High Levels of Serum HER-2/neu and YKL-40 Independently Reflect Aggressiveness of Metastatic Breast Cancer

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ABSTRACT

Purpose: **To evaluate serum levels of HER2 (an epithelial growth factor) and YKL-40 (a growth factor participating in inflammation and remodeling of the extracellular matrix) in relation to outcome in patients with their first diagnosis of recurrent breast cancer.**

Design: **Serum HER2 and YKL-40 levels were measured in 100 patients referred with their first metastatic manifestation of breast cancer before first line anthracycline-based therapy and related to response to therapy, metastatic pattern, time to progression, and overall survival. During the observation period of 64–84 months, 89 patients died of breast cancer.**

Results: **The patients had higher serum HER2 and YKL-40 levels than healthy females ($P < 0.0001$). Serum HER2 was elevated in 32% of the patients and serum YKL-40 in 30%. These patients were more sick ($P < 0.01$) and more often had parenchymal involvement ($P < 0.0005$), especially liver metastases ($P < 0.00005$). In multivariate Cox analysis, high serum levels of HER2 or YKL-40 or lack of estrogen receptors independently doubled the relative risk of progression and dying ($P < 0.001$) even after accounting for other independent prognostic variables, such as axillary nodal involvement at primary diagnosis, liver metastases, and more than two metastatic sites. Fewer patients with high serum HER2 or YKL-40 or lack of estrogen receptors responded with a complete remission on chemotherapy ($P = 0.005$, 0.036, and 0.006). In these patients, high serum YKL-40 was a stronger predictor of survival than high serum HER2 or lack of estrogen receptors.**

Conclusions: **High serum HER2 and YKL-40 independently identified subgroups of patients with metastatic breast cancer with a poor prognosis.**

INTRODUCTION

It is a truism that a better understanding of the biology of cancer should lead to improvements in preventive and therapeutic strategies and in diagnosis of patients with breast cancer. Research over the past several decades has identified the dynamic nature of turnover in normal and diseased tissues and in an ever-expanding array of matrix functions. Networks of interacting extracellular matrix proteins, integrins, and growth factors collaborate to profoundly influence gene expression and the major cellular programs, including growth, migration, differentiation, and survival. Molecularly targeted therapy for advanced solid tumors directed toward receptors for growth factors and other signaling and regulatory molecules has recently proven successful (1–3). The HER2 (or c-erbB-2 or HER-2/neu) oncogene, which encodes the tyrosine kinase HER2 receptor, belongs to a family of epithelial growth factor receptors structurally related to the epidermal growth factor receptor (4). HER2 is a tyrosine kinase receptor composed of a cytoplasmic domain with tyrosine kinase activity, a transmembrane domain, and an ECD,[2] which is shed from the cell surface. The phosphorylation of tyrosine residues initiates complex signaling pathways that ultimately lead to cell division. The ECD of HER2 is a glycoprotein that can be quantified using an ELISA (5). Women with breast cancer that overexpress HER2 resulting in increased serum levels of the ECD of HER2 have an aggressive form of the disease with a significantly shortened disease-free and overall survival (6–13). Targeting of HER2 in metastatic breast cancer by a recombinant monoclonal antibody, Trastuzumab, against the ECD of the HER2 protein has proven successful. In combination with chemotherapy, Trastuzumab prolonged time to disease progression and survival (1), but it is also capable of producing durable objective responses as a single agent (2).

YKL-40 (human cartilage glycoprotein 39) is a member of family 18 glycosyl hydrolases (14–16). It is a heparin and chitin-binding lectin (16, 17) without chitinase activity (14, 18). The biological function of YKL-40 is not known in detail, but YKL-40 is a growth factor for connective tissue cells (19, 20) and a potent migration factor for endothelial cells (21). Furthermore, the pattern of its expression in normal and disease states suggests a function in inflammation and remodeling of the extracellular matrix (22–25). YKL-40 is secreted in large amounts *in vitro* by the MG63 human osteosarcoma cell line (26) and is expressed selectively by murine mammary tumors

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[2] The abbreviations used are: ECD, extracellular domain; ER, estrogen receptor; OR, odds ratio; CI, confidence interval; CR, complete remission; HR, hazards ratio.

initiated by neu/ras oncogenes (15). The gene for YKL-40 has been sequenced (27), and a search of the YKL-40 protein sequence against the dbest database at the National Center for Biotechnology Information using the BLAST program has shown that YKL-40 is expressed by several types of cancer, such as colon, breast, ovarian, uterine, prostate, kidney, lung, oligodendroglioma, glioblastoma, and germ cell tumors. Gene expression microarray analyses have shown that the most differentially expressed gene in papillary thyroid carcinoma, glioblastoma multiforme, and extracellular myxoid chondrosarcoma was *YKL-40* (28–30). Serum YKL-40 levels in patients with glioma were related to tumor grade and burden (29). We have reported previously that increased serum levels of YKL-40 are related to poor survival in patients with metastatic breast cancer (31) and colorectal cancer (32, 33). In patients with colorectal cancer, multivariate analysis showed that elevated serum CEA and YKL-40 independently predicted short survival both preoperative and at 6-months postoperative (32, 33). In the present study, we evaluated the influence of serum HER2 and YKL-40 and ER status on outcome in patients with their first diagnosis of recurrent breast cancer and a possible interplay on metastatic pattern, disease-free, and overall survival.

MATERIALS AND METHODS

One hundred female patients (aged 29–66 years) with their first sign of recurrent metastatic breast cancer were included in a prospective, observational study of the effects of first-line, anthracycline-based therapy between June 1991 and August 1993. The patients were in good performance (performance status ≤ 2) with a life expectancy > 3 months and previous chemotherapy limited to one adjuvant regimen without anthracyclines. None of the patients had severe renal, hematological, hepatic, or cardiac dysfunction or metabolic bone disease. No patients used glucocorticosteroids. A serum sample was collected from all patients at the time of inclusion and within 1 week of start of chemotherapy. At the primary diagnosis of breast cancer, the patients were classified as ER positive if the quantity of biochemical assayed ER was ≥ 10 fmol/mg cytosol protein or if a minimum of 10% of the cells in immunohistochemical analysis was positive. At inclusion, all patients had a medical history, clinical examination, full blood count, and a biochemical screen of renal and liver function, a chest X-ray, whole body bone scintigraphy, and ultrasonic verification of suspected supra-diaphragmatic lymph nodes. An ultrasonic finding of enlarged lymph nodes was followed by a fine needle aspiration for demonstration of malignant cells. Lung metastases were confirmed by plain chest X-ray in doubtful cases supported by a computer tomographic evaluation. Malignancy in a pleural effusion was confirmed by the demonstration of malignant cells. Abnormal biochemistry indicating liver or bone marrow involvement leads to ultrasonic examination of the liver with biopsy or bone marrow aspiration for verification of malignant involvement. An abnormal bone scintigraphy was always followed by a plain roentgenological examination of suspected areas, in doubtful cases supported by a computer tomographic examination. Only if these were abnormal, bone involvement was considered. Recurrent breast cancer at the supraclavicular lymph nodes was considered metastatic. A CR was defined as the disappearance of clinical and laboratory evidence of disease for a minimum of 8 weeks. In the case of osseous metastasis, CR was determined by clear evidence of complete bone recalcification. Development of any new lesions, including central nervous system metastases, or reactivation of previous disease areas marked the end of remission. Two patients who died within 8 weeks of starting chemotherapy were included with those having progressive disease. Patients were followed until death or ≥ 5 years. Time to death or disease progression was measured from the date of starting epirubicin therapy.

Treatment summary is given in Table 1. Eighty patients had first-line mono-therapy with epirubicin (130 mg/m^2) every 3rd week aiming at a cumulative dose not exceeding 1000 mg/m^2, and 20 patients had epirubicin every 6 weeks alternating with four courses of cyclophosphamide (3 grams/m^2), aiming at a low cumulative dose of 500 mg/m^2 epirubicin.

The doses were adjusted according to the WBC and platelet counts on the day of treatment and according to the previous course. Second- and third-line therapy after progressive disease on first-line chemotherapy is also seen in Table 1. Patients with ER-positive tumors had antiestrogen therapy mostly with 30 mg/day of Tamoxifen. Patients with ER-negative tumors had chemotherapy that in this pretaxane era consisted mostly of cyclophosphamide 3 grams/m^2 or cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) every 3rd week until progression. Patients with metastasis causing pain or discomfort had local irradiation. The study was performed in agreement with the Helsinki II declaration. The research protocol was approved by the local ethical committee, and informed written consent was required.

Biochemical Analysis. Blood samples were taken between 9 and 11 a.m. within 1 week before start of chemotherapy. Serum was separated from cellular elements by centrifugation within half an hour after blood sampling and stored at $-20°C$ until analysis. All analysis was performed at the end of the study after a minimum follow-up of 5 years. Serum YKL-40 was determined by an in-house RIA using rabbit antibody raised against human YKL-40. Purified human YKL-40 was used for standard and tracer. The sensitivity of the RIA was 20 μg/liter (34). We have also measured the serum YKL-40 levels in our 100 patients with a commercial available ELISA (Quidel Corp., Santa Clara, CA) and found the same results using this assay. Serum HER2 was determined by a sandwich enzyme immunoassay (Oncogene Science, Bayer Corp., Cambridge, MA; Ref. 35). The sensitivity of the HER2 assay was 1 μg/liter. The normal range of serum HER2 and YKL-40 concentrations was determined in 78 apparently healthy females with a median age of 51 years (range 29–66 years). They were not taking any medicine and had no clinical signs or symptoms of cancer, joint, liver, metabolic, or hormonal disease (36). The median serum YKL-40 level in the 78 healthy women was 97 μg/liter (range 38–238 μg/liter; upper 90th and 95th percentile 168 and 204 μg/liter). The median serum HER2 level was 8 μg/liter (range 4–14 μg/liter; upper 90th and 95th percentile 10 and 11 μg/liter).

Statistical Analysis. The serum YKL-40 and HER2 values were scored as normal or elevated by these normal reference regions arbitrarily aiming at separating approximately one-third of the patients in the high level group comparable with the number of 29 patients lacking ERs at the diagnosis of breast

Table 1 Clinical characteristics and treatment summary

	All patients	Normal HER2 ≤15 μg/liter	High HER2 >15 μg/liter	Normal YKL-40 ≤168 μg/liter	High YKL-40 >168 μg/liter
Number of patients	100	68	32	70	30
Median years old (range) at first metastatic recurrence	51	50 (29–66)	52 (36–60)	50 (29–66)	55 (39–63)
Median months from primary diagnosis to metastatic recurrence (range)	25	23 (1–149)	27 (1–116)	24 (8–149)	25 (1–136)
ER lacking at primary diagnosis	29	20	9	19	10
Previous therapy (number = % of patients)					
None	31	21	10	21	10
Endocrine therapy	22	15	7	14	8
Irradiation	34	23	11	23	11
Chemotherapy (CMF)	37	23	14	26	11
Epirubicin as mono-therapy					
Numbers	80	56	24	55	25
Cumulative epirubicin (median mg/m^2)	1000	1000	1000	1000	1000
Combined therapy					
Numbers	20	12	8	15	5
Cumulative epirubicin (median mg/m^2)	500	500	500	500	282
Cumulative cyclophosphamide (median g/m^2)	8.3	8.3	7.9	8.3	4.9
Duration of first-line therapy					
Median months (range)	5.2	5.2 (0.2–13)	5.0 (0.7–8)	5.3 (0.7–13)	5.2 (0.2–8)
Second- and third-line therapy					
None	24/58	13/35	11/23	12/37	12/21
Irradiation towards the breast and thorax	17/7	16/6	1/1	15/7	2/–
Endocrine therapy	42/23	28/19	14/4	32/17	10/6
Chemotherapy	23/16	17/11	6/5	17/13	6/3

cancer. We chose a cutoff value for HER2 of 15 μg/liter because it was higher than the highest concentration seen in healthy subject and yielded a high level group of 32 patients. For YKL-40, the upper 90th percentile (*i.e.,* 168 μg/liter) was used as cutoff level yielding a high level group of 30 patients. Median values were compared using the Mann-Whitney unpaired test with two-tailed significance. Significance of ORs was estimated with the χ^2 test with two-tailed significance. The end point for survival analysis was breast cancer-related death. The Kaplan-Meier estimate was used to calculate survival curves. Comparison of cumulative survival distributions between subgroups was made with the Log-rank test. The HRs or relative risks of factors for prognosis or survival were assessed by a forward stepwise (conditional likelihood ratio) Cox proportional hazards regression models with categorical indicator covariates. The SPSS statistical software for Windows version 10 was used.

RESULTS

The median follow-up time was 79 months (range 64–84 months). Median months to progression and death were 11 and 21 months. Only 11 patients (11%) were still alive when the study period ended, and only 7 had no signs of disease. Five-year progression-free and overall survival were 8 and 16%. Disease-free and overall survival were equal in the two chemotherapy treatment groups ($P = 0.41$ and 0.57), and because a detailed analysis of clinical parameters revealed the same in each treatment group, all of the patients were evaluated together. Table 1 gives the clinical characteristics and treatment summary for the 100 patients in relation to serum levels of YKL-40 and HER2. Patients were comparable with regard to various treatment variables. Patients with high serum YKL-40 level were about 5 years older than patients with normal level ($P = 0.03$).

Patients with "local regional recurrence" had disease restricted to supraclavicular lymph nodes ($n = 36$), some of which also had skin or breast affection ($n = 9$), and patients with "distant recurrence" had metastases to bones only ($n = 33$), lungs without liver involvement ($n = 13$), and liver ($n = 18$). Patients with "local regional recurrence" had a median survival of 33 months with 17% free of progressive disease after 5 years contrasting a median survival of 18 months for patients with "distant recurrence" ($P = 0.016$) with no progression-free survivors after 5 years ($P = 0.04$). The subgroup of patients with liver metastases had the shortest survival, with a median survival of only 9 months ($P < 0.00001$) and only 1 patient alive after 5 years. Patients with lung metastases without liver metastases had a similar short survival, with a median survival of 10 months and 1 alive after 5 years.

Fig. 1 illustrates the individual serum concentrations of HER2 (Fig. 1*A*) and YKL-40 (Fig. 1*B*) in the 100 patients with recurrent breast cancer and in the 78 age-matched healthy females. The median serum HER2 in the breast cancer patients was significantly ($P < 0.00001$) higher than in healthy women. Elevated serum HER2 (*i.e.,* >15 μg/liter) was seen in 32% of the patients, and 88% (28 of 32) of these had "distant recurrence." Patients with liver metastases had the highest serum concentrations of HER2 (Fig. 1*A*; Table 2), and all patients with serum HER2 > 90 μg/liter had liver metastases. Only 4 patients with "local regional recurrence" had elevated serum HER2. The patient in this group with the highest serum HER2 concentration had a massive tumor burden with diffuse inoperable tumor infiltrations in both breasts extending to the skin of the thorax and neck region and extensive lymph node involvement and a short survival of 13 months.

The median serum YKL-40 concentration in the breast

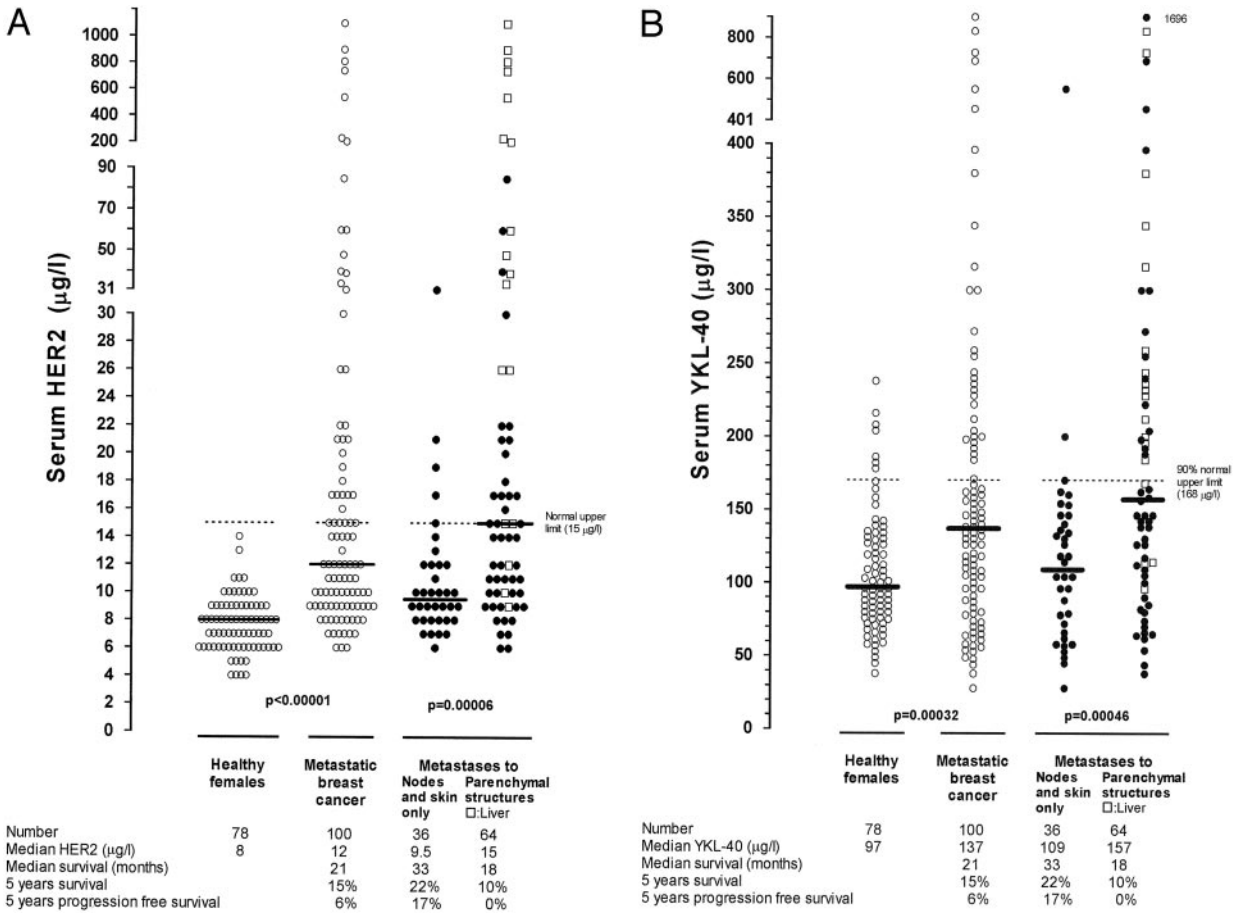

Fig. 1 Individual serum concentrations of HER2 (*A*) and YKL-40 (*B*) in 78 healthy females and 100 patients with advanced breast cancer. The breast cancer patients are subdivided into those with "local regional recurrence," *i.e.*, disease restricted to supraclavicular lymph nodes ($n = 36$), some of which also had skin or breast affection ($n = 9$) and patients with "distant recurrence" with metastases to bones only ($n = 33$), lungs (without liver; $n = 13$), and liver ($n = 18$). □, concentrations in patients with liver metastases. *Scale bars*, median values. *Dotted lines*, serum concentrations of HER2 above normal (Fig. 1*A*) or serum YKL-40 concentrations above the upper 90th percentile for healthy females (Fig. 1*B*). The *P*s indicate significance of difference between the median concentrations (Mann-Whitney test). The number of patients, median serum concentrations, median, and 5-year survival and 5-year progression-free survival are also listed.

cancer patients was significantly higher ($P = 0.00032$) than in healthy women. Elevated serum YKL-40 concentrations (*i.e.*, >168 µg/liter, the 90% percentile level in healthy women) were found in 30%, and 93% (28 of 30) of these patients had "distant recurrence." Patients with liver metastases had the highest serum concentrations of YKL-40 (Fig. 1*B*; Table 2), and only 4 of the 18 patients with liver metastases had normal serum YKL-40. Only 2 patients with "local regional recurrence" had elevated serum YKL-40, and they had a short survival of only 10 months. The patient in this group with the highest serum YKL-40 of 552 µg/liter (HER2 of 8 µg/liter) had an extensive tumor in the breast and mediastinum.

In Fig. 2, the individual serum concentrations of HER2 are plotted against YKL-40. Only 16 patients had overexpression of both factors. Only 2 of the 18 patients with liver metastases had normal serum HER2 and YKL-40, and 1 of these patients with the lowest HER2 had a single liver lesion and obtained a CR on therapy and is still alive 11 years after first recurrence.

In Table 2, the serum concentrations of HER2 and YKL-40 are given in relation to metastatic site and prognostic characteristics of the disease. There were no differences in either factor whether or not the patients had tumors that lacked ERs or had axillary nodal involvement at the primary diagnosis. Some patients with high serum HER2 or YKL-40 had bone involvement or lung metastases without liver involvement (Fig. 1, *A* and *B*), but the median concentrations in these groups of patients were within the normal range (Table 2). Increased serum HER2 and YKL-40 were found in patients with parenchymal metastases, particularly liver metastases, in patients with more than two different metastatic sites and in patients with symptomatic disease at recurrence. The ORs and corresponding *P*s for various disease characteristics dependent on normal or high HER2 and YKL-40 levels are also given in Table 2. A high serum HER2 predicted cancer outside the lymph nodes (OR = 6.2) and liver metastases (OR = 8.6). A high serum YKL-40 level predicted cancer outside the lymph nodes (OR = 13) and liver metastases (OR = 14), more than two metastatic sites (OR = 4.7), and symptomatic disease at recurrence (OR = 2.8). A CR was

Table 2 Serum HER2 and YKL-40 concentrations and disease characteristic

Disease characteristic	No. involved	Serum HER2 (μg/liter) (median and range)				Serum YKL-40 (μg/liter) (median and range)			
		Involved	Not involved	*P*	OR (95% CI) (*P*)[a]	Involved	Not involved	*P*	OR (95% CI) (*P*)[a]
Lymph node involvement at primary diagnosis	60	12 (6–740)	12 (6–1610)			139 (38–1696)	133 (28–552)		
Receptor negative at primary diagnosis	29	9 (6–740)	12 (6–1610)			138 (58–832)	136 (28–1696)		
Parenchymal involvement (outside lymph nodes and skin only)	64	15 (6–1610)	9.5 (6–31)	0.00006	6.2 (2.0–20) (0.001)	157 (38–1696)	109 (28–552)	0.0005	13 (2.9–60) (0.0001)
Bone metastases (+ marrow = 11) without liver or lung metastases	33	14 (6–60)	11 (6–1610)			140 (44–688)	134 (28–1696)		
Lung metastases without liver metastases	13	11 (6–85)	12 (6–1610)			138 (38–1696)	136 (28–832)		
Liver metastases	18	44 (9–1610)	11 (6–85)	0.00001	8.6 (2.7–27) (0.0001)	230 (96–832)	126 (28–1696)	0.00005	14 (4.2–50) (0.00001)
More than two different metastatic sites	13	26 (6–740)	11 (6–1610)	0.03		200 (69–832)	130 (28–1696)	0.008	4.7 (1.4–16) (0.008)
Symptomatic disease (PS 1 + 2)	23	15 (8–1610)	11 (6–900)	0.006		168 (72–832)	118 (28–1696)	0.001	2.8 (1.1–7.4) (0.034)
Complete remission not induced	61	14 (6–1610)	10 (6–40)	0.006	4.0 (1.5–11) (0.005)	146 (38–832)	118 (28–1696)	0.06	2.8 (1.1–7.3) (0.036)

[a] High *vs* low level.

Fig. 2 Relation between serum HER2 and YKL-40 concentrations. *Dotted lines*, the applied cutoff values. *Black dots*, patients with normal HER2 and YKL-40 levels. *Solid stars*, patients with elevated serum concentrations of either HER2 or YKL-40. □, patients with liver metastases. Both *X* and *Y* axes are broken to indicate very high concentrations.

reached for 39 patients after anthracycline-based chemotherapy. Almost all patients with a CR were in the normal serum HER2 level group (33 of 39, OR = 4) or the normal serum YKL-40 level group (32 of 39, OR = 2.8; Table 2) or had ER-positive tumors [24 of 29, OR = 4.4 (95% CI 1.5–13), $P = 0.006$]. The 6 patients with high serum HER2 (6 of 32) with a CR had the same survival as the 33 patients with normal serum HER2 with a CR. The 7 patients with high serum YKL-40 (7 of 30) with a CR had a shorter survival (median 39 months) than the 32 patients with normal YKL-40 levels with a CR (median 55 months, $P = 0.038$). Only two of the 16 patients with high levels of both serum HER2 and YKL-40 had a CR contrasting 52% (28 of 54) of patients with normal serum levels of both factors.

Serum HER2 and YKL-40 Levels and ER Status in Relation to Time to Progression and Death. Serum HER2 and YKL-40 were comparably predictive for time to progression. The median time to progression for patients with high serum HER2 was 7 months compared with 12 months for patients with normal serum HER2 [HR = 2.1 (95% CI: 1.4–3.3), $P = 0.0007$]. The median time to progression for patients with high serum YKL-40 was 8 months compared with 12 months for patients with normal serum YKL-40 [HR = 2.08 (95% CI: 1.3 – 3.2), $P = 0.001$]. All 7 patients free of disease

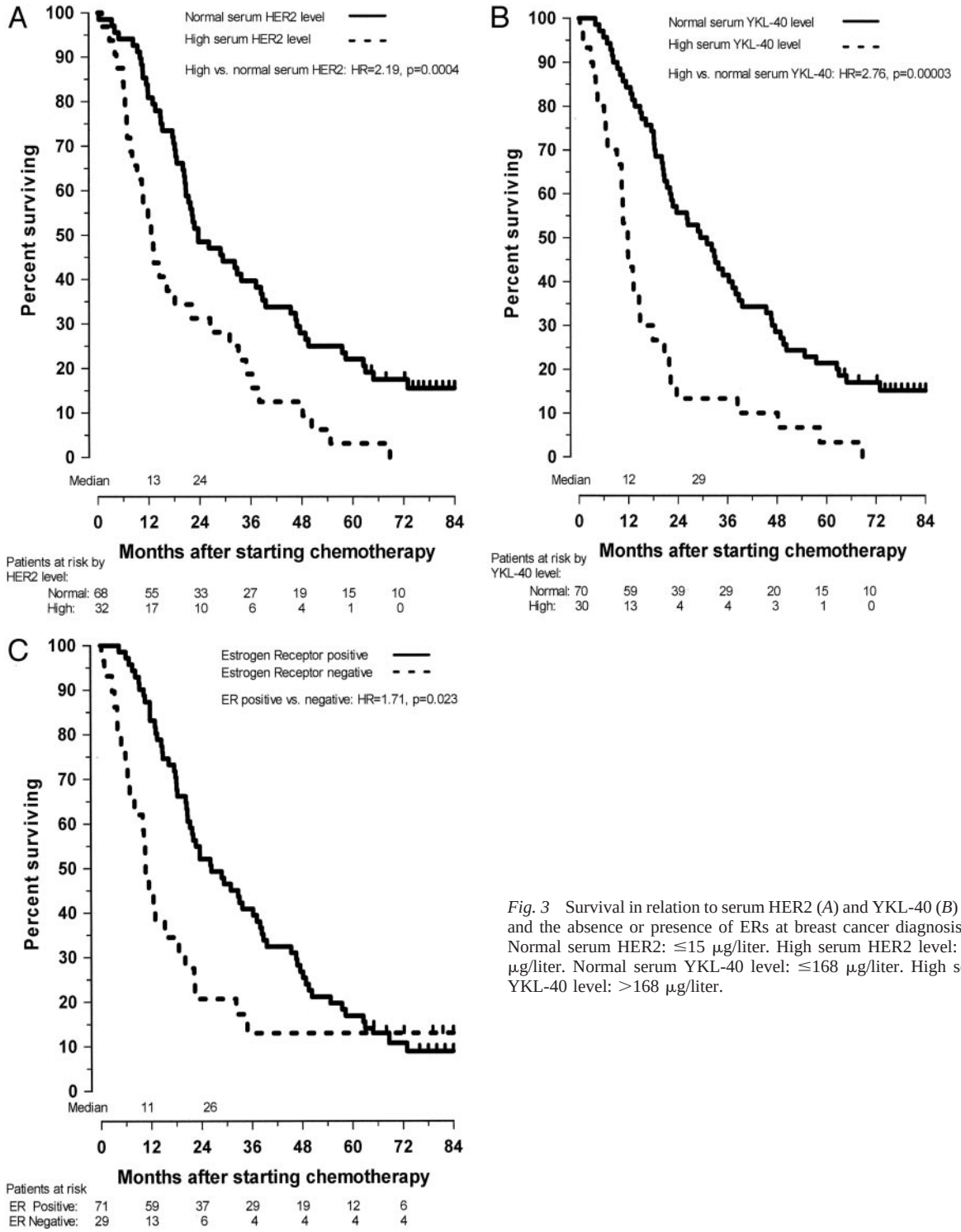

Fig. 3 Survival in relation to serum HER2 (*A*) and YKL-40 (*B*) level and the absence or presence of ERs at breast cancer diagnosis (*C*). Normal serum HER2: ≤15 μg/liter. High serum HER2 level: >15 μg/liter. Normal serum YKL-40 level: ≤168 μg/liter. High serum YKL-40 level: >168 μg/liter.

at follow-up were patients with normal serum HER2 and YKL-40 levels at recurrence. The time to progression reflected the survival time. Fig. 3, *A* and *B* show the survival curves depending on serum HER2 and YKL-40 levels. Patients with high serum levels of HER2 and YKL-40 had a shorter survival time than patients with normal levels. The median survival time after start of first-line epirubicin therapy at first metastatic recurrence was 24 months for the patients with normal serum HER2 level compared with 13 months for patients with high serum HER2 [HR = 2.19 (95% CI: 1.4–3.4), $P = 0.0004$; Fig.

Table 3 Survival dependent on receptor content and normal or high levels of serum YKL-40 and HER2

Receptor content at primary diagnosis	Serum YKL-40 level	Serum HER2 level	No.	Median survival in months (95% CI)	2- and 5-years actuarial survival
Positive (n = 71)	Normal	Normal	39	39 (23–54)	67%, 28%
	Normal		51	34 (25–42)	65%, 22%
		Normal	48	33 (19–47)	58%, 23%
		High	23	16 (9–24)	40%, 4%
	High		20	13 (10–17)	20%, 5%
	High	High	11	12 (9–15)	18%, 9%
Negative (n = 29)	Normal	Normal	15	19 (10–28)	33%, 27%
	Normal		19	15 (7–24)	32%, 21%
		Normal	20	13 (5–21)	25%, 20%
		High	9	6 (5–8)	11%, 0%
	High		10	4 (0–9)	0%
	High	High	5	4 (1–7)	0%

Table 4 Relative risk of progression and dying for serum HER2 and YKL-40 level and ER status in a forward stepwise Cox regression model

Relative risk or HR	High HER2 level (>15 μg/liter, n = 32)	×	High YKL-40 level (>168 μg/liter, n = 30)	×	Lack of ER at primary diagnosis (n = 29)
Of progression					
HR (95% CI)	1.92 (1.2–3.0)	×	1.96 (1.2–3.1)	×	2.16 (1.3–3.5)
P	0.005		0.005		0.002
Of dying					
HR (95% CI)	1.93 (1.2–3.1)	×	2.57 (1.6–4.1)	×	2.18 (1.3–3.5)
P	0.006		0.0002		0.002

3*A*]. The median survival time was 29 months for the patients with normal serum YKL-40 and only 12 months for patients with high serum YKL-40 [HR = 2.76 (95% CI: 1.8–4.3), P = 0.00003; Fig. 3*B*]. All of the patients who were still alive after 5 years had normal serum levels of HER2 and YKL-40 at first recurrence. There was a greater difference in the actuarial fraction of patients alive at 2 years depending on low or high levels of serum YKL-40 (13 *versus* 56%) than serum HER2 (31 *versus* 48%). At recurrence, patients with tumors lacking ERs at primary diagnosis had a more aggressive tumor than patients with ER-positive tumors with a shorter time to progression [median 6 *versus* 12 months, HR = 1.8 (95% CI: 1.1–2.9), P = 0.01]. As illustrated in Fig. 3*C*, they had a shorter median survival [11 *versus* 26 months, HR = 1.71 (95% CI: 1.1–2.7, P = 0.023)] with a marked difference at 2 years (21 *versus* 52%) but equal 5 years survival yielding "banana-shaped" curves. Dependent on ERs, the patients could further be subdivided dependent on serum HER2 and YKL-40 levels. In Table 3, the median survival and actuarial percentage surviving at 2 and 5 years dependent on combinations of these three risk factors are depicted. There is a great variation ranging from a median survival of 39 months in patients with the absence of all risk factors to 4 months in the few patients with the presence of all risk factors and a corresponding 2 years survival various from 67 to 0%. In Table 4, this various independent influence on the relative risk of progression and dying from these prognostics are calculated in a forward stepwise Cox multivariate regression analysis. It shows that high serum levels of HER2 and YKL-40 and lack of ERs are independent and highly significant indicators of aggressive tumor behavior reducing time to progression and survival. The presence of all risk factors increases the relative risk of progression to 8 (95% CI: 1.9–32) and of dying to 11 (95% CI: 2.5–45; the product of the factors).

In Fig. 4, the survival time for patients with the four combinations of YKL-40 or HER2 levels depicted in Fig. 2 is shown. The patients with normal serum levels of both HER2 and YKL-40 (n = 54) had the longest median survival of 32 months with an actuarial 28% still being alive after 5 years. All patients still alive at follow-up were found in this group of patients. Three minor groups of patients had various combinations of high levels of one (n = 16 and 14) or both (n = 16) of the factors. In patients with a normal serum HER2 level (fat lines), a high or normal YKL-40 made a clear separation of the survival curves. In patients with a normal serum HER2, a high serum YKL-40 predicted a poor prognosis with a median survival of 15 months [HR = 2.84 (95% CI: 1.5–5.3), P = 0.0007]. Patients with both high serum HER2 and YKL-40 level had the poorest median survival of only 9 months contrasting 22 months for patients with high HER2 and normal YKL-40 levels (thin lines). The difference in this small group of patients did, however, not reach significance (P = 0.12).

Table 5 gives the relative risk of dying taking one more of various clinical prognostic variables and a high serum HER2 or YKL-40 level or lack of ERs into account in a forward stepwise Cox regression models with categorical indicator covariates. For each prognostic variable, a high serum HER2 or YKL-40 level or the lack of ERs independently doubled the risk of dying after first recurrence (HR = 1.9–2.68). The failure of inducing a CR on epirubicin therapy had the highest independent impact on survival (HR = 7.91). In patients not obtaining a CR, high serum YKL-40 was a stronger predictor of survival than high serum HER2 or lack of ERs. The clinical variables that also

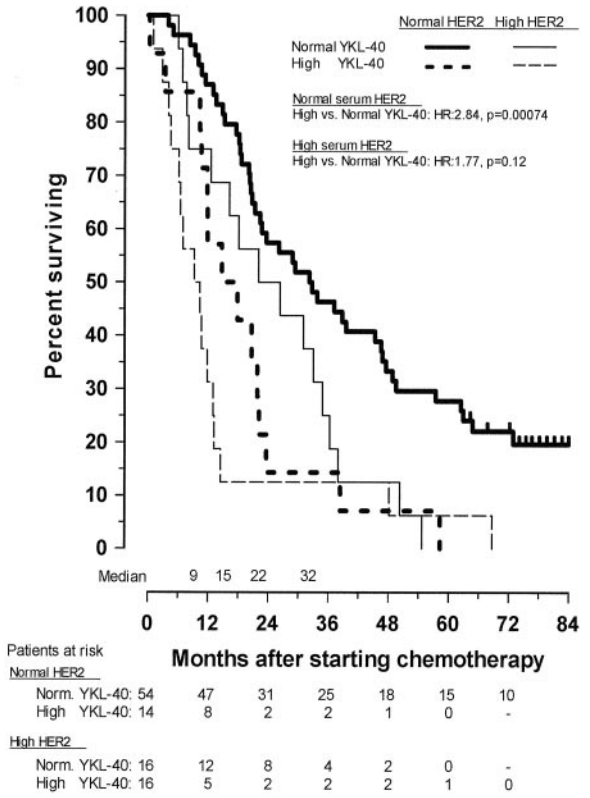

Fig. 4 Survival in relation to serum HER2 and YKL-40 level. With a normal HER2 level (*fat lines*), the YKL-40 level separated the patients into those with a good (*straight fat line*) and bad prognosis (*dotted fat line*). With a high HER2 level (*thin lines*), separation by YKL-40 level did not reach significance probably because of the small numbers of patients.

independently added prognostic information of short survival independent of HER2, YKL-40, and ERs were involvement of the axillary lymph nodes at primary diagnosis, liver metastasis, more than two metastatic sites, symptomatic disease at recurrence, and age ≥ 50. Table 6 shows the results of a forward stepwise Cox regression model, including 10 prognostic variables. Serum HER2, YKL-40, receptor status, nodal status, liver metastases, and more than two metastatic sites all yielded independent prognostic information, whereas this was not the case for age or parenchymal, bone, or lung (without liver) metastases.

DISCUSSION

Much cancer research of the past 2 decades has focused on identifying the molecular and genetic changes that cause malignant transformation. These abnormalities can be attractive targets for the development of new anticancer treatments. One example is the humanized monoclonal antibody, Trastuzumab, that binds with high affinity to the ECD of HER2, thereby blocking its function in signal transduction and has proven successful as single agent (2) or when combined with chemotherapy (1). In chemoresistant gastrointestinal stromal tumors that overexpress the proto-oncogene c-kit leading to kinase activation, selective inhibition of tyrosine kinases has proven successful (3). These findings led us to explore another growth factor, YKL-40, expressed by several types of adenocarcinomas, including breast cancer, and to compare the prognostic value of the serum levels of this protein with serum HER2 levels in patients with their first recurrence of breast cancer.

We found that high serum levels of HER2 and YKL-40 in these patients independently reflected increased aggressiveness and decreased response to anthracycline-based therapy. Patients with high serum levels of HER2 or YKL-40 progressed and died twice as fast as patients with normal serum levels. They were significantly sicker at recurrence and had more extensive disease with more different metastatic sites and frequent liver involvement. In a multivariate Cox analysis, high serum levels of HER2 or YKL-40 or lack of steroid receptors at diagnosis independently doubled the relative risk of progression and dying. This influence was maintained even after accounting for other independent prognostic variables, such as axillary nodal involvement at primary breast cancer diagnosis, lack of steroid receptors, liver metastases, more than two metastatic sites, symptomatic disease at recurrence, and the failure to induce a CR. The failure of inducing a CR on epirubicin therapy had the highest independent impact on survival (HR = 7.91). In these patients, high serum YKL-40 was a stronger predictor of survival than high serum HER2 or lack of ERs. The lack of correlation between serum levels of HER2 and YKL-40 and the independence of their impact on time to progression and survival indicate that these two growth factors have different biological functions. Among patients with a normal serum HER2 level, a subgroup could be identified with a high serum YKL-40 level with a significantly worse prognosis.

The family of epidermal growth factor-receptor tyrosine kinases, which include HER2, has attracted considerable interest in the last decade because many epithelial tumors express increased amounts of these proteins. Immunohistochemical analysis has shown that HER2 is overexpressed in 25–30% of breast cancer patients usually as a result of gene amplification. Overexpression of HER2 has been associated with an adverse prognosis (6, 7), and the serum level of the ECD of the HER2 protein correlates with tissue expression of the HER2 protein in most (9, 37–39) although not in all studies (12, 13). In one study, the serum HER2 level was a better prognostic parameter than the tissue expression of HER2, suggesting that the shedding of the soluble fragments of HER2 into the serum may be a characteristic of the malignant cell (40). In the present study, we found that 32% of the patients with their first recurrence of metastatic breast cancer had serum HER2 concentrations higher than seen in healthy females. High serum HER2 levels reflected disease burden with a shorter time to progression and death, indicating an aggressive form. These observations are in accordance with others (8–13), and only one study found no relation to the clinical course (41). Serum HER2 levels seem to correlate with the patients' prognosis, whatever the stage of disease (13, 42), and is associated with tumor burden and metastatic disease (9, 10, 37, 42–44) and may be useful to monitor breast cancer patients for early recurrence (39, 40, 43). Serum HER2 levels may predict patients' resistance, especially to hormonal therapy and possibly less to chemotherapy (8, 10,

Table 5 Relative risk of dying by serum HER2 and YKL-40 level and ER status and one more prognostic variables in a forward stepwise Cox regression model

Prognostic variable	No. of patients with variable	Relative risk of dying for: Variable present	×	High HER2 (>15 μg/liter) $n = 32$	×	High YKL-40 (>168 μg/liter) $n = 30$	×	ER lacking at diagnosis $n = 29$
Axillary lymph node involvement at primary diagnosis	61	2.34[a]		2.20[b]		2.64[c]		2.68[c]
Liver metastases	18	2.33[d]		1.91[d]		2.13[e]		2.09[e]
More than two metastatic sites	13	4.51[c]		2.05[e]		2.10[e]		2.34[b]
Age = 50 years	56	1.87[d]		1.95[d]		2.55[c]		2.55[a]
Symptomatic disease (PS 1 + 2)	23	2.63[a]		1.90[d]		2.57[c]		2.61[c]
Not obtain a complete remission	61	7.91[c]		[f]		3.11[c]		[f]

[a] $P < 0.0005$.
[b] $P < 0.001$.
[c] $P < 0.0001$.
[d] $P < 0.01$.
[e] $P < 0.005$.
[f] Not significant.

Table 6 The relative risk of dying in a forward stepwise Cox regression model for 10 prognostic variables

Prognostic variables included in the model	No.	Relative risk	(95% CI)	*P*
High serum HER2 (>15 μg/liter)	32	2.21	(1.4–3.6)	0.02
High serum YKL-40 (>168 μg/liter)	30	1.88	(1.1–3.2)	0.02
ER lacking at diagnosis	29	2.68	(1.6–4.4)	0.0001
Axillary lymph node involvement at primary diagnosis	61	2.06	(1.3–3.3)	0.003
Liver metastases	18	1.96	(1.0–3.7)	0.04
More than two metastatic sites	13	3.36	(1.6–6.9)	0.001

11, 43–45). In the present study, normal serum HER2 level predicted sensitivity to anthracycline-based chemotherapy as reflected by the number of patients reaching a CR. Patients with liver metastases had very high serum levels of HER2. Others have also reported increased expression of serum HER2 in patients with liver metastases (9), but the cause is unknown. The role of HER2 in other cancers than breast cancer remains to be elucidated. HER2 is also expressed in other cancers of epithelial origin, like lung, ovarian, colorectal, pancreatic, and prostate carcinoma and primary hepatoma (38, 46). The *HER2* gene is amplified in ~40% of patients with nasopharyngeal carcinoma (47) and in 10% of patients with small cell lung cancer where overexpression was an independent prognostic factor for survival (48).

The findings that serum YKL-40 levels may be useful to identify breast cancer patients with a very aggressive disease and bad prognosis at time of first recurrence are in accordance with a small study of patients with metastatic breast cancer (31) and with two large studies of patients with colorectal cancer (32, 33). It is unknown if serum YKL-40 could be used to monitor patients at follow-up after primary diagnosis of breast cancer as has been found for colorectal cancer (33). YKL-40 is, like HER2, expressed by several types of adenocarcinomas. A search of the YKL-40 protein sequence against the dbest database at the National Center for Biotechnology Information using the BLAST program has shown that the protein is expressed by adenocarcinomas in the colon, breast, ovarian, uterine, prostate, kidney, and lung.

High YKL-40 production in tissues and high serum YKL-40 levels are associated with intense remodeling processes in tissues, such as cartilage (14, 25, 49), breast (31), vascular smooth muscle (16), and liver fibrosis (24), but is not observed in the same tissues in the absence of tissue remodeling and inflammation. Although these observations indicate that the function of YKL-40 is linked with tissue remodeling, the exact function is unknown. It has recently been shown that YKL-40 has growth factor activity for specific cell types involved in tissue remodeling processes (19–21). Malinda *et al.* (21) demonstrated that YKL-40 acts as a chemoattractant for human umbilical vein endothelial cells and stimulates migration of these cells at a level comparable with that achieved with the known endothelial cell chemoattractant basic fibroblast growth factor. They found that YKL-40 modulates vascular endothelial cell morphology by promoting the formation of branching tubules, indicating that YKL-40 may function in angiogenesis by stimulating the migration and reorganization of vascular endothelial cells. Recklies *et al.* (19) have found that YKL-40 increased growth rates of three fibroblastic cell lines derived from human osteoarthritic synovium, fetal lung, and adult skin. YKL-40 was effective in a concentration range similar to insulin-like growth factor 1, and YKL-40 and insulin-like growth factor worked synergistically in stimulating the growth of the fibroblasts. De Ceuninck *et al.* (20) have demonstrated that YKL-40 in physiological concentrations increased the number of chondrocytes and synovial cells and proteoglycan

synthesis. We found very high serum levels of YKL-40 in breast cancer patients with liver metastases. In the liver, the hepatic stellate cells play a central role in both synthesis and degradation of extracellular matrix, but whether the breast cancer cells or hepatic stellate cells are the major source of YKL-40 in liver metastases is unknown. Ongoing immunohistochemical analysis has shown that some patients operated for primary breast cancer have YKL-40-positive breast cancer cells.[3] Immunohistochemical analysis of liver biopsies from patients with nonmalignant liver disease have shown that YKL-40 is found in areas with fibrogenesis (24), and patients with liver fibrosis have elevated serum YKL-40 (24, 50, 51). Liver metastases from patients with breast cancer have not yet been evaluated for YKL-40 expression. Liver metastases from human colon carcinoma cells have been shown to be closely associated with hepatic stellate cells, and the tumor induced peritumoral accumulation and activation of hepatic stellate cells (52, 53).

All cells rely on a complex interplay of both extracellular and intracellular signals to control their metabolism, growth, and differentiation. We recently demonstrated that aggressive breast cancer was closely associated with extracellular matrix building (54). Breast cancer induces a strong fibroproliferative response with synthesis of type I collagen, reflecting breast cancer activity, aggressiveness, expansion, and metastases (54). It is unknown if YKL-40 has some function in the production of an altered extracellular matrix surrounding the cancer cells by playing a regulating role in this cell–matrix interaction. Although high serum levels of YKL-40 may indicate a poor prognosis for patients with breast cancer, the mechanism and function of YKL-40 in cancer are essentially unknown. The elucidation of YKL-40 function in cancer may be an important objective of future studies, as YKL-40 may play an important role in cancer expansion and invasiveness. The YKL-40-positive cancer cells may have a different phenotype than the YKL-40-negative cancers, and thereby, YKL-40 may reflect differences in the biology of various cancer cells.

Serum HER2 and YKL-40 and absence of ERs at diagnosis exerted a comparable and mutually independent biological response modification on breast cancer aggressiveness as reflected by metastatic pattern, responsiveness to anthracycline therapy, progression, and fatal outcome. Interestingly, high serum HER2 and high serum YKL-40 independently identified subgroups of patients with metastatic breast cancer with a poor prognosis.

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