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Anil K. Hurmale¹
Sudheer K. Choudhary¹
Satish K. Bhaskar²
Suresh K. Jatawa¹
Mahavir Yadav¹
Archana Tiwari¹

Overexpression of chitinase like protein YKL-40 in leukemia patients

Authors' addresses:

¹ School of Biotechnology, Rajiv Gandhi Proudhyogiki Vishwavidhyalaya, Airport Bypass Road, Bhopal, India.

² Anand Niketan, A democratic school 159, Induds Empire, Bawadiya Kalan Trilanga, Bhopal- India 462039

Correspondence:

Anil K. Hurmale

School of Biotechnology, Rajiv Gandhi Proudhyogiki Vishwavidyalaya State Technological University of Madhya Pradesh, Airport Bypass Road, Bhopal, India.
e-mail: biotechhurmale@gmail.com

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ABSTRACT

YKL-40 is a member of mammalian chitinase (CHI3L1), expressed and secreted by several types of solid tumor cells, inflammatory cells and stem cells. The precise physiological role of YKL-40 in cancer is still not clear and it is suggested that it play a role in cancer cell proliferation, differentiation, metastatic potential, cell attachment and migration, reorganization and tissue remodeling. The aim of the study was to check the appearance of YKL-40 in leukemic cells and over-expression of YKL-40 in the plasma of leukemia patients in comparison to healthy controls, and find whether YKL-40 could serve as a peripheral biomarker for leukemia. The study was conducted between July 2012 and March 2013 and included 67 volunteers, 55 having leukemia at the stage of diagnosis of the disease and 12 normal healthy volunteers. YKL-40 levels were determined in all plasma samples using the YKL-40 enzyme-linked immunosorbent assay (ELISA) kit and expression of YKL-40 was observed by using immunocytochemical (ICC) analysis. YKL-40 plasma levels differed significantly between patients with leukemia and the normal healthy volunteers ($P < 0.001$) and YKL-40 was positively expressed in all four types of leukemia (AML, ALL, CLL and CML) specimens.

Key words: YKL-40, leukemia, biomarker, ELISA, immunocytochemistry.

Introduction

YKL-40 is an inflammatory glycoprotein associated with mammalian chitinase-like proteins (CHI3L1). It is expressed and secreted by some types of solid tumor cells, inflammatory cells and stem cells. The plasma/serum YKL-40 level is often elevated in patients with disease depicted by inflammation, like some types of infections compared to normal healthy volunteers (Cintin *et al.*, 2002; Johansen *et al.*, 2004). The physiological role of YKL-40 in cancer is not clear (Johansen, 2006) and it has been suggested that it play a role in differentiation, overgrowth cancer cell, cell attachment, metastatic potential, cell migration, tissue remodeling and tissue reorganization (De Ceuninck *et al.*, 2001; Recklies *et al.*, 2002; Ling & Recklies, 2004).

It was first time reveal in whey secretions of non-lactating

cows. Human YKL-40 contains a single polypeptide chain of 383 amino acids, molecular mass of 40.4 kDa (Hakala *et al.*, 1993) and an isoelectric point about 7.6 (Renkema *et al.*, 1998).

Amino acid sequence analysis reveals that YKL-40 belongs to the glycosyl hydrolase [chitinase protein] family 18 (Henrissat & Bairoch, 1993), but it does not has chitinase function. On the basis of its three N-terminal amino acids tyrosine (Y), lysine (K) and leucine (L) and its molecular mass of 40 kDa, the protein was named YKL-40 (Johansen *et al.*, 1992). In the human, the gene encoding YKL-40 glycoprotein was discovered in 1997 (Rehli *et al.*, 1997). It is located on chromosome 1q32.1, has a size of 7.948 kb and contain of 10 exons. Two splice forms of the YKL-40 gene are reported: isoform 1 that contains exon 1-10 and isoform 2, where exon 8 is spliced out (Rehli *et al.*, 2003).

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Several clinical analysis of patients with distinct types of cancer revealed that the higher concentration of YKL-40 in serum also seems to correlate with poor prognosis and short survival in some types of cancers including ovary, breast, glioblastoma melanoma and colorectal cancer (Cintin *et al.*, 2002; Johansen *et al.*, 2003; Johansen, 2006; Roslind *et al.*, 2008; Hogdall *et al.*, 2009). YKL-40 protein expression has not been evaluated in tissue from patients with leukemia and lymphoma. In myeloma, YKL-40 arises from cells in the bone marrow microenvironment surrounding the myeloma cells (Mylin *et al.*, 2009; Schultz & Johansen, 2010).

The purpose of this study was to explore the clinical usefulness of YKL-40 in leukemia and to examine the plasma level of YKL-40 in leukemic patients (ALL, AML, CLL, and CML) and normal volunteers using enzyme linked immunosorbent assay (ELISA) and also studied the expression of YKL-40 by immunocytochemistry assay.

Patients and Methods

Patients

The study included 67 volunteers, 55 having leukemia at the stage of diagnosis of the disease and 12 normal healthy volunteers. The healthy subjects were not on medication, and had no signs or clinical symptoms of cancer, joint, liver, metabolic or endocrine diseases. Out of 55 leukemic patients, 18 patients had acute lymphocytic leukemia (ALL), 6 had acute myeloid leukemia (AML), 4 had chronic lymphocytic leukemia and 27 had chronic myeloid leukemia (CML). A written informed consent was obtained from patients before study entry, which was approved by the review board of Regional Cancer Hospital, Bhopal. From every patients and healthy volunteers, 5 ml of blood were taken by a vacutainer system with EDTA.

Analysis of YKL-40 plasma levels

YKL-40 levels were determined in all plasma samples using the YKL-40 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer instructions (BlueGene Biotech, Shanghai). The ELISA assay for the quantitative measurement of YKL-40 utilized a polyclonal anti-YKL-40 antibody and YKL-40-HRP conjugate. The assay sample and buffer were incubated together with YKL-40-HRP conjugate in pre-coated plate for one hour. Then the wells were incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction formed a blue colored complex. Finally, a stop solution was added to stop

the reaction, which was then turned the solution yellow. The intensity of color was measured spectrophotometrically at 450 nm in a microplate reader. Concurrent standards of known YKL-40 were run and a standard curve was plotted relating the intensity of the color (O.D.) to the concentration of YKL-40. The detection limit of the YKL-40 ELISA assay was 20.0 ng/ml

Immunocytochemistry for YKL-40/CHI3L1

Immunocytochemical (ICC) analysis for YKL-40 antigen was carried out on randomly selected samples of each leukemic group. ICC analysis was performed directly on slides with blood smear. The ICC procedures were performed by fixation and permeabilization in chilled methanol-acetone mixture at -20°C. Endogenous peroxidase blocking was performed using 3% H₂O₂ in PBS and blocking of non-specific binding was done by 5% BSA in PBS. The antibodies used in this study were GP39 rabbit polyclonal IgG (diluted 1:1000) and goat anti-rabbit IgG-HRP (diluted in 1% BSA, 1:1000). The color of immunostaining was developed by chromogenic substrate (TMB substrate solution). After color development, the slides were analyzed under magnification 40x by using inverted light microscope (Leica, Germany).

Results

Average values of the YKL-40 plasma level from patients with different types of leukemia and from normal healthy volunteers are shown in Table 1. The geometric mean and median of plasma YKL-40 level in leukemia patients was 180.55 (AML 170.91, ALL 168.65, CLL 211.21, and CML 186.09) and in normal volunteers was 108.25 and 173.03 (AML 171.90, ALL 171.27, CLL 194.23 and 173.03) (Table 1 and Table 2). Immunocytochemistry analysis showed that YKL-40 was positively expressed in all four types of leukemia (AML, ALL, CLL and CML) specimens (Figure 1).

Distribution of YKL-40 levels and determination of cut-off value

Plasma YKL-40 levels in each group were normally distributed. The geometric mean and median YKL-40 levels for the normal control groups were 108.25 ng/ml and 112.25 ng/ml, respectively (range 72.40–153.81 ng/ml). These values are consistent with those given in the manufacturer's instructions (Blue Gene Biotech).

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Table 1. Descriptive statistics of YKL-40 plasma levels in the different groups.

Subgroup	Size	Mean (ng/ml)	SE	Max (ng/ml)	Min (ng/ml)	CI of Mean	Range	Median (ng/ml)	90%
Healthy	12	108.25	1.04	153.81	72.40	15.49	81.41	112.25	112.00
AML	6	170.31	10.63	200.17	131.62	27.32	68.55	171.90	196.77
ALL	18	168.65	11.27	270.83	109.45	23.79	161.38	171.27	203.28
CLL	4	211.21	27.25	288.91	167.47	86.72	121.44	194.23	248.49
CML	27	186.09	9.26	317.93	117.06	19.05	200.87	173.03	212.06
All Leukemia	55	180.55	6.33	317.93	109.45	12.07	208.48	173.03	203.65

SE= Standard Error, CI= Confidence Interval

Table 2. ANOVA (Duncan's and Dunnett's Test) for YKL-40 plasma levels of leukemia patients versus normal healthy volunteers.

Group Name	N	Mean (ng/ml)	SD	SEM	Difference of Mean	q	P<0.05
AML	6	170.91	26.04	19.535	60.40	3.027	Yes
ALL	18	168.65	47.85	6.138	62.66	4.213	Yes
CLL	4	211.21	54.50	27.250	102.96	4.469	Yes
CML	27	186.90	48.16	9.268	77.84	5.622	Yes
All Leukemia	55	180.55	46.69	6.336	73.03	6.336	Yes

N = Number of Patients, SD = Standard Deviation, SEM = Standard Error of Mean, Yes = statistically significant differences.

The 90th percentile of YKL-40 values for the normal control group, 112.00 ng/ml (Prism GraphPad 5.03), was used as the cut-off value for the analyses (Figure 2). YKL-40 plasma levels differed significantly between patients of leukemia (Leukemia) and the normal healthy volunteers (ANOVA, $P < 0.001$). From Duncan's and Dunnett's test it was found that the mean of YKL-40 plasma levels in the leukemia patients (AML, ALL, CLL and CML) was significantly higher than the means found in the normal healthy volunteers ($P < 0.05$).

Analysis of Variance (ANOVA)

Using *t*-test and one way ANOVA analysis it was found that the differences in the mean values of plasma YKL-40 level between the leukemia patients and normal healthy volunteers was greater than would be expected by chance. There is a statistically significant differences $P < 0.05$ ($t = 5.139$) (Table 2).

The differences in the mean values between the chronic (CLL and CML) and acute (ALL and AML) leukemia were not statically significant ($P = 0.116$) due to random sampling variability ($t = 1.597$).

Discussion

Over the last two decades, promising data have established that YKL-40, a secreted glycoprotein, elevated in

a broad spectrum of human diseases including liver damage, asthma, diabetes, some inflammatory diseases, cardiac disorders and cancer (Shao et al., 2009; Francescone et al., 2011).

In the present study, we demonstrated that plasma YKL-40 level was elevated in all four types of leukemia: AML 6 (100%) out of 6, ALL 15 (83%) out of 18, CLL 4 (100%) out of 4 and CML 27 (100%) out of 27 (Figure 2 and Figure 3). It is also consistent with previous findings that plasma/serum YKL-40 level is increased in several types of cancer and solid tumors with a variety of histological types (Johansen et al., 2003; Mitsuhashi et al., 2009) such as it was overexpressed in 42 (58.3%) out of 74 epithelial ovarian cancer specimens (Yang et al., 2010).

Pretreatment YKL-40 was above the cut-off level in 75% (78 of 104) of patients with squamous cell carcinoma (SCC) of the cervix and 78% (29 of 37) of those with adenocarcinoma (Mitsuhashi et al., 2009). In colorectal cancer, the median preoperative serum/plasma YKL-40 level in 603 patients was 180 ng/mL (range 56-2709 ng/mL) and 16% of the Dukes' A patients, 26% with Dukes' B, 19% with Dukes' C and 39% with Dukes' D had overexpression of YKL-40 protein (Dehn et al., 2003). YKL-40 secretion was also found in human lactating mammary gland (Rosling et al., 2008).

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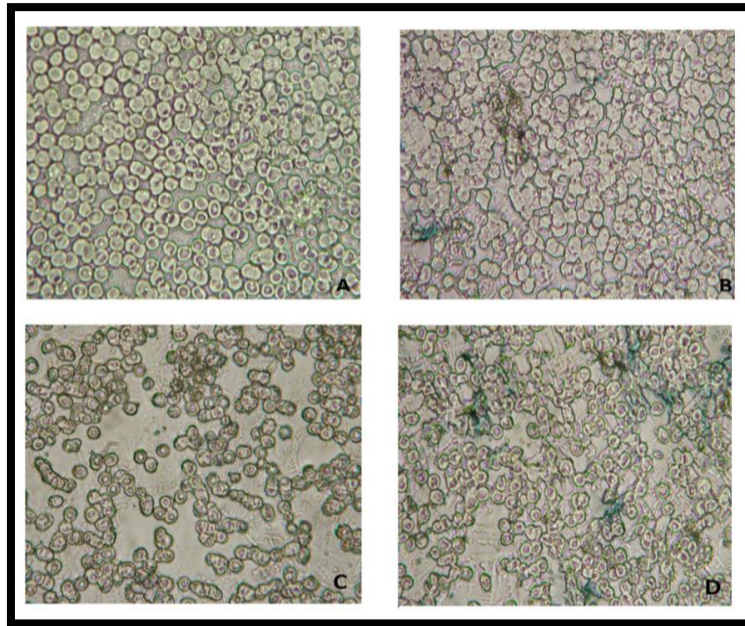


Figure 1. Immunocytochemical analysis of all four leukemia [(A) AML (B) ALL (C) CLL and (D) CML] blood smear shows the positive expression and indicate the presence of chitinase like protein YKL-40.

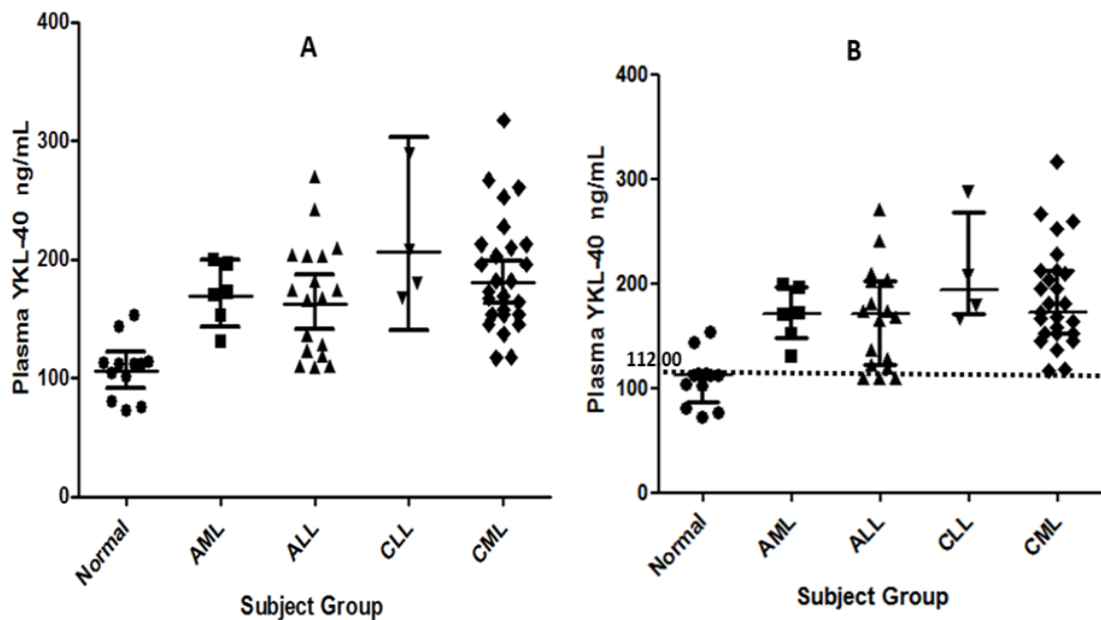


Figure 2.(A) Graph showing the point plot of plasma YKL-40 mean value of normal healthy volunteers and patients with different types of leukemia. Long horizontal lines - mean values; Short horizontal lines - standard deviations; (B) Point plot of plasma YKL-40 median value with interquartile in patients with leukemia and control healthy group patients. Horizontal dotted line - 90th percentile of healthy controls.

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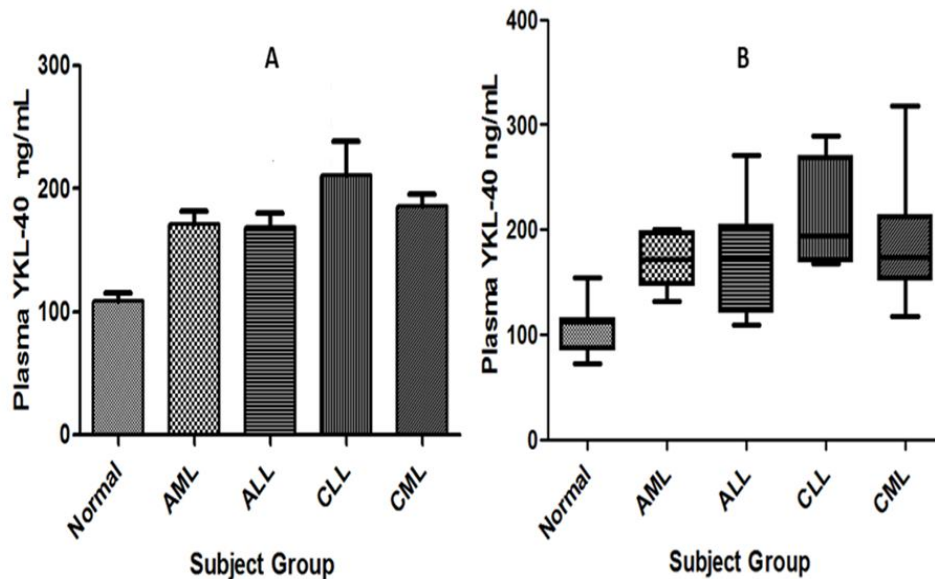


Figure 3: (A) Diagram of mean with SEM of plasma YKL-40 levels (B) Mean and SD with error bars.

In our study, we found that YKL-40 was positively expressed in all four blood smear of leukemia (AML, ALL, CLL and CML) using immunocytochemistry analysis. For this study randomly selected, one blood sample of each group (AML, ALL, CLL and CML) were used for immunocytochemical expression of YKL-40 on blood smear slides (Figure 1) and it was confirmed that biologically active YKL-40 protein produced by cancerous cells in all four types of leukemia patients.

In previous study, the expression of YKL-40 was found in synovial cells, articular cartilage chondrocytes and raise considerably in a variety of tumors and cell lines derived from kind of tumors, such as tumors of the bone, brain, breast, lung (Johansen et al., 2004), and ovary (Johansen, 2006; Hogdall et al., 2009). At the cellular level, YKL-40 protein expression is high in embryonic and fetal tissues characterized by rapid proliferation and marked differentiation and in tissues undergoing morphogenetic changes (Johansen et al., 2007; Kim et al., 2007). Overexpression of CHI3L1 on the protein level in HEK 293 cell line was confirmed by immunofluorescence and Western blot analysis (Kavsan et al., 2011) and its expression was absent in subset of glioblastomas termed “proneural”, which comprise up to 30% of all glioblastomas, due to promoter hypermethylation (Noushmehr et al., 2010).

Our study shows that plasma level of YKL-40 have a high sensitivity for leukemia, and determination of plasma YKL-

40 can be used as a prognostic marker for the existence of leukemia. We confirmed that, overexpression of plasma YKL-40 was frequently noticed in patients with leukemia. Consequently, we assume that higher-expression of YKL-40 protein in leukemiaous or leukemic patients may promote tumorigenesis or cancer progression and the plasma level of YKL-40 was elevated through increasing extracellular matrix degradation.

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