, Roels H (1990) The *neu*-oncogene product as a predictive factor for haematogenous metastases in breast cancer patients. Int J Cancer 45:55–58
20. De Potter CR, Eeckhout I, Geerts M-L, Schelfhout A-M, Roels H (1994) Keratinocyte induced chemotaxis in the pathogenesis of Paget's disease of the breast. Histopathology 24: 349–356
21. De Potter CR, Schelfhout A-M, Verbeeck P, Lakhani SR, Brünken R, Schroeter CA, Van den Tweel JG, Schauer AJ, Sloane JP (1995) *neu*-overexpression correlates with extent of disease in large cell ductal carcinoma in situ of the breast. Hum Pathol 26:(in press)
22. Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA (1987) *erb*B-2 is a potent oncogene when overexpressed in NIH/3T3 Cells. Science 237:178–182
23. Drebin JA, Link VC, Weinberg RA, Greene MI (1986) Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. Proc Natl Acad Sci USA 83:9129–9133
24. Drebin JA, Link VC, Greene MI (1988) Monoclonal antibodies specific for the *neu* oncogene product directly mediate anti-tumor effects in vivo. Oncogene 2:387–394
25. Dobashi K, Davis JG, Mikami Y, Freeman JK, Hamuro J, Greene MI (1991) Characterization of a *neu*/c-*erb*B-2 protein-specific activating factor. Proc Natl Acad Sci USA 88: 8582–8586
26. Fukushige S-I, Matsubara K-I, Yoshida M, Sasaki M, Suzuki T, Semba K, Toyoshima K, Yamamoto T (1986) Localization of a novel v-*erb*B-related gene, c-*erb*B-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol Cell Biol 6:955–958
27. Gabbiani G, Kapanci Y, Barazzone P, Franke WW (1981) Immunochemical identification of intermediate sized filaments in human neoplastic cells. A diagnostic aid for the surgical pathologist. Am J Pathol 104:206–216
28. Guérin M, Gabillot M, Mathieu M-C, Travagli J-P, Spielmann M, Andrieu N, Riou G (1989) Structure and expression of c-*erb*B-2 and EGF receptor genes in inflammatory and non-inflammatory breast cancer: prognostic significance. Int J Cancer 43:201–208
29. Gullick WJ, Berger MS, Bennett PLP, Rothbard JB, Waterfield MD (1987) Expression of the c-*erb*B-2 protein in normal and transformed cells. Int J Cancer 40:246–254
30. Gullick WJ, Love SB, Wright C (1991) c-*erb*B-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved nodes. Br J Cancer 63:434–438
31. Gusterson BA, Machin LG, Gullick WJ, Gibbs NM, Powles TJ, Price P, McKinna A, Harrison S (1988) Immunohistochemical distribution of c-*erb*B-2 in infiltrating and in situ breast cancer. Int J Cancer 42:842–845
32. Haldane JS, Hird V, Hughes CM, Gullick WJ (1990) c-*erb*B-2 oncogene expression in ovarian cancer. J Pathol 162:231–237
33. Hall PA, Hughes CM, Staddon SL, Richman PI, Gullick WJ, Lemoine NR (1990) The c-*erb*B-2 proto-oncogene in human pancreatic cancer. J Pathol 161:195–200
34. Heatley M, Maxwell P, Whiteside C, Toner PG (1993) c-*erb*B-2 oncogene product expression depends on tumour type and is

related to oestrogen receptor and lymph node status in human breast carcinoma. Pathol Res Pract 189:261–266
35. Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, Yansura D, Abadi N, Raab H, Lewis GD, Shepard HM, Kuang W-J, Wood WI, Goeddel DV, Vandlen RL (1992) Identification of heregulin, a specific activator of p185^{erbB2}. Science 256:1205–1210
36. Huang SS, Huang JS (1992) Purification and characterization of the *neu*/*erb*B2 ligand-growth factor from bovine kidney. J Biol Chem 267:11508–11512
37. Kokai Y, Cohen JA, Drebin JA, Greene MI (1987) Stage- and tissue-specific expression of the *neu* oncogene in rat development. Proc Natl Acad Sci USA 84:8498–8501
38. Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA (1989) Isolation and characterization of *ERB*B3, a third member of the *ERB*B/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc Natl Acad Sci USA 86:9193–9197
39. Lammie GA, Barnes DM, Millis RR, Gullick WJ (1989) An immunohistochemical study of the presence of c-*erb*B-2 protein in Paget's disease of the nipple. Histopathology 15: 505–514
40. Lehväslaiho H, Lehtola L, Sistonen L, Alitalo K (1989) A chimeric EGF-R-*neu* proto-oncogene allows EGF to regulate *neu* tyrosine kinase and cell transformation. EMBO J 8:159–166
41. Lovekin C, Ellis IO, Locker A, Robertson JFR, Bell J, Nicholson R, Gullick WJ, Elston CW, Blamey RW (1991) c-*erb*B-2 oncoprotein expression in primary and advanced breast cancer. Br J Cancer 63:439–443
42. Lupu R, Colomer R, Zugmaier G, Sarup J, Shepard M, Slamon D, Lippman ME (1990) Direct interaction of a ligand for the *erb*B2 oncogene product with the EGF receptor and p185^{erbB2}. Science 249:1552–1555
43. Marks JR, Humphrey PA, Wu K, Berry D, Bandarenko N, Kerns BJ, Iglehart JD (1994) Overexpression of p53 and HER-2/*neu* proteins as prognostic markers in early stage breast cancer. Ann Surg 219:332–341
44. McCann A, Dervan PA, Johnston PA, Gullick WJ, Carney DN (1990) c-*erb*B-2 oncoprotein expression in primary human tumors. Cancer 65:88–92
45. McCann AH, Dervan PA, O'Regan M, Codd MB, Gullick WJ, Tobin BMJ, Carney DN (1991) Prognostic significance of c-*erb*B-2 and estrogen receptor status in human breast cancer. Cancer Res 51:3296–3303
46. Meissner K, Rivière A, Haupt G, Löning T (1990) Study of *neu*-protein expression in mammary Paget's disease with and without underlying breast carcinoma and in extramammary Paget's disease. Am J Pathol 137:1305–1309
47. Mizukami Y, Nonomura A, Yamada T, Kurumaya H, Hayashi M, Koyasaki N, Taniya T, Noguchi M, Nakamura S, Matsubara F (1990) Immunohistochemical demonstration of growth factors, TGFα, TGFb, IGF-I and *neu* oncogene product in benign and malignant human breast tissues. Anticancer Res 10:1115–1126
48. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31:11–24
49. Mooi WJ, Peterse JL (1992) Progress in molecular biology of breast cancer. Eur J Cancer 28:623–625
50. Muss HB, Thor AD, Berry DA, Kute T, Liu ET, Koerner F, Cirrincione CT, Budman DR, Wood WC, Barcos M, Henderson IC (1994) c-*erb*B-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. N Engl J Med 330:1260–1266
51. Paone JF, Baker RR (1981) Pathogenesis and treatment of Paget's disease of the breast. Cancer 48:825–829
52. Paterson MC, Dietrich KD, Danyluk J, Paterson AH, Lees AW, Jamil N, Hanson J, Jenkins H, Krause BE, McBlain WA (1991) Correlation between c-*erb*B-2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Res 51:556–567
53. Peles E, Bacus SS, Koski RA, Lu HS, Wen D, Ogden SG, Ben Levy R, Yarden Y (1992) Isolation of the *neu*/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell 69:205–216
54. Peles E, Ben-Levy R, Tzahar E, Liu N, Wen D, Yarden Y (1993) Cell-type specific interaction of *neu* differentiation factor (NDF/heregulin) with *neu*/HER-2 suggests complex ligand-receptor relationships. EMBO J 12:961–971
55. Plowman GD, Whitney GS, Neubauer MG, Green JM, McDonald VL, Todaro GJ, Shoyab M (1990) Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. Proc Natl Acad Sci USA 87:4905–4909
56. Plowman GD, Culouscou J-M, Whitney GS, Green JM, Carlton GW, Foy L, Neubauer MG, Shoyab M (1993) Ligand-specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. Proc Natl Acad Sci USA 90:1746–1750
57. Plowman GD, Green JM, Culouscou J-M, Carlton GW, Rothwell VM, Buckley S (1993) Heregulin induces tyrosine phosphorylation of HER4/p180*erb*B4. Nature 366:473–475
58. Poller DN, Snead DRJ, Roberts EC, Galea M, Bell JA, Gilmour A, Elston CW, Blamey RW, Ellis IO (1993) Oestrogen receptor expression in ductal carcinoma in situ of the breast: relationship to flow cytometric analysis of DNA and expression of the c-*erb*B-2 oncoprotein. Br J Cancer 68:156–161
59. Porter PL, Garcia R, Moe R, Corwin DJ, Gown AM (1991) c-*erb*B-2 oncogene protein in in situ and invasive lobular breast neoplasia. Cancer 68:331–334
60. Read LD, Keith D, Slamon DJ, Katzenellenbogen BS (1990) Hormonal modulation of HER-2/*neu* protooncogene messenger ribonucleic acid and p185 protein expression in human breast cancer cell lines. Cancer Res 50:3947–4951
61. Richter King C, Kraus MH, Aaronson SA (1985) Amplification of a novel v-*erb*B-related gene in human mammary carcinoma. Science 229:974–976
62. Schechter AL, Stern DF, Vaidyanathan L, Decker SJ, Drebin JA, Greene MI, Weinberg RA (1985) The *neu* oncogene: an *erb*-B-related gene encoding a 185,000-Mr tumour antigen. Nature 312:513–516
63. Schechter AL, Hung M-C, Vaidyanathan L, Weinberg RA, Yang-Feng TA, Francke U, Ullrich A, Coussens L (1985) The *neu* gene: an *erb*B-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science 229:976–978
64. Schneider PM, Hung M-C, Chiocca SM, Manning J, Zhao X, Fang K, Roth JA (1989) Differential expression of the c-*erb*B-2 gene in human small cell and non-small cell lung cancer. Cancer Res 49:4968–4971
65. Schroeter CA, De Potter CR, Rathsmann K, Willighagen RGJ, Greep JC (1992) c-*erb*B-2 positive breast tumours behave more aggressively in the first years after diagnosis. Br J Cancer 66:728–734
66. Semba K, Kamata N, Toyoshima K, Yamamoto T (1985) A v-*erb* related protooncogene, c-*erb*B-2, is distinct from c-*erb*B-1/epidermal growth factor receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc Natl Acad Sci USA 82:6497–6501
67. Seshadri R, Matthews C, Dobrovic A, Horsfall DJ (1989) The significance of oncogene amplification in primary breast cancer. Int J cancer 43:270–272
68. Singleton TP, Niehans GA, Gu F, Litz CE, Hagen K, Qiu Q, Kiang DT, Strickler JG (1992) Detection of c-*erb*B-2 activation in paraffin-embedded tissue by immunohistochemistry. Hum Pathol 23:1141–1150
69. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177–181
70. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF (1989) Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science 244:707–712

71. Sliwkowski MX, Schaefer G, Akita RW, Lofgren JA, Fitzpatrick VD, Nuijens A, Fendley BM, Cerione RA, Vandlen RL, Carraway III KL (1994) Coexpression of *erb*B2 and *erb*B3 proteins reconstitutes a high affinity receptor for heregulin. J Biol Chem 269:14661–14665
72. Tal M, Wetzler M, Josefberg Z, Deutsch A, Gutman M, Assaf D, Kris R, Shiloh Y, Givol D, Schlessinger J (1988) Sporadic amplification of the HER2/*neu* protooncogene in adenocarcinomas of various tissues. Cancer Res 48:1517–1520
73. Tarakhovsky A, Zaichuk T, Prassolov V, Butenko ZA (1991) A 25 kDa polypeptide is the ligand for p185*neu* and is secreted by activated macrophages. Oncogene 6:2187–2196
74. Van De Vijver MJ, Mooi WJ, Wisman P, Peterse JL, Nusse R (1988) Immunohistochemical detection of the *neu* protein in tissue sections of human breast tumors with amplified *neu* DNA. Oncogene 2:175–178
75. Van de Vijver MJ, Peterse JL, Mooi WJ, Wisman P, Lomans J, Dalesio O, Nusse R (1988) *neu* protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N Engl J Med 319:1239–1245
76. Varley JM, Swallow JE, Brammar WJ, Whittaker JL, Walker RA (1987) Alterations to either c-*erb*B-2 (*neu*) or c-*myc* proto-oncogenes in breast carcinomas correlate with poor short-term prognosis. Oncogene 1:423–430
77. Venter DJ, Tuzi NL, Kumar S, Gullick WJ (1987) Overexpression of the c-*erb*B-2 oncoprotein in human breast carcinomas: immunohistological assessment correlates with gene amplification. Lancet 2:69–72
78. Weiner DB, Nordberg J, Robinson R, Nowell PC, Gazdar A, Greene MI, Williams WV, Cohen JA, Kern JA (1990) Expression of the *neu* gene-encoded protein (P185^{neu}) in human non-small cell carcinomas of the lung. Cancer Res 50:421–425
79. Wen D, Suggs SV, Karunagaran D, Liu N, Cupples RL, Luo Y, Janssen AM, Ben-Baruch N, Trollinger DB, Jacobsen VL, Meng S-Y, Lu HS, Hu S, Chang D, Yang W, Yanigahara D, Koski RA, Yarden Y (1994) Structural and functional aspects of the multiplicity of *neu* differentiation factors. Mol Cell Biol 14:1909–1919
80. Wilbur DC, Barrows GH (1993) Estrogen and progesteron receptor and c-*erb*B-2 oncoprotein analysis in pure in situ breast carcinoma: an immunohistochemical study. Mod Pathol 6:114–120
81. Wolber RA, Dupuis BM, Wich MR (1991) Expression of c-*erb*B-2 oncoprotein in mammary and extramammary Paget's disease. Am J Clin Pathol 96:243–247
82. Wright C, Angus B, Nicholson S, Sainsbury JRC, Cairns J, Gullick WJ, Kelly P, Harris AL, Horne CHW (1989) Expression of c-*erb*B-2 oncoprotein: a prognosis indicator in human breast cancer. Cancer Res 49:2087–2090
83. Wright C, Mellon K, Neal DE, Johnston P, Corbett IP, Horne CHW (1990) Expression of c-*erb*B-2 protein product in bladder cancer. Br J Cancer 62:764–765
84. Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, Saito T, Toyoshima K (1986) Similarity of protein encoded by the human c-*erb*B-2 gene to epidermal growth factor receptor. Nature 319:230–234
85. Yamanaka Y, Friess H, Kobrin MS, Büchler M, Kunz J, Beger HG, Korc M (1993) Overexpression of HER2/*neu* oncogene in human pancreatic carcinoma. Hum Pathol 24:1127–1134
86. Yarden Y, Weinberg RA (1989) Experimental approaches to hypothetical hormones: detection of a candidate ligand of the *neu* protooncogene. Proc Natl Acad Sci USA 86:3179–3188
87. Yu D, Hung M-C (1991) Expression of activated rat *neu* oncogene is sufficient to induce experimental metastasis in 3T3 cells. Oncogene 6:1991–1996
88. Yu D, Hamada J, Zhang H, Nicolson GL, Hung M-C (1992) Mechanisms of c-*erb*B2/*neu* oncogene-induced metastasis and repression of metastatic properties by adenovirus 5 E1A gene products. Oncogene 7:2263–2270
89. Zeillinger R, Kury F, Czerwenka K, Kubista E, Sliutz G, Knogler W, Huber J, Zielinski C, Reiner G, Jakesz, Staffen A, Reiner A, Wrba F, Spona J (1989) HER-2 amplification, steroid receptors and epidemal growth factor receptor in primary breast cancer. Oncogene 4:109–114
90. Zhou D, Battifora H, Yokota J, Yamamoto T, Cline MJ (1987) Association of multiple copies of the c-*erb*B-2 oncogene with spread of breast cancer. Cancer Res 47:6123–6125
91. Zhou DJ, Ahauja H, Cline MJ (1989) Proto-oncogene abnormalities in human breast cancer: c-*erb*B-2 amplification does not correlate with recurrence of disease. Oncogene 4:105–108