available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

HER-3 in colorectal tumourigenesis: From mRNA levels through protein status to clinicopathologic relationships

Petros D. Grivas[a], *Anna Antonacopoulou*[a], *Vassiliki Tzelepi*[b,c], *Georgia Sotiropoulou-Bonikou*[b], *Zinovia Kefalopoulou*[b,c], *Athanasios G. Papavassiliou*[d], *Haralabos Kalofonos*[a,*]

[a]*Division of Oncology and Clinical Oncology Laboratory, Department of Internal Medicine, Medical School, University of Patras, 26504 Patras, Greece*
[b]*Department of Anatomy and Histology-Embryology, Medical School, University of Patras, 26504 Patras, Greece*
[c]*Department of Pathology, Medical School, University of Patras, 26504 Patras, Greece*
[d]*Department of Biological Chemistry, Medical School, University of Athens, 11527 Athens, Greece*

ARTICLE INFO

Article history:
Received 4 June 2007
Accepted 13 August 2007
Available online 24 October 2007

Keywords:
HER-3
pHER-3
Colorectal cancer
Tumourigenesis
Immunohistochemistry

ABSTRACT

Introduction: Colorectal cancer is a major cause of cancer mortality in the Western world. Although HER-3 signalling is known to be implicated in colorectal carcinogenesis, the significance of its expression, localisation and phosphorylation remains elusive.
Methods: Quantitative RT-PCR for HER-3 mRNA and immunohistochemistry for HER-3 and phosphorylated HER-3 (pHER-3) protein were performed in normal tissue, adenomas and carcinomas from 140 patients with colorectal cancer.
Results: HER-3 was detected both in the cytoplasm and nucleus, whereas pHER-3 was observed in the nucleus and membrane of cells. A possible switch in HER-3 topography from the nucleus to the cytoplasm during colorectal tumourigenesis is suggested. The expression of pHER-3 did not differ significantly in normal tissue, adenomas and carcinomas, but was related to disease stage. HER-3 mRNA overexpression was significantly associated with decreased time to disease progression. It was also correlated with higher median age, left colon and rectal tumour sites and lymph node involvement.
Conclusion: We postulate that HER-3 is critically involved in colorectal tumourigenesis and its expression/phosphorylation might be of prognostic significance.

1. Introduction

Colorectal cancer is the fourth most common malignancy in the Western world and the second most frequent cause of cancer-related mortality. Despite recent advances in the field of clinical research, the efficacy of chemotherapy in the treatment of colorectal cancer has reached a plateau. Exploring the molecular mechanisms underlying cellular transformation and tumour progression has led to the identification of key molecules that are appropriate targets for sophisticated therapy with biological compounds.

The c-erbB family of receptor tyrosine kinases holds a critical role in the pathogenesis of colorectal cancer. It comprises four homologous members, ErbB-1, (EGFR), ErbB-2 (HER-2\neu), ErbB-3 (HER-3) and ErbB-4 (HER-4) that share common domains. Each receptor bears a cysteine-rich

* *Corresponding author:* Tel.: +30 2610 999535; fax: +30 2610 994645.
E-mail address: kalofon@med.upatras.gr (H. Kalofonos).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2007.08.019

extracellular domain, a transmembrane component and an intracellular region with tyrosine kinase activity. Despite their structural homology these receptors differ in their ligand specificities.[1] HER-2 is regarded as an orphan receptor since no HER-2 ligand has been identified yet.[2] Ligand binding results in homo-/heterodimerisation of the receptors, which are then phosphorylated in the intracellular domain, thus leading to a cascade of signalling pathways that govern critical cellular processes. HER-2 acts as the preferred heterodimeric partner of ligand-occupied complexes of the three other HER proteins. HER-2 containing heterodimers are potent activators of signalling due to increased stability and decreased degradation.[3]

Although these receptors have major physiologic roles and wide expression in epithelial, neuronal and mesenchymal tissues, they are also implicated in the pathogenesis of several carcinomas.[4] Overexpression of HER-2 has been associated with poor clinical outcome in breast cancer and increased expression of EGFR is correlated with advanced disease in colorectal, head and neck, stomach and lung cancer.[5,6]

HER-3 is a 180-Kd transmembrane glycoprotein that is encoded by a gene located on chromosome 12q13. Although HER-3 is catalytically deficient due to its impaired kinase activity,[7] it is capable of forming signalling-competent heterodimers with other HER members. It has been shown that the most potent receptor heterodimer is HER-2–HER-3,[3] frequently causing resistance to anticancer targeted therapy. EGFR–HER-3 dimers and HER-2–HER-3 dimers activate signalling pathways including mitogen-activated protein kinase (MAPK), stress-activated protein kinase (protein kinase C, Jak/Stat) and phosphoinositide-3 kinase (PI-3K)/Akt/m-TOR pathways.[1]

HER-3 has been found to be expressed in various organs, such as breast, lung, pancreas and stomach, but also in several tumours.[8–10] HER-3 was reported to be overexpressed in 20–30% of breast carcinomas,[11] while its expression in colorectal cancer ranges from 36 to 89%.[12,13] There is limited information regarding its prognostic role in colorectal cancer and data are controversial. Kapitanovic et al.[14] showed that HER-3 expression is related to decreased overall survival, a finding that was not confirmed by other studies.[12]

HER-2-mediated trans-phosphorylation of the kinase-inactive HER-3 seems to be related to drug resistance in HER-2 overexpressing breast carcinomas treated with trastuzumab (anti-HER-2).[15] Tyrosine trans-phosphorylated HER-3 is able to activate the PI-3K/Akt pathway, resulting in increased cell proliferation, growth, survival, adhesion and motility.[16] In non-small cell lung carcinoma (NSCLC), overexpression of HER-3, which seems to be transactivated by EGFR, is suggested to predict sensitivity to erlotinib (anti-EGFR).[17,18] In pancreatic and colorectal cancer cell lines, inactivation of Akt by erlotinib is mediated by HER-3 and contributes to erlotinib sensitivity.[19] These data suggest that HER-3 could serve as a useful tumour biomarker and that investigation of both phosphorylated and unphosphorylated HER-3 expression might be part of a comprehensive anticancer approach.

To shed light into the role of HER-3 in colorectal carcinogenesis, we studied HER-3 and phosphorylated HER-3 (pHER-3) protein expression by immunohistochemistry as well as HER-3 mRNA expression by real time RT-PCR in colorectal tumours. Potential correlations with clinicopathologic parameters including survival were also explored.

2. Patients and methods

2.1. Patients selection and evaluation

The present study comprised 140 surgical specimens of primary colorectal adenocarcinomas. Of these, 134 were sequential colectomy specimens resected from an equal number of patients at the University Hospital of Patras, Greece, between 1994 and 2006 and six were biopsy specimens. Furthermore, 110 adjacent normal mucosa specimens and 24 adenomas from the same cohort of patients were included in the study. Of 140 specimens, 54 were evaluated by RT-PCR and 119 by immunohistochemistry. Twenty one specimens were evaluated only by RT-PCR, due to inadequate material for immunohistochemistry. Data from the pathology records and clinical follow up were readily available for all patients. This study received ethical approval from the Local Research Ethics Committee at University Hospital of Patras according to the principles laid down by Declaration of Helsinki.

The patients' clinicopathologic characteristics are displayed in Table 1. The median follow up period was 53 months (7–153 months). During this period of time 46 deaths and 60 relapses were observed. The 3-year survival rate was 80.5% and the 5-year survival rate was 57.1%.

2.2. Immunohistochemistry

Immunohistochemistry was performed on 4-μm thick formalin-fixed, paraffin-embedded tissue sections. Antigen retrieval was performed for 15 min in 1 mM EDTA-NaOH (pH 8.0) in a microwave oven. Endogenous peroxidase was blocked with 3% H_2O_2 for 15 min at room temperature. For protein blocking, sections were incubated with 1% BSA (bovine serum albumin Fraction V, Serva Electrophoresis, Germany) for 30 min at room temperature. The sections were next incubated with primary antibodies, anti-ErbB-3 [Rabbit polyclonal, 1:150 (sc-285, Santa Cruz, Ca, USA)] or anti-phospho-HER-3/

Table 1 – Patients' clinicopathologic characteristics

Patients	140	
Age median (range)	66 (30–90)	
Stage	B	53
	C	75
	D	12
Grade	I	26
	II	100
	III	14
Gender	Male	91
	Female	49
Histologic type	Non mucinous	127
	Mucinous	13
Primary site	Right colon	35
	Left colon	58
	Rectal	47

ErbB3 [Rabbit monoclonal 1:100, Tyr1289 (21D3, Cell Signaling Technology, USA)], for 1 h at room temperature, followed by sequential 30-min incubation with Dako EnVision Labelled Polymer (Dako, CA, USA). Diaminobenzidine (Dako, CA, USA) was used as the chromogen. Nuclei were counterstained with haematoxylin. In negative control slides, the same method was performed and the primary antibody was substituted by 10% TBS.

2.3. Staining evaluation

Each slide was individually evaluated and scored in a blind fashion by two independent observers. Discrepancies in scoring between the observers were resolved by additional review of the slide and discussion until a consensus was achieved. Cytoplasmic and nuclear immunostaining of HER-3 were evaluated separately. Areas with the highest density of positive cells were selected at low power (×40) magnification. The number of positive stained cells and the total number of cells was determined by visual inspection of six different fields per section at 400× magnification. For each field, a percentage of positive cells was calculated and the average of those was taken. The intensity of staining was also evaluated and scored as 1+ (weak), 2+ (moderate) and 3+ (strong). For cytoplasmic HER-3 staining, intensity scores of 2+ and 3+ were considered overexpression. There was no variation in the intensity of nuclear staining and only cases with more than 25% of cells expressing nuclear HER-3 were considered positive. pHER-3 immunostaining was observed in the nucleus and cases with more than 5% of cells expressing pHER-3 were considered positive. Membranous pHER-3 immunostaining was regarded as positive when more than 1% of cells stained for anti-pHER-3 in the membrane.

2.4. Total RNA isolation and quantification

Total RNA was extracted from 54 of the formalin-fixed paraffin-embedded primary colorectal carcinoma tumour tissue specimens and 29 normal tissue controls, as described elsewhere.[20] Briefly, tissue sections were deparaffinised using xylene and lyzed with proteinase K in a Tris-HCl, EDTA and sodium dodecyl sulfate (SDS) containing buffer. RNA was subsequently purified using phenol-chloroform-isoamyl alcohol extraction and precipitated by isopropanol in the presence of sodium acetate and glycogen. Isolated RNA was treated with DNase I. DNA-free total RNA was then quantified using Ribogreen (Molecular Probes, Leiden, The Netherlands) and the MX3000p (Stratagene, La Jolla, USA) according to the manufacturer's instructions.

2.5. cDNA synthesis

First strand cDNA was synthesised from 1.6 μg total RNA from each sample using random nonamers (ITE, Crete, Greece) and 50 U Stratascript reverse transcriptase (Stratagene, La Jolla, USA). In addition, Human Reference RNA (Stratagene, La Jolla, USA) was used as a calibrator sample to allow adjustment for run-to-run variation. A no enzyme control was also included to ensure lack of DNA contamination.

2.6. RT-PCR

Quantification of HER-3 mRNA levels was performed using Brilliant Quantitative PCR Core Reagents (Stratagene, La Jolla, USA) with gene-specific primers (F:CAAGAGTGGACGGCAGAGT, R:GCCTTACAATGTGGGCATG and Taqman probe (6-carboxy-fluorescein-CTGTGACAGATCATATGCTGGCCA-BHQ1). Moreover, expressed Alu-Sq repeat levels were quantified using SYBR Green I intercalation dye in Brilliant Sybr Green QPCR Master Mix (Stratagene, La Jolla, USA) (Dr. Vandesompele, Gent University Hospital, Belgium). Primers were synthesised by the Foundation for Research and Technology-Hellas (Crete, Greece) and the probe by DNA Technology A/S (Aarhus C., Denmark). Reactions contained 5-carboxy-x-rhodamine (ROX) as a passive reference dye and cDNA equivalent to 100 ng of total RNA. In addition, a standard curve was included in each run for assay validation. PCR reactions were performed in triplicate in MX3000 (Stratagene) under the following conditions: 10 min at 95 °C (enzyme activation) followed by 45 cycles of 30 s at 95 °C (template denaturation), 1 min at 60 °C (annealing) and 30 s at 72 °C (extension). The relative expression level of HER-3 was deducted from the cycle number in the exponential phase of the PCR reaction, at which fluorescence was detected above background level, after normalisation to the cycle number obtained for the calibrator sample. Furthermore, HER-3 mRNA levels were normalised to Alu-Sq levels. Alu-Sq was validated to be a stably expressed repeat sequence in normal and tumour tissue of different grades and stages.

2.7. Statistical analysis

Intergroup comparisons, regarding correlation of clinicopathologic parameters with expression levels of HER-3 and pHER-3 were performed using Kruskal–Wallis and Mann–Whitney nonparametric tests for continuous or ordinal variables, and Chi square test for nominal variables. Because of multiple comparisons, these tests were followed by a post hoc Bonferroni test. Comparisons between related groups were performed using Wilcoxon paired samples test. Spearman rank correlation was used to detect any potential correlations between HER-3 protein and mRNA levels and between HER-3 and pHER-3 protein levels. Kaplan–Meier procedure was used to compare the survival and the time to disease progression rates. Cox hazard regression model was applied to assess the prognostic value of HER-3 protein and mRNA levels and pHER-3 protein levels in conjunction with clinicopathologic parameters. Data were analysed using the SPSS statistical package (SPSS©, Release 14.0.1, Chicago, IL, USA). The level of significance was set at p-value < 0.05.

3. Results

HER-3 and pHER-3 protein expression was initially assessed by immunohistochemistry in 119 carcinomas, 110 adjacent normal mucosa samples and 24 adenomas. HER-3 was detected in the cytoplasm and in the nucleus and was evaluated separately, while distinct complete membranous HER-3 staining was not observed.

Table 2 – HER-3 cytoplasmic staining intensity

	n total	Weak n (%)	Moderate n (%)	Strong n (%)
Normal	110	23 (20.9%)	57 (51.8%)	30 (27.3%)
Adenomas	24	1 (4.2%)	8 (33.4%)	15 (62.4%)
Carcinomas	119	25 (21%)	61 (51.3%)	33 (27.7%)

All normal tissue, adenoma and carcinoma specimens displayed cytoplasmic HER-3 protein expression in >50% of cells. The results regarding HER-3 intensity staining in the cytoplasm are shown in Table 2. When HER-3 expression was measured quantitatively, cytoplasmic expression of HER-3 was statistically significantly lower in normal tissue than in adenomas ($p < 0.0001$) and carcinomas ($p < 0.0001$). However, there was no significant difference between adenomas and carcinomas ($p = 0.088$).

Nuclear staining for HER-3 was positive in 90 out of 110 normal mucosa samples (81.8%), in 13 out of 24 adenomas (54.2%) and in 27 out of 119 carcinomas (22.7%). Nuclear expression of HER-3 was statistically significantly higher in normal tissue than in adenomas ($p = 0.033$) and carcinomas ($p < 0.0001$). Furthermore, adenomas expressed significantly higher levels of nuclear HER-3 compared to carcinomas ($p = 0.01$).

In both normal and carcinoma specimens, cytoplasmic expression of HER-3 was statistically significantly correlated with the nuclear HER-3 expression ($r = 0.348$, $p < 0.0001$ and $r = 0.241$, $p = 0.008$, respectively). Statistical analysis revealed no correlation between either cytoplasmic or nuclear HER-3 expression in carcinomas and any of the clinicopathologic parameters under evaluation (Table 3). Neither cytoplasmic nor nuclear HER-3 (data not shown) expression in carcinomas was correlated with overall survival as analysed by Kaplan–Meier procedure. Representative images of both cytoplasmic and nuclear HER-3 in normal mucosa, adenomas and carcinomas are depicted in Fig. 1.

Membranous staining for pHER-3 was found in seven out of 110 normal mucosa (6.3%), ten out of 24 adenomas (41.7%) and ten out of 119 carcinoma specimens (8.4%). Membranous staining of pHER-3 displayed great heterogeneity and inconsistent appearance in all specimens; therefore, it was not included in the statistical analysis. Notably, pHER-3 staining was repetitively detected in smooth muscle tissue in malignant, adenoma and normal mucosa specimens.

pHER-3 was consistently observed in the nucleus. Nuclear pHER-3 positivity ($\geqslant$5% of cells expressing pHER-3) was observed in 22 out of 110 normal mucosa specimens (20%), in 12 out of 24 adenomas (50%) and in 27 out of 119 (22.7%) carcinomas. Nuclear pHER-3 expression did not differ significantly between normal tissue, adenomas and carcinomas, albeit there was a trend towards increased expression in adenomas compared to normal tissue ($p = 0.057$). In carcinomas, levels of nuclear pHER-3 were significantly different between stage D and stage B disease ($p = 0.011$). However, nuclear pHER-3 expression was not associated with other clinicopathologic parameters (Table 4) or with overall survival (Fig. 2). Neither cytoplasmic nor nuclear HER-3 expression was correlated with nuclear pHER-3 expression in carcinomas, adenomas and normal tissue samples. Representative images of nuclear and membranous pHER-3 in normal mucosa, adenomas and carcinomas are depicted in Fig. 1.

Table 3 – Clinicopathologic parameters and HER-3 expression in carcinomas

Feature	n = 119	Cytoplasmic HER-3		P value	Nuclear HER-3		P value
Duke's stage		(+)[a] n = 103	(–)[a] n = 16		(+)[b] n = 27	(–)[b] n = 92	
B	44	38	6	0.99	12	32	0.27
C	68	59	9		15	53	
D	7	6	1		0	7	
Grade							
I	21	18	3	0.90	3	18	0.36
II	87	75	12		20	67	
III	11	10	1		4	7	
Gender							
male	77	65	12	0.35	17	60	0.83
female	42	38	4		10	32	
Histologic type							
non mucinous	107	93	14	0.73	24	83	0.84
mucinous	12	10	2		3	9	
Primary site							
right colon	31	25	6	0.51	7	24	0.84
left colon	49	43	6		10	39	
rectal	39	35	4		10	29	
Age median (range)		67 (30–90)	65.5 (44–79)	0.65	66(30–77)	67(40–90)	0.53

a For cytoplasmic HER-3: (+): overexpression, (–): no overexpression.
b For nuclear HER-3: (+): positive expression, (–):negative expression.

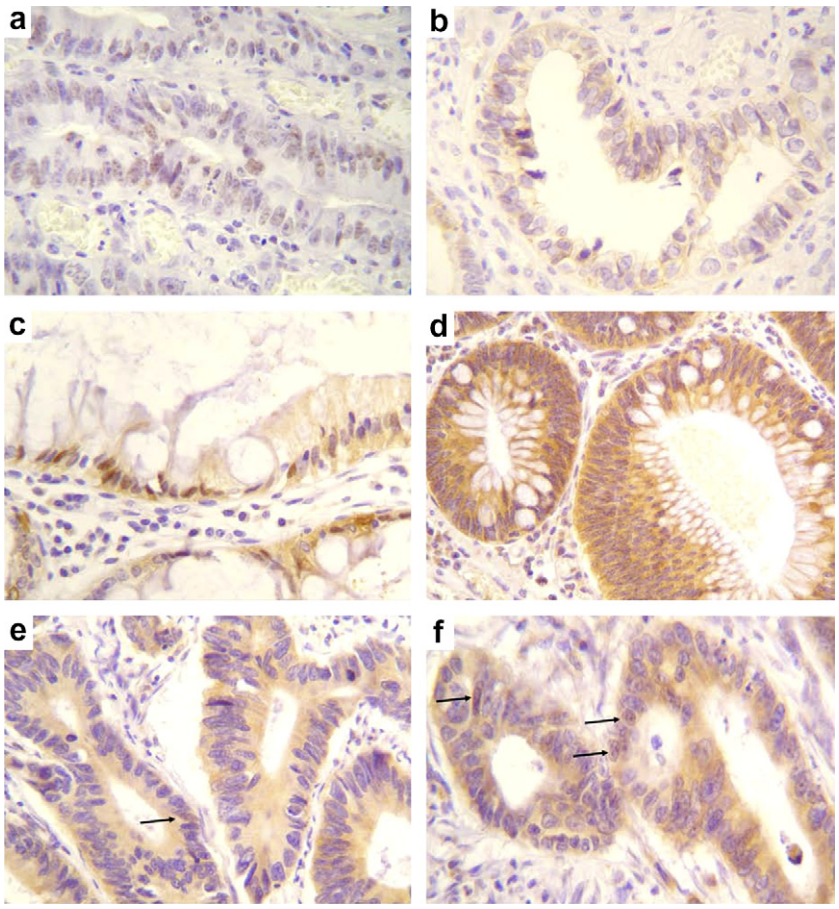

Fig. 1 – (a) pHER-3 nuclear expression in carcinomas. (b) pHER membranous expression in carcinomas. (c) HER-3 nuclear and weak cytoplasmic expression in normal tissue. (d) HER-3 cytoplasmic expression in adenomas. (e, f) HER-3 cytoplasmic and sparse nuclear (arrows) expression in carcinomas. (a–f: 400×).

Table 4 – Clinicopathologic parameters and pHER-3 nuclear expression in carcinomas

Feature	*n* = 119	pHER-3		*P* value
Duke's stage		(+)[a] *n* = 27	(–)[a] *n* = 92	
B	46	7	39	0.043
C	66	16	50	
D	7	4	3	0.011 (B versus D)
Grade				
I	20	4	16	0.17
II	88	18	70	
III	11	5	6	
Gender				
male	76	15	61	0.31
female	43	12	31	
Histologic type				
non mucinous	107	26	81	0.21
mucinous	12	1	11	
Primary site				
right colon	33	5	28	0.21
left colon	49	15	34	
rectal	37	7	30	
Age median (range)		70 (50–90)	66.5 (30–83)	0.06

a (+): positive expression, (–): negative expression.

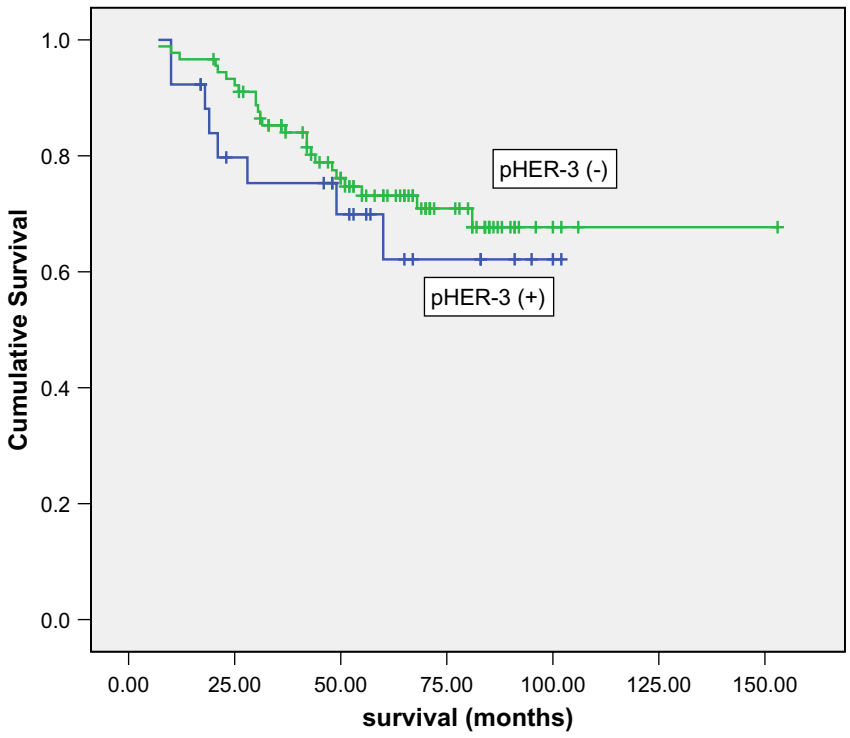

Fig. 2 – Kaplan–Meier curves for overall survival of patients with positive (+) and negative (–) pHER-3 expression (log rank test: $p = 0.45$).

Table 5 – Clinicopathologic parameters and HER-3 mRNA expression in tumour samples

Feature	$n = 54$	pHER-3		P value
Duke's stage		(+)[a] $n = 27$	(–)[a] $n = 27$	
B	17	6	11	0.08
C	27	13	14	
D	10	8	2	
Grade				
I	13	6	7	0.48
II	34	16	18	
III	7	5	2	
Gender				
male	39	20	19	0.76
female	15	7	8	
Histologic type				
non mucinous	49	25	24	0.64
mucinous	5	2	3	
Primary site				
right colon	14	3	11	0.046
left colon	25	15	10	0.013 (right *versus* left and rectal)
rectal	15	9	6	
Age median (range)		71 (42–80)	64 (40–75)	0.016

a (+): overexpression, (–): no overexpression.

Employing RT-PCR, we investigated the relative expression of HER-3 mRNA in 54 tumour samples and 29 adjacent normal mucosa samples. HER-3 mRNA expression in tumour samples ranged from 0.0115 to 0.1714 with a median value of 0.0355, while in normal samples it ranged from 0.012 to 0.338 with a median value of 0.0411. HER-3 RNA levels did not differ significantly between tumour and paired normal samples ($p = 0.159$). However, HER-3 mRNA levels in tumour samples were significantly elevated in samples with positive lymph nodes compared to samples with negative lymph nodes (median values 0.043694 and 0.026406, respectively; $p = 0.016$).

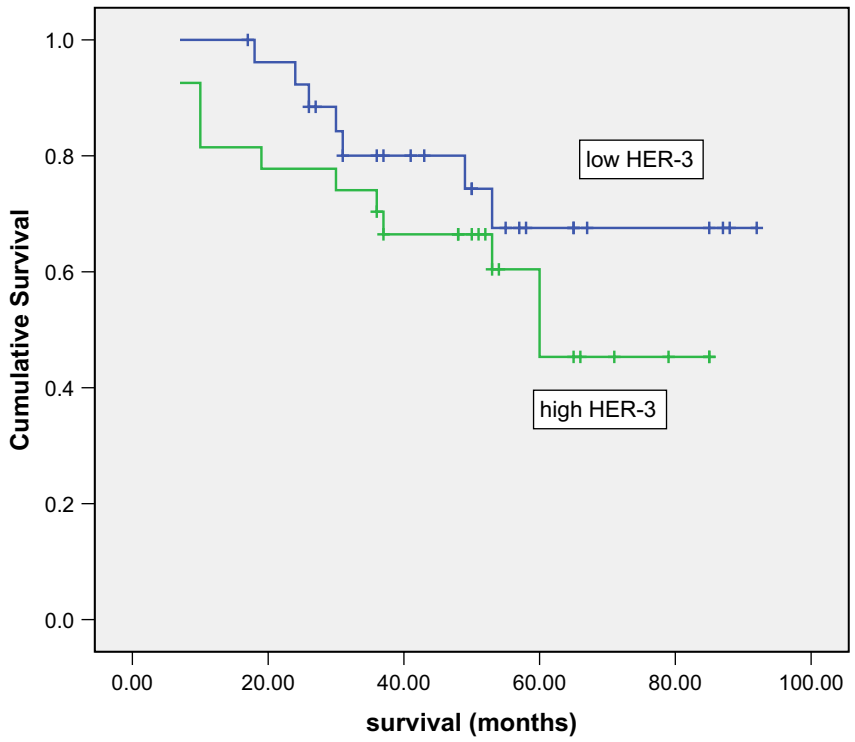

Fig. 3 – Kaplan–Meier curves for overall survival of patients with high and low HER-3 mRNA levels (log rank test, $p = 0.23$).

For statistical purposes, values higher than the median value were considered high expression and values lower than the median value were considered low. The median age of patients who expressed high levels of HER-3 mRNA in tumour samples was greater than of those with low levels of expression (71 *versus* 64, $p = 0.016$). Furthermore, low HER-3 mRNA levels were associated with right colon tumours ($p = 0.013$, Table 5) compared to tumours located in the left colon and rec-

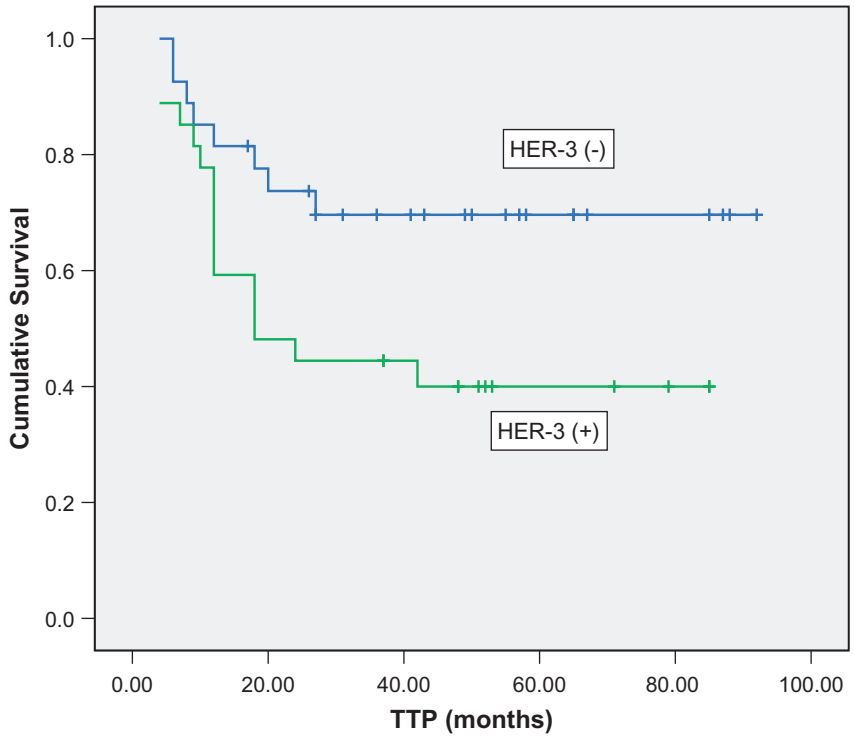

Fig. 4 – Kaplan–Meier curves for time to progression (TTP) of patients with high (+) and low (–) HER-3 mRNA levels (log rank test, $p = 0.039$).

tum. There was no correlation between HER-3 mRNA levels in tumour samples and survival as analysed by Kaplan–Meier procedure (Fig. 3). However, they were significantly correlated with time to disease progression (log rank test: $p = 0.039$, Fig. 4) and with probability of disease relapse. Particularly, tumours with high HER-3 mRNA levels exhibited doubled postoperative recurrence rate than those with low levels (59.2% *versus* 29.6%, $p = 0.028$).

We also investigated the correlation between HER-3 mRNA and protein levels in tumour samples and adjacent normal mucosa from 33 patients. In carcinomas, mRNA levels were significantly correlated with cytoplasmic HER-3 protein levels ($r = 0.334$, $p = 0.031$), but not with nuclear HER-3 protein levels ($r = 0.083$, $p = 0.6$).

Stepwise regression analysis based on the Cox proportional-hazards model revealed that only tumour stage ($p < 0.001$) and grade ($p = 0.004$) constituted independent prognostic factors.

4. Discussion

Although HER-3 seems to have a crucial role in oncogenic signalling, its role in colorectal carcinogenesis remains elusive. In this study, cytoplasmic and nuclear expression of HER-3 protein was evaluated in 119 colorectal carcinomas, 110 adjacent normal mucosa specimens and 24 adenomas. With regard to cytoplasmic staining, we observed HER-3 overexpression in 79.1% of normal mucosa samples, in 95.8% of adenomas and in 79% of carcinomas. These results are in accordance with previous studies, where HER-3 cytoplasmic overexpression in carcinomas ranged from 68.75% to 89%.[13,14,21] Lee et al.[12] detected membranous HER-3 expression at a rate of 36%, but in our study distinct complete membranous HER-3 was not detected. Cytoplasmic expression of HER-3 in normal tissue was significantly lower than in adenomas and carcinomas. It appears that cytoplasmic HER-3 levels increase from the early stages of colorectal tumourigenesis.

Statistical analysis revealed no correlation between cytoplasmic HER-3 expression in carcinomas and any of the clinicopathologic parameters under evaluation. Cytoplasmic HER-3 expression was previously reported to be associated with age and grade of differentiation[22] but not with other clinicopathologic parameters. Discrepancies between results from diverse studies may occur due to heterogeneity of patient populations and variations in study methodology and analysis.

In our study, nuclear HER-3 was found to be overexpressed in 81.8% of normal mucosa samples, in 54.2% of adenomas and in 22.7% of carcinomas. Nuclear expression of HER-3 in normal tissue was significantly higher than in adenomas and carcinomas. Furthermore, nuclear expression of HER-3 in adenomas was significantly higher than in carcinomas. These findings suggest a possible progressive reduction in nuclear HER-3 levels during colorectal tumourigenesis. Although no data have been previously reported regarding nuclear HER-3 in colorectal cancer, in prostate cancer high levels of nuclear HER-3 were associated with malignant prostate tissues.[23] This may be due to the diverse roles HER-3 plays in different tissues and in various temporal frames during normal development and pathogenesis of malignant diseases. Moreover, dynamic balance of HER-3 phosphorylation status may regulate transcription of target genes in a spatial and temporal manner. The aforementioned data regarding cytoplasmic and nuclear HER-3 levels pose a possible switch in HER-3 topography from the nucleus to the cytoplasm during colorectal tumourigenesis.

Statistical analysis revealed no correlation between nuclear HER-3 expression in carcinomas and any of the clinicopathologic parameters under evaluation. In a previous study, nuclear HER-3 was correlated with high Gleason grading score in prostate cancer.[23] Neither cytoplasmic nor nuclear HER-3 expression in carcinomas was correlated with overall survival. These findings are in concert with previously published data[12,22] but in disagreement with data reported by Kapitanovic et al., who claimed that HER-3 overexpression is associated with shorter overall survival.

A limited number of data are available concerning nuclear localisation of HER family members and HER-3 in particular.[24–27] Nuclear localisation of HER-3 in normal and malignant human mammary epithelial cells has been reported. It was suggested that HER-3 shuttles between nuclear and non-nuclear compartments, depending on the presence of exogenous stimuli, such as heregulin. Moreover, HER-3 was detected in the nucleolus indicating a potential involvement in the regulation of ribosomal biosynthesis and cell differentiation. Another study[23] provided evidence that nuclear localisation of unphosphorylated HER-3 in prostate cancer cell lines and tissues modulates androgen receptor activity and it is associated with risk of disease progression. Nuclear localization of HER-3, which was also observed in our study, may suggest its potential role as a transcription (co)factor similarly to other HER family members.[24]

Nuclear pHER-3 expression did not differ significantly between normal mucosa, adenomas and carcinomas. Although there was a trend for nuclear pHER-3 to increase in adenomas compared to normal tissue, this difference failed to reach statistical significance probably due to the low number of adenomas included in the study. These data imply that phosphorylation of HER-3 may be an important pathogenetic event during adenoma development and larger studies are required to confirm this hypothesis. In tumour, adenomas and normal mucosa samples, neither cytoplasmic nor nuclear HER-3 expression was correlated with nuclear pHER-3 expression. These findings suggest that phosphorylation of HER-3 protein may be independent of HER-3 levels in either the nucleus or the cytoplasm. Nuclear pHER-3 expression was significantly higher at stage D compared to stage B disease. Levels of nuclear pHER-3 were not associated with other clinicopathologic parameters or with overall survival. To the best of our knowledge, this is the first study to evaluate the expression of pHER-3 in colorectal cancer.

HER-3 mRNA expression was examined in 54 tumour samples and 29 adjacent normal mucosa samples and was detected in all samples. Although there was a great variation in HER-3 mRNA levels, there was no significant difference between normal and malignant tissue. In another study,[28] HER-3 mRNA was detected by Northern blot analysis – which is less sensitive than RT-PCR – in 55% of primary or metastatic human colorectal carcinomas but in only 22% of normal colon mucosa and 32% of normal liver samples. In our study, the median age of patients who expressed high levels of HER-3

mRNA in tumour samples was greater than of those with low levels of expression. Low HER-3 mRNA expression was associated with right colon carcinomas. This finding merits further investigation, taking into account the differential biological characteristics in right- and left-sited colon tumours; in particular, the levels of oestrogen receptor-β,[29] which is known to interact with the HER signalling pathways. HER-3 mRNA levels in tumour samples were also found to be significantly related to lymph node involvement. Since lymph node status is of utmost prognostic significance in colorectal cancer, HER-3 mRNA expression might serve as a useful prognostic biomarker in this malignancy. Although there was no correlation between HER-3 mRNA expression and overall survival, HER-3 mRNA expression was significantly correlated with time to disease progression and probability of disease relapse. As far as we are concerned, this is the first study providing data that HER-3 mRNA expression is predictive of progression-free survival in colorectal cancer patients. Conclusive evidence from further studies with higher numbers of patients is necessary to confirm our results.

HER-3 appears to have an instrumental role in colorectal tissue signalling. Additional studies are required to clarify its prognostic significance in colorectal cancer. Novel therapies targeting EGFR have recently been used in colorectal tumours[30] and resistance to anti-EGFR compounds may involve HER-3. The predictive value of HER-3 levels in response to anti-EGFR treatment would be validated and potentially included in the criteria used for patient selection. This might render HER-3 a useful biomarker of treatment response, hence leading to individualisation of anticancer treatment.

Conflict of interest statement

None declared.

Acknowledgements

We acknowledge the Committee of Research, University of Patras, for the funding of our study. Study sponsors had no involvement in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; in the decision to submit the manuscript for publication. We also thank Dr. Vassiliki Bravou, M.D., PhD for her useful comments.

REFERENCES

1. Karamouzis MV, Badra FA, Papavassiliou AG. Breast cancer: the upgraded role of HER-3 and HER-4. *Int J Biochem Cell Biol* 2007;**39**:851–6.
2. Harris RC, Chung E, Coffey RJ. EGF receptor ligands. *Exp Cell Res* 2003;**284**:2–13.
3. Citri A, Skaria KB, Yarden Y. The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Exp Cell Res* 2003;**284**:54–65.
4. Tzahar E, Yarden Y. The ErbB-2/HER2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. *Biochim Biophys Acta* 1998;**1377**:M25–37.
5. Mendelsohn J. The epidermal growth factor receptor as a target for cancer therapy. *Endocr Relat Cancer* 2001;**8**:3–9.
6. Mendelsohn J, Baselga J. Epidermal growth factor receptor targeting in cancer. *Semin Oncol* 2006;**33**:369–85.
7. Guy PM, Platko JV, Cantley LC, Cerione RA, Carraway 3rd KL. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. *Proc Natl Acad Sci U S A* 1994;**91**:8132–6.
8. Prigent SA, Lemoine NR, Hughes CM, Plowman GD, Selden C, Gullick WJ. Expression of the c-erbB-3 protein in normal human adult and fetal tissues. *Oncogene* 1992;**7**:1273–8.
9. Poller DN, Spendlove I, Baker C, et al. Production and characterization of a polyclonal antibody to the c-erbB-3 protein: examination of c-erbB-3 protein expression in adenocarcinomas. *J Pathol* 1992;**168**:275–80.
10. Tanner B, Hasenclever D, Stern K, et al. ErbB-3 predicts survival in ovarian cancer. *J Clin Oncol* 2006;**24**:4317–23.
11. Badra FA, Karamouzis MV, Ravazoula, et al. Non-palpable breast carcinomas: correlation of mammographically detected malignant-appearing microcalcifications and epidermal growth factor receptor (EGFR) family expression. *Cancer Lett* 2006;**244**:34–41.
12. Lee JC, Wang ST, Chow NH, Yang HB. Investigation of the prognostic value of coexpressed erbB family members for the survival of colorectal cancer patients after curative surgery. *Eur J Cancer* 2002;**38**:1065–71.
13. Maurer CA, Friess H, Kretschmann B, et al. Increased expression of erbB3 in colorectal cancer is associated with concomitant increase in the level of erbB2. *Hum Pathol* 1998;**29**:771–7.
14. Kapitanovic S, Radosevic S, Slade N, et al. Expression of erbB-3 protein in colorectal adenocarcinoma: correlation with poor survival. *J Cancer Res Clin Oncol* 2000;**126**:205–11.
15. Sergina NV, Rausch M, Wang D, et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. *Nature* 2007;**445**:437–41.
16. Hellyer NJ, Cheng K, Koland JG. ErbB3 (HER3) interaction with the p85 regulatory subunit of phosphoinositide 3-kinase. *Biochem J* 1998;**333**(Pt 3):757–63.
17. Engelman JA, Cantley LC. The role of the ErbB family members in non-small cell lung cancers sensitive to epidermal growth factor receptor kinase inhibitors. *Clin Cancer Res* 2006;**12**:4372s–6s.
18. Fujimoto N, Wislez M, Zhang J, et al. High expression of ErbB family members and their ligands in lung adenocarcinomas that are sensitive to inhibition of epidermal growth factor receptor. *Cancer Res* 2005;**65**:11478–85.
19. Buck E, Eyzaguirre A, Haley JD, Gibson NW, Cagnoni P, Iwata KK. Inactivation of Akt by the epidermal growth factor receptor inhibitor erlotinib is mediated by HER-3 in pancreatic and colorectal tumor cell lines and contributes to erlotinib sensitivity. *Mol Cancer Ther* 2006;**5**:2051–9.
20. Taron M, Rosell R, Felip E, et al. BRCA1 mRNA expression levels as an indicator of chemoresistance in lung cancer. *Hum Mol Genet* 2004;**13**:2443–9.
21. Rajkumar T, Gooden CS, Lemoine NR, Gullick WJ, Goden CS. Expression of the c-erbB-3 protein in gastrointestinal tract tumours determined by monoclonal antibody RTJ1. *J Pathol* 1993;**170**:271–8.
22. Kountourakis P, Pavlakis K, Psyrri A, et al. Prognostic significance of HER3 and HER4 protein expression in colorectal adenocarcinomas. *BMC Cancer* 2006;**6**:46.
23. Koumakpayi IH, Diallo JS, Le Page C, et al. Expression and nuclear localization of ErbB3 in prostate cancer. *Clin Cancer Res* 2006;**12**:2730–7.
24. Lo HW, Xia W, Wei Y, Ali-Seyed M, Huang SF, Hung MC. Novel prognostic value of nuclear epidermal growth factor receptor in breast cancer. *Cancer Res* 2005;**65**:338–48.

25. Lin SY, Makino K, Xia W, et al. Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol* 2001;**3**:802–8.
26. Offterdinger M, Schofer C, Weipoltshammer K, Grunt TW. c-erbB-3: a nuclear protein in mammary epithelial cells. *J Cell Biol* 2002;**157**:929–39.
27. Srinivasan R, Gillett CE, Barnes DM, Gullick WJ. Nuclear expression of the c-erbB-4/HER-4 growth factor receptor in invasive breast cancers. *Cancer Res* 2000;**60**:1483–7.
28. Ciardiello F, Kim N, Saeki T, et al. Differential expression of epidermal growth factor-related proteins in human colorectal tumors. *Proc Natl Acad Sci U S A* 1991;**88**:7792–6.
29. Jassam N, Bell SM, Speirs V, Quirke P. Loss of expression of oestrogen receptor beta in colon cancer and its association with Dukes' staging. *Oncol Rep* 2005;**14**:17–21.
30. Kalofonos HP, Grivas PD. Monoclonal antibodies in the management of solid tumors. *Curr Top Med Chem* 2006;**6**:1687–705.