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Investigation of the prognostic value of coexpressed erbB family members for the survival of colorectal cancer patients after curative surgery

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Abstract

Epidermal growth factor receptor (EGFR), erbB2, erbB3 and erbB4 are four transmembrane glycoproteins belonging to the subtype I tyrosine kinases. They share structure homologies and are believed to direct cellular growth through the ligand-stimulated tyrosine phosphorylation of intracellular substrate. The overexpression of these tyrosine kinases has been linked to various cancers. To examine the role of the erbB family in the neoplastic transformation of the human colon, we analysed the protein expression of these four members by immunohistochemistry in paraffin-embedded specimens from 125 resected colorectal cancers. Our data showed that for EGFR expression, 62 (50%) were scored as '+', and 2 (2%) as '++'. For erbB2 expression, 39 (31%) were classified as '+', and 5 (4%) as '++'. For erbB3 expression, 43 (34%) were scored as '+', and 3 (2%) as '++'. A significantly higher percentage of overexpressed erbB3 was observed in early stage carcinomas (Dukes' stage A or B) (50%) than in advanced stage cancers (Dukes' stage C or D) (15%) ($P<0.0001$). For erbB4 expression, 22 (18%) were scored as '+', and 5 (4%) as '++'. Early stage patients had a lower percentage of erbB4 overexpression than the late stage ones (18% versus 28%). Concomitant overexpression of erbB2 and erbB3 occurred in 21% (16/78) of the early stage carcinomas, whereas it occurred in only 2% (1/47) of the late stage ones ($P=0.003$). Conversely, simultaneous overexpression of erbB2 and erbB4 occurred in 17% (8/47) of the late stage carcinomas but in only 4% (3/78) of the early stage ones ($P=0.02$). Overexpression of EGFR, erbB2, erbB3 or erbB4 alone was not significantly associated with a shortened survival. However, patients with a simultaneous overexpression of erbB2 and erbB4 had a shorter overall survival time than others in the univariate analysis ($P=0.01$). This significance disappeared after adjustment for Dukes' staging in the Cox model. In conclusion, overexpressed erbB3 was common in early stage colorectal cancers, but its prevalence was significantly reduced in late stage ones. The percentage of its coexpression with erbB2 was significantly higher in early stage than in late-stage cancers. Heterodimerisation between erbB2 and erbB4 may play a role in the late stages of carcinogenesis.

Keywords: Colorectal cancer; Subtype I receptor tyrosine kinase; Epidermal growth factor receptor; erbB2; erbB3; erbB4; Heterodimer

1. Introduction

The epidermal growth factor receptor (EGFR), erbB2 (also known as HER-2), erbB3 (HER-3) and erbB4 (HER-4) are a group of subtype I tyrosine kinases sharing structural homologies, especially at the intracellular domain. They are believed to direct cellular growth through the ligand-stimulated tyrosine phosphorylation of intracellular substrate [1]. Expression of EGFR has been investigated in different types of cancer [2–6] and its overexpression has been found to be related to a poor prognosis in breast [2], endometrial [5] and cervical cancers [6]. Recent studies have suggested that the *erbB2* oncogene product is involved in the development of human malignant tumours such as breast [7–9], ovary [9] and gastric carcinomas [10], and its overexpression in these kinds of cancers has been shown to be associated with an earlier recurrence and shortened

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survival. Both the *erbB3* and *erbB4* gene products were discovered to be receptors for heregulin or neu differentiated factor (NDF) [11,12]. These interactions can promote tumour progression. One study had shown that the *erbB3* gene is amplified in a subset of human adenocarcinomas [13]. However, the relationship of the expression of the erbB3 and erbB4 proteins to the growth of human cancers remains largely unknown [11–15]. Recent analyses of interreceptor interactions within the erbB family revealed that NDF-occupied erbB3 or erbB4 are able to form heterodimers with erbB2 and thereby accelerate its phosphorylation on tyrosine residues and promote cancer growth [11,16]. It was further shown that the affinity of erbB4 to NDF is much higher than the affinity of erbB3 to this ligand [16]. Similar heterodimers of erbB2 with EGFR not only bind epidermal growth factor, but also display elevated ligand affinity and kinase activity [17]. Whether the simultaneous overexpression of erbB family members could result in the progression of carcinogenesis remains largely unexplored.

Colorectal cancer is one of the most common types of cancer in Western societies and Taiwan. Colorectal cancers is the second most common cause of cancer mortality in the Western world and is ranked third in Taiwan. However, there have been only a few studies exploring the expression of the subtype I receptor tyrosine kinases in colorectal cancers and their roles in the progression of carcinogenesis [4,18–25]. This study was designed to investigate the expression of the erbB family and interreceptor interactions in colorectal cancers and observe their roles in predicting patients' prognosis.

2. Patients and methods

2.1. Patients and tumour specimens

125 paraffin-embedded colorectal cancer specimens resected at the Department of Surgery, National Cheng Kung University Hospital from 1989 to 1993 were studied. These specimens belonged to consecutive patients collected from chronological hospital records both retrospectively and non-selectively. None of the patients had received chemotherapy or radiation therapy before surgery. The patients were staged with a modified Dukes system (Astler–Coller modification) [26] as follows: 78 patients (62%), early stage colorectal cancers: (5 patients (4%): Dukes' class A, T1NoMo International Union Against Cancer (UICC)-TNM classification) [27,28]; 73 patients (58%), Dukes' class B, T_{2-4} NoMo); and 47 patients (38%) late-stage colorectal cancers: 29 patients (23%), Dukes' C, $TxN_{1-3}Mo$; 18 patients (14%), Dukes' D, TxNxM1). Postoperative adjuvant chemotherapy with 5-fluorouracil (5-FU) plus leucovorin (LV) was routinely administered to the patients with Dukes' stage C or D. All patients were regularly followed-up at the outpatient clinic after operation and survival data as of September 1998 were ascertained through hospital records. The median length of follow-up was 71 months (range 1–123 months).

2.2. Immunohistochemical staining

After an initial review of all the available haematoxylin and eosin-stained slides of surgical specimens, representative paraffin blocks for each case were selected for immunohistochemical study. They were then submitted for deparaffinisation. Monoclonal anti-erbB2 and EGFR antibodies (Triton Diagnostics, Alameda, CA, USA) were selected on the basis of their advantage in detecting the gene product in routinely processed tissue [29]. The immunostaining dilution and cross-reactivity have been previously described in Ref. [30]. Monoclonal anti-erbB3 (RTJ.2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was raised against cytoplasmic domains of the human erbB3 [31]. erbB4 was studied by using rabbit polyclonal antibody (Santa Cruz Biotechnology) raised against a peptide corresponding to amino acids 1291–1308 mapping at the carboxyl terminus of human erbB4. Optimal dilution for erbB3 (1:50) and erbB4 (1:20) was determined using colonic mucosa [32] and human kidney [12] as controls. Sections were first washed for 5 min with phosphate-buffered saline (pH7.2) and blocked with 0.1 mol/1 HCl for 20 min at room temperature. Then they were covered with 3% normal horse serum for 15 min. Primary antibodies to EGFR (1:2), erbB2 (1:10), erbB3 and erbB4 were incubated for 2 h at room temperature. The StrAviGen Super Sensitive MultiLink kit (BioGenex) was used to detect the resulting immune complex. The procedures of blocking, linkage and labelling of the binding reaction were carried out as per the manufacturer's instructions. The peroxidase activity was visualised by the 3,3′ diamino-benzidine tetrahydrochloride (Sigma Chemical Co, St. Louis, M0, USA) except that EGFR were demonstrated by aminoethyl carbazole substrate kit (Zymed Laboratory, San Francisco, CA, USA). Finally, sections was counterstained with haematoxylin.

EGFR, erbB2, erbB3, and erbB4 were mainly stained at the cell membrane, and occasionally in the cytoplasm. Only the membranous reaction was considered as positive. In evaluation of the expression of receptors, immunoreactivity was semiquantitatively graded. '−' indicated no immunoreactive cells. Those with reactivity in less than 20% of tumour cells and weak staining intensity were defined as '+'. Those with diffuse reactivity in no less than 20% of tumour cells and strong staining intensity were classified as '++'.

2.3. Statistical analysis

Association of the staining of various receptor tyrosine kinases with clinicopathological parameters was analysed using the Chi-square test. The interrelationship between subclass members was analysed with Fishers Exact Test. A P value of 0.05 or less was considered significant.

Survival was described using the Kaplan–Meier curve. The log-rank test was used to test for differences in the time to survival between subgroups. A multivariate analysis using the Cox's proportional hazards model was performed to investigate the independence of the risk factors identified as significant in the univariate analysis.

3. Results

The demographic and tumour characteristics of the 125 patients are summarised in Table. 1. Among these patients, 61 (49%) were negative for EGFR expression; 62 (50%) were scored as '+', and 2 (2%) as '++' (Fig. 1a and Table 2). For erbB2 expression (Fig. 1b), 81 (65%) were negative; 39 (31%) were classified as '+', and 5 (4%) as '++'. For erbB3 (Fig. 1c), 79 (63%) were negative; 43 (34%) were classified as '+', and 3 (2%) as '++'. For erbB4 (Fig. 1d), 98 (78%) were negative; 22 (18%) were classified as '+', and 5 (4%) as '++'. Due to the relatively small numbers of patients with '++' expression of EGFR, erbB2, erbB3 or erbB4, the patients were divided into two groups in the data analysis, negative versus positive '+' or '++'. The data showed erbB2 expression was associated with histological differentiation ($P=0.003$). 38% (43/112) of well and moderately-differentiated colon cancers showed '+' or '++' expression of the erbB2 gene product compared with 8% (1/13) of the poorly differentiated or mucinous adenocarcinomas (Table 3). The erbB3 expression was inversely significantly associated with tumour staging ($P<0.0001$) (Table 4). Otherwise, correlations between other clinicopathological indicators and the expression of these four subtype I tyrosine kinases were not statistically significant.

50% (39/78) of early stage patients had erbB3 overexpression compared with only 15% (7/47) of the late stage patients ($P<0.0001$). Early stage patients had a lower percentage of erbB4 overexpression than the late stage patients (18% versus 28%). With regard to the patterns of coexpression, concomitant overexpression of erbB2 and erbB3 occurred in 21% (16/78) of the early stage patients, but occurred in only 2% (1/47) of

Table 1
Frequency of prognostic factors investigated in colorectal carcinomas

Prognostic factors	No. of patients (%)
Dukes' category	
A	5 (4)
B	73 (58)
C	29 (23)
D	18 (14)
pT stage	
pT1	5 (4)
pT2	24 (19)
pT3	15 (12)
pT4	81 (65)
pN stage	
pN0	83 (66)
pN1	23 (18)
pN2 or pN3	19 (15)
Differentiation	
Moderate or well	112 (90)
Poor	13 (10)
Location	
Proximal colon	44 (35)
Rectum + sigmoid colon	81 (65)
Sex	
Male	71 (57)
Female	54 (43)
Age (years)	
<65	75 (60)
$\geqslant 65$	50 (40)

Table 2
Expression of subtype I receptor tyrosine kinases in 125 colorectal cancer patients

Immunoreactivity	EGFR	erbB2	erbB3	erbB4
	n (%)	*n* (%)	*n* (%)	*n* (%)
–	61 (49)	81 (65)	79 (63)	98 (78)
+	62 (50)	39 (31)	43 (34)	22 (18)
++	2 (2)	5 (4)	3 (2)	5 (4)

Table 3
Correlation of erbB receptor family expression with tumour differentiation

Parameter	Tumour differentiation		*P* value
	1[a]	2[b]	
EGFR (–)	53 (47)	8 (62)	0.39
(+ and ++)	59 (53)	5 (38)	
ErbB2 (–)	69 (62)	12 (92)	0.003
(+ and ++)	43 (38)	1 (8)	
ErbB3 (–)	70 (63)	9 (69)	0.77
(+ and ++)	42 (38)	4 (31)	
ErbB4 (–)	88 (79)	10 (77)	1.0
(+ and ++)	24 (21)	3 (23)	

EGFR, epidermal growth factor receptor.
[a] Well + moderately differentiated adenocarcinoma.
[b] Poorly differentiated + mucinous adenocarcinoma.

the late stage patients ($P=0.003$) (Table 5). Conversely, simultaneous overexpression of erbB2 and erbB4 occurred in 17% (8/47) of the late-stage patients and in only 4% (3/78) of the early-stage patients ($P=0.02$). Only 2 early-stage patients and 1 late-stage patient had simultaneous overexpression of erbB2, erbB3 and erbB4.

Table 4
Correlation of erbB receptor family expression with tumour staging

Parameter	Tumour staging		*P* value
	Early stage[a] *n* (%)	Late stage[b] *n* (%)	
EGFR (−)	35 (45)	26 (55)	0.27
(+ and ++)	43 (55)	21 (45)	
ErbB2 (−)	49 (63)	32 (68)	0.57
(+ and ++)	29 (37)	15 (32)	
ErbB3 (−)	39 (50)	40 (85)	<0.0001
(+ and ++)	39 (50)	7 (15)	
ErbB4 (−)	64 (82)	34 (72)	0.26
(+ and ++)	14 (18)	13 (28)	

EGFR, epidermal growth factor receptor.
[a] Dukes' A + Dukes' B.
[b] Dukes' C + Dukes' D.

Overexpression of EGFR, erbB2, erbB3 or erbB4 alone was not significantly associated with overall survival (data not shown). However, in univariate analysis, patients with simultaneous overexpression of erbB2 and erbB4 had a shorter overall survival (Fig. 2). However, this did not emerge as an independent prognostic factor, after adjustment for Dukes' staging in the multivariate analysis.

4. Discussion

The proportion of overexpressed EGFR in colorectal cancers differs in reports in the literature: 30% in Jong's series [22], 53.7% in Nakae's series [19] and 77.1% in Yasui's series [4], but the percentages are mostly in the range of 30–80%. In one report published by Mayer [18], all of the cases of colorectal cancer were positive for EGFR expression. However, their definition for 'positive staining' was based on results of cytoplasmic staining, which was different from others' and our

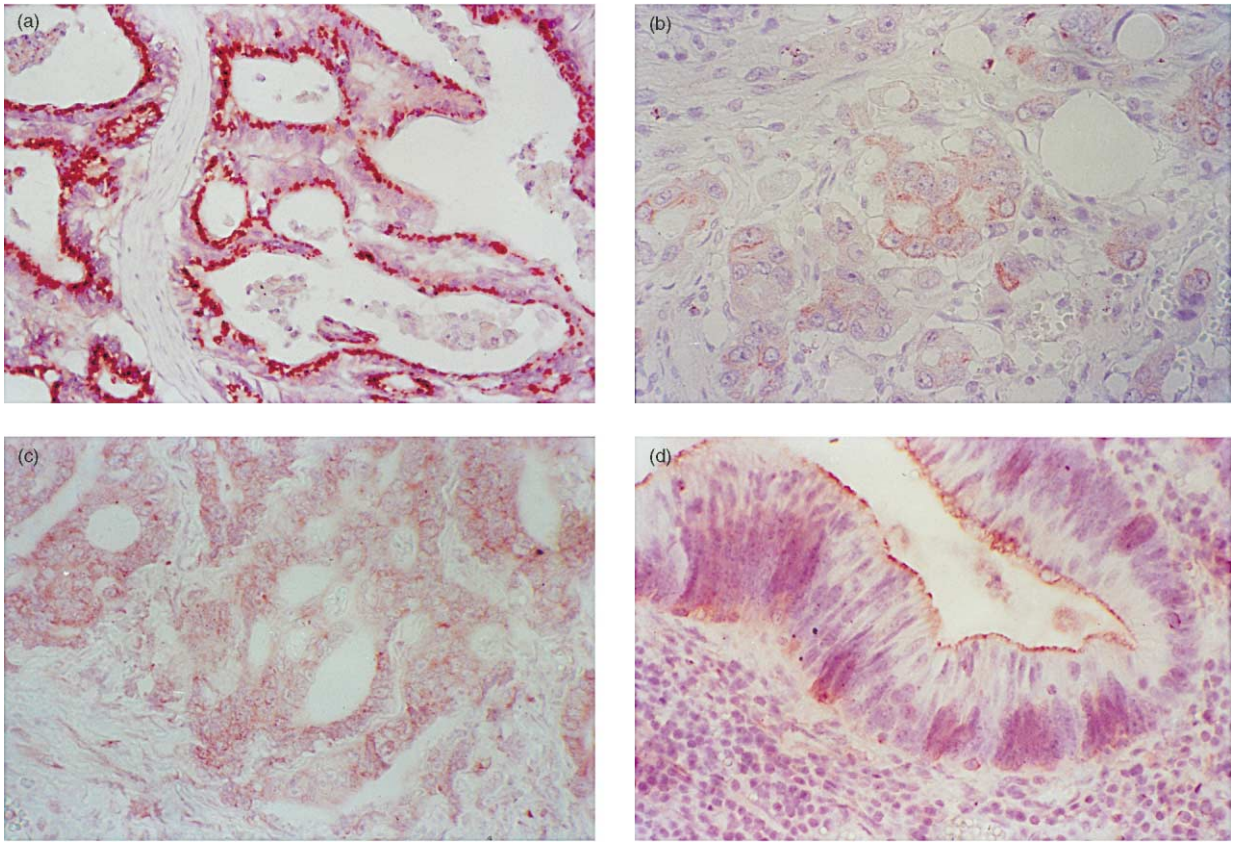

Fig. 1. (a) Epidermal Growth Factor Receptor (EGFR) immunostaining is revealed at the cell membrane and cytoplasm of a well-differentiated adenocarcinoma (original magnification 10×3). (b) The erbB2 is weakly stained at the cell membrane of poorly-differentiated carcinoma cells (original magnification 100×3). (c) Membranous and weakly cytoplasmic staining of erbB3 is present in a moderately differentiated adenocarcinoma and fibroblast cells (original magnification 100×3). (d) erbB4 is predominantly present at the luminal side of the cell membrane of the well-differentiated adenocarcinoma (original magnification 100×3).

Table 5
Concomitant overexpression of erbB family in colorectal cancer patients

	Early stage[a] *n* (%)	Late stage[b] *n* (%)	*P* value
erbB2 and erbB3	16/78 (21)	1/47 (2)	0.003
erbB2 and erbB4	3/78 (4)	8/47 (17)	0.02

[a] Dukes' A + Duke's B.
[b] Dukes' C + Duke's D.

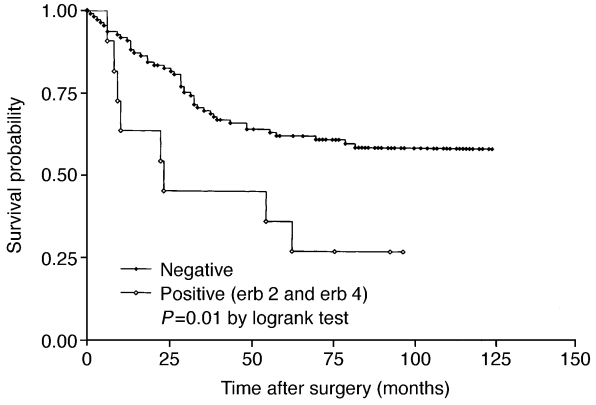

Fig. 2. Survival of colorectal cancer patients (after curative surgery) with coexpression of erbB2 and erbB4 compared with the patients without coexpression.

definition—where only a membranous reaction was regarded as positive. As for erbB2 expression in colorectal cancers, a 56.8% positive rate was reported in Nakae's series [19], 20% in D'Emilia's series [23], 89% in Maurer's series [20], and 35% in our series. There have been few studies on erbB3 expression, in colorectal cancers or in other cancers. Maurer and colleagues detected an 83% positive rate of erbB3 expression in colorectal cancer by immunohistochemistry using the RTJ 1 monoclonal antibody [20]. Rajkumar and colleagues revealed a 69% positive rate in colorectal cancers [31], and Chow and colleagues revealed a 41.1% positive rate in bladder cancers using the same antibody and same method [33]. 37% of our patients showed positive expression. 50% of the early-stage patients showed overexpression; yet only 15% of the late-stage patients showed positive expression. Regarding erbB4 expression in the clinical cancer sample, only Chow and colleagues reported a 10.7% positive rate in bladder cancer [33]. In our series, 22% showed overexpression of erbB4.

In this study, erbB2 overexpression was found to be associated with histological differentiation of colorectal cancer. This is in agreement with the results of Yamanaka and colleagues in pancreatic cancer patients [34]. The 'Neu differentiation factor', one of ligands binding to the heterodimer of erbB2 and erbB3 or erbB2 and erbB4, exerts a potent effect on cell differentiation [35]. Therefore, it is possible that the overexpression of erbB2 may induce the differentiation of colorectal and pancreatic carcinoma cells, thereby contributing to the morphogenesis of tubular structures in these tumours.

Overexpression of EGFR or erbB2 oncoproteins has been reported to be associated with a poorer prognosis in human breast cancers [2,7,8]. However, their roles in predicting prognosis are still controversial for other cancers, including lung [36,37] and ovarian cancers [38]. The role of EGFR in relation to the prognosis of colorectal cancer patients is also controversial, only two [21,25] of several immunohistochemical studies [18,19,21,24,25] have demonstrated a positive correlation between EGFR expression and pathological criteria of tumour aggressiveness. Moreover, both studies could not demonstrate that EGFR was an independent prognostic factor. Nakae and colleagues [19] found that the incidence of erbB2 protein overexpression in Dukes' D was significantly higher than that in Dukes' A to C colorectal cancers. However, D'Emilia and colleagues [23] obtained the opposite finding. Both studies could not demonstrate an independent role of erbB2 in predicting prognosis. In our series, no association was found between the overexpression of EGFR or erbB2 and prognosis.

The role of erbB3 or erbB4 in predicting the prognosis of colorectal cancer patients has not been reported. In our series, overexpression of erbB3 or erbB4 was not an independent prognostic factor for colorectal cancer patients. Overexpression of erbB3 occurred in half of the early-stage colon cancers and in only 15% of the late-stage cancers. Conversely, the incidence of overexpression of erbB4 tended to be higher in late stage than in early cancers. Quite interestingly, concomitant overexpression of erbB2 and erbB3 occurred in 21% of the early-stage patients and was rare in late-stage patients. Conversely, simultaneous overexpression of erbB2 and erbB4 occurred with a higher frequency in late-stage patients than in early-stage ones. This suggested that heterodimer formation of erbB2 and erbB3 may play an important role in the early-stages of carcinogenesis. However, in the late stages, heterodimerisation between erbB2 and erbB4 may have a more important role to play. As the affinity of erbB4 to NDF is much higher than the affinity of erbB3 to this ligand [16], the mitogenic and transforming potential of a heterodimer of erbB2 and erbB4 may be more potent than that of erbB2 and erbB3 or other heterodimers. Interestingly, in light of this theory, patients with overexpression of erbB2 and erbB4 had a worse prognosis. However, this overexpression did not emerge as an independent factor after adjustment for Dukes' staging in the multivariate analysis.

In conclusion, overexpressed erbB3 was common in early-stage colorectal cancers, but its prevalence was significantly reduced in late-stage ones. The percentage

of its coexpression with erbB2 was significantly higher in early stage than that in-late stage cancers. Heterodimerisation between erbB2 and erbB4 appeared to play a role in the late stages of carcinogenesis.

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