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Report

High serum YKL-40 levels in patients with primary breast cancer is related to short recurrence free survival

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Summary

YKL-40 is a growth factor for connective tissue cells and stimulates migration of endothelial cells. YKL-40 is secreted by cancer cells, and elevated serum YKL-40 in patients with metastatic breast cancer and colorectal cancer is associated with a poorer prognosis as compared to patients with normal serum YKL-40. In the present study we evaluated the associations of preoperative serum YKL-40 in 271 patients with primary breast cancer in relation to relapse-free survival and overall survival. The median follow-up time was 5.9 years. There were 77 relapses and 69 patients died. The median serum YKL-40 concentration in the patients was 57 μg/l (range 22–688 μg/l) and significantly elevated ($p < 0.0001$) compared to serum YKL-40 in healthy females. Nineteen percent of the patients had high serum YKL-40 (i.e., >95 percentile of healthy females). Patients with high serum YKL-40 had shorter relapse-free interval (hazard ratio (HR) = 1.77, 95% confidence interval (CI): 1.06–2.95, $p = 0.028$) and overall survival (HR = 1.78, 95% CI: 1.04–3.05, $p = 0.036$) than patients with normal serum YKL-40. Serum YKL-40 was higher ($p = 0.005$) in lymph node positive patients as compared to lymph node negative patients. Multivariate analysis including lymph node status, estrogen receptor status, tumor size, age, menstrual status and serum YKL-40 showed that serum YKL-40 was an independent prognostic variable of relapse-free survival (HR = 1.73, 95% CI: 1.03–2.91, $p = 0.039$). Our results show that serum YKL-40 in patients with primary breast cancer at time of operation is only elevated in a small group of patients, but these patients have a shorter recurrence free interval. Further studies are required to determine the biological function of YKL-40 in breast cancer.

Introduction

YKL-40, a member of the mammalian family 18 glycosyl hydrolases [1–3], is secreted in large amounts *in vitro* by the MG63 human osteosarcoma cell line [4] and expressed selectively by murine mammary tumors initiated by neu/ras oncogenes [2]. The gene for YKL-40 has been sequenced [5] and a search of the YKL-40 protein sequence against the *dbest* database at the NCBI using the BLAST program has shown that YKL-40 is expressed by several types of cancer (breast, colon, kidney, lung, ovarian, prostate, uterine, oligodendroglioma, glioblastoma and germ cell tumors). Gene expression microarray analysis has shown that YKL-40 was the most differentially expressed gene in glioblastoma multiforme [6]. YKL-40 is a heparin- and chitin-binding lectin [3, 7] without chitinase activity [1, 7]. Regulatory studies of the protein are sparse and receptor analyses have not been described. The biological function of YKL-40 in cancer diseases is not known in detail, but it has been shown that YKL-40 is a growth factor for connective tissue cells [8, 9] and a potent migration factor for endothelial cells [10]. YKL-40 is also secreted

by macrophages [5, 7, 11–14], neutrophils [15], arthritic chondrocytes [1, 14], and hepatic stellate cells (E. Efsen, personal communication) and the pattern of its expression in normal and disease states suggest a function in inflammation and remodeling of the extracellular matrix [13, 16–20].

Serum levels of YKL-40 in patients with glioma are related with tumor grade and burden [6] and we have reported that increased serum levels of YKL-40 are found in patients with metastatic breast cancer [21, 22] and in pre- and postoperative samples from patients with colorectal cancer [23, 24]. Interestingly, it was found that high serum YKL-40 levels were related to short recurrence-free interval and poor survival of these patients and that the serum YKL-40 level was an independent prognostic variable. We also found that high serum YKL-40 levels in patients with first relapse of breast cancer was a better predictor of short relapse free interval and short survival after first line chemotherapy than estrogen receptor (ER) status and high serum levels of HER2 (also named c-erbB-2 or Her-2/neu) [22].

In the present study we evaluated the value of preoperative serum levels of YKL-40 in patients with primary breast cancer in relation to relapse-free survival and overall survival.

Patients and methods

Breast cancer patients. Two hundred and seventy one female patients (median age = 58 years, range 26–83 years) who underwent surgery at John Radcliff Hospital, Oxford, England between October 1990 and December 1994 for histologically verified primary breast cancer were included. None of the patients had evident distant metastases. The patients were entered into the study consecutively provided that a preoperative serum sample was available. Sixty-six of the patients (24%) underwent modified radical mastectomy and 42 received additional adjuvant radiotherapy. Two hundred and eight (76%) had lumpectomy of whom 188 received breast irradiation. All patients had axilla dissection and axillary lymph node status was confirmed histologically. If lymph node involvement was found then adjuvant radiotherapy was delivered to the locoregional lymph nodes. Nineteen patients did not receive irradiation due to (1) radiation therapy planned but the patient progressed while receiving chemotherapy, (2) good prognostic features of the tumor, thus tamoxifen was given alone with radiation therapy in reserve or (3) the patient decided not to receive irradiation. Adjuvant systemic treatment consisted of six cycles of intravenous cyclophosphamide, methotrexate and 5-fluorouracil (CMF) delivered every 3 weeks for pre-menopausal women with positive lymph nodes ($n = 78$). CMF was given as monotherapy in ER negative patients ($n = 23$) or followed by tamoxifen (20 mg/daily) for 5 years in ER positive patients ($n = 55$). Postmenopausal women ($n = 165$) were treated with tamoxifen (20 mg/daily) for 5 years regardless of hormonal receptor status. The patients were classified as ER positive if the quantity of biochemical assayed ER was greater than or equal to 10 fmol/mg cytosol protein.

All patients were seen in the follow-up every 3 months for the first 18 months and every 6 months thereafter. Treatment for confirmed recurrent disease was delivered by endocrine manipulation of soft tissue or skeletal metastases or by chemotherapy for visceral disease and failed endocrine therapy. The actual cause of death was not available for all patients, thus recording of survival was based on death from all causes. The median time of observations was 5.9 years. 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 required. Further information about the patients and the study are described elsewhere [25].

Healthy volunteers. The normal range of serum YKL-40 was determined in 63 apparently healthy English females (median age of 41 years, range 30–58 years) who were attending the blood bank at the John Radcliff Hospital, Oxford, England.

Biochemical analysis. Blood samples from the patients were taken within 1 week before operation. Blood samples from the patients and controls were drawn into dry tubes, allowed to clot at 4°C for maximal 2 h and the sera separated from cellular elements by centrifugation at 1600*g* at 4°C for 10 min. The sera were stored at −80°C until analysis. All analysis were performed at the end of the study after a minimum follow up of 5 years. Serum YKL-40 was determined by ELISA (Quidel Corporation, Santa Clara, CA, USA). The YKL-40 assay is a sandwich enzyme immunoassay in a microtiter stripwell format. The Fab fragment of a monoclonal anti-YKL-40 antibody conjugated to biotin binds to streptavidin on the strip and captures YKL-40 in a standard, control or sample. A polyclonal anti-YKL-40 antibody

Table 1. Relation between serum YKL-40 levels and patient ($n = 271$) and tumor characteristics

	Serum YKL-40 µg/l	
	Median (range)	*p*-value
Lymph node status		
Negative ($n = 163$)	51 (22–326)	0.005
Positive ($n = 108$)	62 (22–688)	
ER status		
Positive[a] ($n = 177$)	60 (22–688)	0.08
Negative ($n = 94$)	49 (22–279)	
Tumor size		
≤2 cm ($n = 142$)	55 (22–688)	0.47
>2 cm ($n = 129$)	58 (22–471)	
Tumor histology		
Ductal ($n = 215$)	54 (22–688)	0.15
Non-ductal ($n = 56$)	66 (24–457)	
Tumor grade[b]		
I ($n = 36$)	49 (22–688)	0.74
II ($n = 95$)	59 (22–360)	
III ($n = 60$)	52 (22–471)	
Age		
<40 ($n = 18$)	37 (22–104)	<0.01
40–50 ($n = 69$)	39 (22–360)	
>50 ($n = 184$)	65 (22–688)	
Menopausal status		
Pre ($n = 87$)	39 (22–360)	<0.0001
Post ($n = 184$)	65 (22–688)	

[a] ≤10 fmol/mg protein.
[b] Only ductal carcinomas.

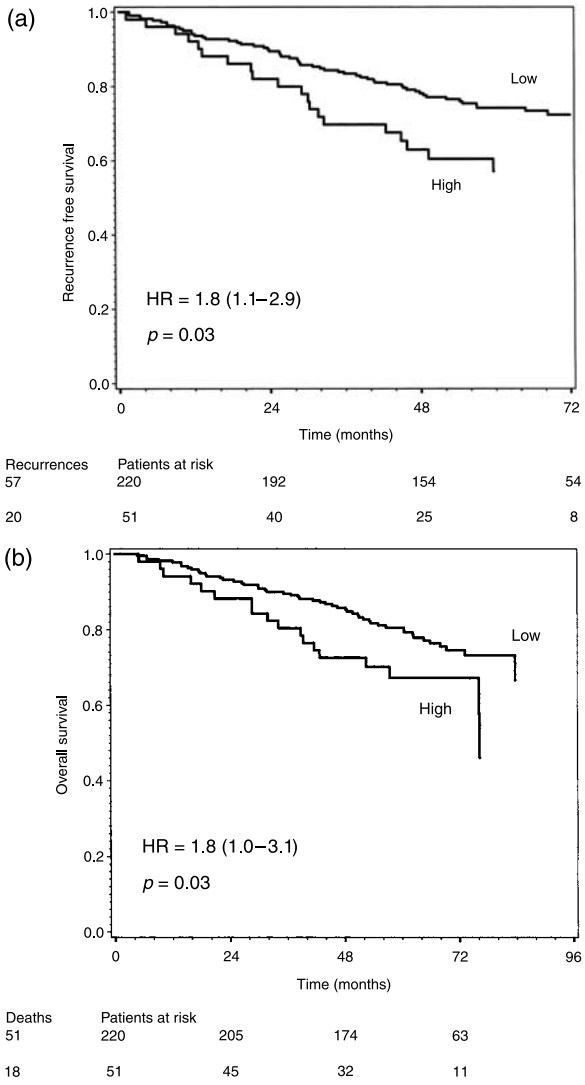

Figure 1. Survival curves showing the association between serum YKL-40 levels and recurrence-free interval (a) and between serum YKL-40 levels and overall survival (b) in 271 patients with primary breast cancer. Patients were divided into two groups according to high versus normal (age adjusted) serum level of YKL-40 obtained preoperatively. The strata are: (1) patients having normal serum YKL-40 level preoperatively ('Low'); and (2) patients having elevated serum YKL-40 preoperatively ('High'). The cut-off limit used for the determination of high versus normal serum YKL-40 was the 95% percentile of the serum YKL-40 concentration in healthy females. The *p*-value shown is for the log–rank test for equality of strata. The number of events (recurrence or death) for stratum 1 and 2, and the number of patients at risk in each stratum at 0, 24, 48 and 72 months are shown below the graph, the first line for low levels and second line for high levels.

conjugated to alkaline phosphatase binds to the captured YKL-40. Bound enzyme activity is detected with *p*-nitrophenyl phosphate as substrate [19]. The sensitivity of the ELISA is 10 µg/l. The intra-assay and interassay coefficient of variation are <4.3 and <3.7%, respectively.

Statistical analysis. The SAS® software package (version 6.12; SAS Institute, Cary, NC) was used to manage the patient data and to perform all statistical analyses. Linear regression was used to estimate the age dependence of serum YKL-40 levels in the healthy controls. A normal reference region was calculated as described by Royston [26] on the log transformed serum YKL-40 values of the healthy controls adjusting for age, and the 95% percentile was chosen for the upper limit. The serum YKL-40 levels in the patients were scored as normal or elevated (by the normal age adjusted serum YKL-40 level as described above). The age range of our healthy subjects was

Table 2. Independent prognostic variables of relapse-free survival and overall survival from the multivariate Cox regression analyses

	Relapse-free survival			Overall survival		
	HR	(95% CI)	*p*-value	HR	(95% CI)	*p*-value
Lymph node status						
Negative	1.0			1.0		
Positive	2.46	(1.54–3.94)	0.0002	3.26	(1.95–5.44)	<0.0001
ER status						
Negative	1.0			1.0		
Positive[a]	0.51	(0.32–0.80)	0.004	0.48	(0.30–0.78)	0.003
Tumor size						
≤2 cm	1.0			1.0		
>2 cm	3.23	(1.94–5.39)	<0.0001	2.53	(1.48–4.33)	0.0007
Age (years)						
+1 year	1.01	(0.97–1.04)	0.67	1.03	(0.99–1.07)	0.13
Menopausal status						
Pre	1.0			1.0		
Post	0.37	(0.11–1.23)	0.11	0.60	(0.14–2.54)	0.49
Pre-menopausal						
40 years ≤ age < 50 years	1.0			1.0		
Age < 40 years	3.49	(1.42–8.60)	0.007	1.85	(0.56–6.12)	0.31
Serum YKL-40[b]						
Normal	1.0			1.0		
High	1.73	(1.03–2.91)	0.039	1.77	(1.03–3.06)	0.040

HR, relative hazard ratio; CI, confidence interval.
[a] ≤10 fmol/mg protein.
[b] Age corrected (95% upper limit of healthy females). Seventy-seven patients had a relapse and 69 patients died during the follow-up.

30–58 years, but we have found in another study of 260 healthy Danish controls with age up to 79 years using a RIA to measure serum YKL-40 that serum YKL-40 increased linearly with age [23, 27]. The primary end-point for survival analysis was recurrence (local or metastatic) and the secondary death of all causes. The Kaplan–Meier method was used to estimate survival probabilities, and the log–rank test was used to test for equality of strata. The Cox proportional hazards model was used for analysis of continuous covariates as well as for multivariate analysis. The assumption of proportional hazards was verified graphically where applicable. Rank statistics were used to calculate correlation coefficients and to test hypothesis on location. Tests of independence were done using the χ^2-test. The significance level was set to 5%.

Results

The median serum YKL-40 concentration in the patients was 57 μg/l (range 22–688 μg/l) and significantly elevated ($p < 0.0001$) as compared with the level in the healthy females 27 μg/l (range 20–122 μg/l). Fifty-one (19%) of the patients had serum YKL-40 levels above the upper 95% limit (age-corrected) of the healthy females. A correlation was found between serum YKL-40 levels and age in the patients ($\rho = 0.35$, $p < 0.0001$) and the healthy females ($\rho = 0.35$, $p = 0.005$).

Table 1 shows the associations between serum YKL-40 levels and patient axillary lymph node status, ER status, tumor size, tumor histology, tumor grade, age of the patient and menopausal status. Patients with axillary lymph node metastasis had significantly

($p = 0.005$) higher serum YKL-40 as compared with lymph node negative patients, whereas there was no difference in serum YKL-40 levels between ER positive and negative patients, tumor size, and tumor histology and grade. Forty-nine percent of the breast cancer patients with elevated serum YKL-40 levels had positive lymph nodes (25/51, $p = 0.14$) and 76% (39/51, $p = 0.09$) were ER negative. Postmenopausal women and older women (>50 years) had significantly higher serum YKL-40 levels ($p < 0.0001$) as compared with premenopausal and younger women.

Serum YKL-40 levels in relation to relapse-free survival and overall survival. The median follow-up period was 5.9 years. During this period there was a total of 77 (28%) relapses and 69 (25%) patients died. High serum levels of YKL-40 (i.e., >age-corrected 95 percentile of healthy females) at time of operation predicted shorter recurrence-free interval as compared with patients with normal serum YKL-40 (hazard ratio (HR) = 1.77, 95% confidence interval (CI): 1.06–2.95, $p = 0.028$). The Kaplan–Meier plot is shown in Figure 1(a). Furthermore, a significant relation was found between high serum levels of YKL-40 at time of breast cancer operation and short overall survival (HR = 1.78, 95% CI: 1.04–3.05, $p = 0.036$). The Kaplan–Meier plot is shown in Figure 1(b).

Table 2 shows multivariate Cox regression analysis of independent prognostic variables of relapse-free survival and overall survival including the serum YKL-40 level, axillary lymph node status, ER status, tumor size, age, and menopausal status. High serum level of YKL-40 (i.e., >age-corrected 95 percentile of healthy females) was an independent prognostic variable of short relapse-free survival (HR = 1.73, 95% CI: 1.03–2.91, $p = 0.039$) and for overall survival (HR = 1.77, 95% CI: 1.03–3.06, $p = 0.040$) in this multivariate Cox analysis.

Discussion

This is the first report on preoperative serum YKL-40 levels in patients with primary breast cancer. We found elevated serum levels of YKL-40 in 19% of these patients and higher serum YKL-40 in patients with metastasis to axillary lymph nodes compared to the YKL-40 levels in lymph node negative patients. There were no relationships between serum YKL-40 levels and ER status, tumor size or histology. The percentage of patients with primary breast cancer with elevated serum YKL-40 preoperatively is lower compared to breast cancer patients at time of first relapse where 24–61% of patients with metastases to bone, lung or liver have elevated serum YKL-40 levels [21, 22].

Interestingly the present study showed that high preoperative serum YKL-40 levels in patients with primary breast cancer reflected increased breast cancer aggressiveness since patients with high serum YKL-40 had shorter relapse-free survival and overall survival than patients with normal serum YKL-40. Furthermore multivariate Cox analysis including the classical parameters with known prognostic value in breast cancer patients showed that the serum YKL-40 level did give additional information of relapse-free survival. Serum YKL-40 is statistically significant at the 5% level for the primary endpoint (recurrence free survival) as well as the secondary endpoint (overall survival). However these endpoints are not independent and therefore the results can be regarded as an enhanced data description and the Bonferroni correction for multiple testing will be too conservative [28]. The statistical power of this study is relatively low as the group of patients with elevated serum YKL-40 levels is small and therefore a confirmatory study is needed. The results of the present study are in accordance with studies of patients with metastatic breast cancer [21, 22] and patients with colorectal cancer [23, 24]. A high serum YKL-40 level in breast cancer patients at time of first recurrence after first line chemotherapy and radiotherapy has been shown to predict shorter time to progression and shorter survival compared to patients with normal serum YKL-40 levels at time of first recurrence [22]. It was also shown that the serum YKL-40 level was an independent prognostic variable of short time to progression and death in a multivariate Cox analysis including axillary nodal involvement at primary diagnosis, ER status, liver metastases, more than two metastatic sites, symptomatic disease at recurrence and serum HER2 levels [22]. High preoperative serum YKL-40 levels in patients with colorectal cancer is also a prognostic parameter of short relapse-free interval and short survival and this is independent of Dukes' stage, age, tumor localization and serum CEA [23]. Recently it has also been shown in curatively operated colorectal cancer patients that a high postoperative serum YKL-40 level during the follow-up was a strong independent predictor of short recurrence-free interval and short survival [24].

YKL-40 is overexpressed by several types of cancer, including breast, colon, ovarian, uterine, prostate,

kidney and lung. Although high serum levels of YKL-40 may be associated with poor prognosis in patients with primary breast cancer and in metastatic breast cancer and colorectal cancer the precise biological function of YKL-40 in cancer diseases is unknown. We speculate that the YKL-40 positive cancer cells may have a different phenotype than the YKL-40 negative cancer cells, and thereby YKL-40 may reflect differences in the biology of various cancer cells. It has recently been shown that YKL-40 has growth factor activity for cell types involved in inflammation and tissue remodeling processes [8–10]. YKL-40 increased growth rates of three fibroblastic cell lines derived from human osteoarthritic synovium, fetal lung and adult skin. Furthermore, YKL-40 worked synergistically with IGF-1 in stimulating the growth of the fibroblasts [8]. It has also been shown that YKL-40 in physiological concentrations increased the numbers of chondrocytes and synovial cells, and stimulated proteoglycan synthesis [9]. Interestingly, Malinda et al. [10] demonstrated that YKL-40 acts as a chemoattractant for human umbilical vein endothelial cells and stimulates migration of these cells at a level comparable to that achieved by basic fibroblast growth factor. YKL-40 also modulated vascular endothelial cell morphology by promoting the formation of branching tubules [10], indicating that YKL-40 may also function in angiogenesis by stimulating the migration and reorganization of vascular endothelial cells.

In conclusion, serum levels of YKL-40 levels are only elevated in 19% of patients with primary breast cancer at time of operation, but the patients with elevated serum YKL-40 level have a shorter recurrence free interval than patients with normal serum YKL-40. Future studies are required to further elucidate the biological function of YKL-40 in cancer diseases.

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