Serum 5-LOX: a progressive protein marker for breast cancer and new approach for therapeutic target

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Abstract

Lipoxygenase (LOX) pathway has emerged to have a role in carcinogenesis. There is an evidence that both 12-LOX and 5-LOX have procarcinogenic role. We have previously reported the elevated level of serum 12-LOX in breast cancer patients. This study evaluated the serum level of 5-LOX in breast cancer patients and its in vitro inhibition assessment with peptide inhibitor YWCS. The level of 5-LOX was determined by surface plasmon resonance (SPR). The peptide inhibitor of 5-LOX was designed by molecular modeling and kinetic assay was performed by spectrophotometry. The siRNA mediated 5-LOX gene silencing was performed to investigate the effect on proliferation of MDA-MB-231, breast cancer cell line. The serum 5-LOX level in breast cancer (5.69 ± 1.97 ng/µl) was almost 2-fold elevated compared to control (3.53 ± 1.0 ng/µl) (P < 0.0001). The peptide YWCS had shown competitive inhibitory effects with IC50, 2.2 µM and dissociation constant (K_D), 4.92 × 10^-8 M. The siRNA mediated knockdown of 5-LOX, resulted in the decreased gene expression for 5-LOX and increased cell death in MDA-MB-231 cell line and thereby play a key role in reducing tumor proliferation. Thus, it can be concluded that 5-LOX is one of the potential serum protein marker for breast cancer and a promising therapeutic target for the same.

Introduction

Breast cancer is the most common diagnosed malignancy among women in India and other developing countries (1). It is a leading cause of mortality in developing countries, even after the increase in number of women diagnosed with breast cancer, mainly due to late presentation. While mammography is an useful screening procedure, it is often associated with low sensitivity and specificity (2). Circulatory markers like Carcino embryonic antigen and carbohydrate antigen which is mainly used for patient follow-up have low sensitivity and specificity to detect malignancy (3). There is no consensus regarding the threshold or cutoff value to be used. Hence, there is need to work aggressively on identification and development of novel and more accurate non-invasive diagnostic markers for early detection of disease which may be helpful in the development of new therapeutic molecule. If diagnosed early, the patients will be provided with more choice in the selection of treatment options with a corresponding better patient response. The molecular diagnostic study of breast cancer in blood level will be simple and cost effective for early detection of cancer.

Inflammatory response plays a major role in tumorigenesis. The identification of leukocyte, by Rudolf Virchow, in tumor provided first evidence for the relation of inflammation and cancer (4). Arachidonic acid (AA) pathway controls the mechanism of pain and inflammation, as well as homeostatic function of the body (5). The AA pathway is regulated by cyclooxygenase and lipoxygenase enzymes, which have been shown to not only have biological impact on cancer cells, but also have important links in clinical studies of cancer (6). There is evidence that both COX-2 and LOX play a key role in tumorigenesis through stimulating epithelial cell proliferation, inhibiting apoptosis, stimulating antigen suppression and by increasing the production of mutagens (7). Both the enzyme possess two activities, peroxidase and oxidase, thus converting AA to an intermediate prostaglandin and finally to several eicasanoids PGD2, PGE2, PGF2, thromboxane A2 and leukotriene (8). The mammalian LOXs have two principal functions. One is to modify membranes by peroxidation reactions; 12/15-LOX, in homo 15-LOX type 1,
is classically connected with this function. The other is to produce signaling lipid mediators which apply its effects via G protein-coupled plasma membrane-bound receptors; may be the best example is 5-LOX and the Leukotriens (LTs). LTs are biologically active lipids that comprise of various pathological processes, such as inflammation and cancer (8).

LOX metabolites are strong physiological effectors in a variety of cellular responses, associated with normal host defense and inflammation. In particular, LTs are produced through the 5-LOX pathway. Upon cell activation, 5-LOX converts AA to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) and afterward into the epoxide LTA4. The subsequent conversions of LTA4 by LTA4 hydrolase produce LTB4, whereas metabolism by LTC4 synthase yields the cysteinyl-LTs C4, D4 and E4. Human 5-LOX is physiologically expressed in cells of the myeloid lineage, but also in B lymphocytes and pulmonary artery endothelial cells. In our previous study, it has been observed that COX-2 and 12-LOX were elevated in serum of breast cancer patients (9,10). This study focused on determination of serum level of 5-LOX protein in breast cancer patients and also analyzes the effect of 5-LOX protein by YWCS peptide inhibitor. YWCS is a small peptide inhibitor of 12-LOX which was reported in our previous study (11). Owing to the structural similarities in the active site of 12-LOX and 5-LOX (12), the effect of YWCS was assessed on 5-LOX. To the best of our knowledge, we are here, for the first time, reporting the serum level of 5-LOX in breast cancer patient and attempt to inhibit 5-LOX with small peptide inhibitor.

Materials and methods

Patient recruitment

Blood from patients with histologically diagnosed breast cancer (n = 30) before surgery was drawn at Breast Cancer Clinic, All India Institute of Medical Sciences (AIIMS), New Delhi. Institute’s Ethics committee approved the study (IEC/OP-495/06.2014) and a written consent was obtained from the entire participant recruited in the study. The clinical stage was classified according to the American Joint Committee on Cancer (AJCC) tumor-lymph node metastasis (TNM) classification system.

Patient characteristics including age, TNM stage, hormone receptors and menopausal status were recorded. Conditions including presence of pain, lump and nipple discharge were also recorded. Patients with severe infection, active clinical comorbidities, or a history of any other malignancy were excluded. Sera from a set of 30 healthy females were collected for the control group.

Estimation of 5-LOX in serum using surface plasmon resonance (SPR) technology

The level of 5-LOX protein was estimated by SPR analysis by Biacore 3000 instrument (GE Healthcare). Rabbit anti-human 5-LOX monoclonal IgG (Cell Signalling, USA) was covalently immobilized on the carboxymethyl dextran of a CM5 sensor chip by the amine-coupling method using HBS-EP buffer and controls after adjusting potential confounders. The P value of < 0.05 was considered as significant.

Molecular modeling

The crystal structure of human 5-LOX (PDB: 3O8Y, Chain A) (13) was taken from Brookhaven Protein Data Bank (14). Water molecules were removed while iron atom in the active site was retained along with their oxidation state. All the hydrogen atoms were added and their position was optimized by energy minimization with the help of CHARMM force field. Active site pocket of the protein surrounded by mainly non-polar residues Phe177, Tyr181, Phe359, His432, His436, His437, His438, Ala510, Leu414, Ile415, Phe421, Asn425, His432, His436, Ala430 and Leu607 was defined as binding site for docking studies with peptide ligand.

Conformational analysis of the peptide sequence (YWCS) was performed to calculate the probable three dimensional structure of the peptide ligand (15). Peptide sequence was modeled with protonated amino group and deprotonated carboxyl group. Gasteiger-Marsili charges were added and local energy minimization was followed using the conjugate gradient energy minimization method with termination criterion of a root mean square gradient 0.05 kcal/mol/A2. Simulated Annealing Molecular Dynamics of the minimized peptide was carried out through 50 cycles of heating to 700K followed by annealing to 200K. Fifty structures obtained at the end of each cycle were again energy-minimized and superimposed on each other. The structure of peptide with the lowest energy conformation occurring in the major cluster was chosen as the most probable conformation. This conformation of the peptide was docked into the above defined binding pocket of 5-LOX using AutoDock4 through AutoDockTools4 (16). 5-LOX protein was kept rigid during docking process while peptide ligand was treated flexible keeping the backbone atoms of the peptide constrained as they are stereochemically known to be rigid. Fifty independent docking runs were performed with potentials map Lamarckian Genetic Algorithm (17) was used for conformational sampling of the ligand with default parameters to generate the docked poses. AutoDock ranks the generated docked poses based on semi-empirically derived free energy of binding (18) and cluster them (conformations with r.m.s deviation of less than 1.5 Å was clustered together). Docked conformation from the largest cluster with the lowest free energy of binding was taken as the best binding mode and analyzed further. Since, the AutoDock does not allow torsional flexibility in the protein, the docked complex was further energy minimized with similar convergence criterion in order to relax the restriction of keeping the protein fixed during docking process.

Synthesis of peptide YWCS

The peptide, YWCS was synthesized by solid phase method using Fmoc N-terminal protector and Wang resin by automatic peptide synthesizer (PS3, Protein Technology, USA) (11).

Inhibition assay of 5-LOX by YWCS

Kinetic assay by spectrophotometry

The activity assay of purified recombinant 5-LOX was performed in the presence of different concentrations of YWCS (1, 2, 4, 8 and 10 µM) by using the conjugated diene method of biochemical assay. The assay mixture contained 20 µM AA (substrate), 0.2% Tween-20, 50 mM potassium phosphate buffer pH 7.2 and 1.0 µM purified enzyme. The conjugated diene present in hydroperoxy lipid product of the reaction strongly absorbs at
234 nm. Hence, change in absorbance at 234 nm was observed and activity was assessed.

**Binding assay by SPR**

The SPR binding study of YWCS peptide with the recombinant 5-LOX enzyme reveals the binding affinity of the peptide. SPR directly detects mass (concentration) with no need for special radioactive or fluorescent labeling of polypeptides for measurement, offering a great advantage in minimizing time and complexity of the studies (19). The experiment was performed in Biacore 3000 by using CM5 sensor chip, amine coupling kit in HBS-EP running buffer, pH 7.4 (10 mM HEPES (pH 7.4), 0.15 M NaCl and 0.005% surfactant P20, 3 mM EDTA).

The pure recombinant 5-LOX (2.37 μM) protein was immobilized over CM5 sensor chip by amine coupling method in HBS-EP running buffer at the rate of 5 μl/min. Then, three concentrations of pure YWCS (0.22, 0.17 and 0.11 μM) were passed over the immobilized 5-LOX protein to achieve the RU corresponding to the binding value. The dissociation constant (K_D) was calculated by BIAevaluation-3.0 software.

**5-LOX silencing**

Ambion siRNA Target Finder, available online tool was used for predicting the siRNA sequence against 5-LOX. The complementary oligonucleotides having flanking ApaI and EcoRI restriction sites containing the predicted siRNA sequences against 5-LOX mRNA was cloned into expression vectors pSilencer 1-U6. Three LOX-5 siRNA expression vectors namely pU6/226, pU6/321 and (pU6/328) were constructed. The vector containing the siRNA sequence was transfected into MDA-MB-231 cells using FuGENE 6 transfection reagent (Promega, USA) as per the manufacturer’s instructions. The MDA-MB-231 cell line used for the study was procured from National Centre for Cell Science, Pune of ATCC no. 245918 and it was used within 6 months with 30–35 passage numbers. The cells were maintained in DMEM media (Gibco) with 10% fetal bovine serum (Gibco, South American origin) in a humidified condition of 5% CO2 in air and 37°C temperature. Briefly, 0.2 × 10⁶ cells were seeded 1 day before transfection to obtain approximately 80% confluence. On the day of transfection, FuGENE® 6 Transfection Reagent was added to serum-free medium. This was mixed and incubated for 5min. An appropriate amount of vector DNA was added to FuGENE® 6 Transfection Reagent/medium to achieve the proper ratio of reagent to DNA. This was again mixed and incubated for 15min. This mixture (10 μl) was added to the cells and incubated for 24h and western blot was performed to check the level of knockdown. Empty vector and untreated cells served as the control.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay**

Survival of the cells after 5-LOX silencing was estimated by the MTT assay. Briefly, 3 × 10⁶ cells/well were seeded in 96-well plates and incubated with different concentrations of the siRNA for 24, 48 and 72h. MTT solution was added and plates were further incubated for 3h at 37°C. The formazan crystals formed were dissolved by adding DMSO and absorbance was measured at 570 nm. All the concentrations of siRNA used in the experiment were in triplicates and the data was analyzed using one way ANOVA. The results were presented as the mean with SD.

**Results**

**Patient recruitment**

Table 1 shows the baseline data and clinicopathological data of patients.

**Level of 5-LOX in serum of study groups**

The RU value of immobilized antibody was 4468, where 1 RU corresponds to 1 pg/mm². A standard curve was plotted with RU obtained from different concentrations of pure 5-LOX protein. The binding of the 5-LOX ligands was in the linear range. In this study, we examined the concentration of 5-LOX in the serum of breast cancer patients and controls. It was observed that the mean concentration of serum 5-LOX was significantly higher (P < 0.0001) in case of breast cancer patients (5.69 ± 1.97 ng/µl) as compared to controls (3.53 ± 1.0 ng/µl) (Figure 1A). In analysis of covariance, after adjustment for age and menopausal status, serum 5-LOX level was found to be significantly higher (P < 0.0001) in case of breast cancer patients as compared to controls. The relationship between serum 5-LOX level and clinical histopathological features was analyzed and it was found that menopausal status, Her2 status, PR status, ER status, tumor size, node, nipple discharge and lump did not affect the serum level of 5-LOX. There was no significant variation in serum 5-LOX concentration with clinical histopathological features and it was elevated in cancer with all stages compared to control group.

Based on the SPR data, ROC analysis was carried out to measure the utility of 5-LOX as a potential protein marker for breast cancer (Figure 1B). The calculated area under curve for predicting breast cancer was 0.865. Threshold for detecting breast cancer was selected based on the distribution of specificities and sensitivities. Based on our data, a threshold value of ≥ 3.32 ng/µl yields a sensitivity of 80.0% and a specificity of 83.33% to detect breast cancer.

**Molecular docking**

Binding site of 5-LOX is highly non-polar and conserved like human 12-LOX. Docked peptide ligand (Tyr-Trp-Cys-Ser) occupies the hydrophobic binding pocket of 5-LOX and forms several Vander Waals interactions (Figure 2). Hydrophobic side chains of Trp and Tyr of the peptide ligand occupies the non-polar binding region where long alkyl chain of AA has been observed (14) while negatively charged carboxyl terminal move towards positively charged Arg411. The side chain of the Trp of the ligand was surrounded by conserved hydrophobic residues Phe177, Ile406, Leu414 and Leu607. The aromatic ring of the Tyr of the ligand interacts with hydrophobic side chain of Leu420 while hydroxyl group of Tyr forms hydrogen bonded interactions with main chain carboxyl of Leu420. The amino terminal of the ligand forms hydrogen bond with side chain of conserved polar residue Gln557 while, carboxyl

![Figure 1A](https://academic.oup.com/carcin/article-abstract/37/9/912/2450017)

**Figure 1A** Baseline data and clinicopathological data of patients.

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<th>Table 1. Baseline data and clinicopathological features of subjects</th>
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<td><strong>Patients</strong></td>
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terminal of the ligand form hydrogen bonded interactions with guanidium moiety of Arg411 and carbonyl group of Ala410. These hydrogen bonds help to stabilize the peptide ligand in the mainly non-polar binding pocket. This indicates the potential binding mode of the peptide ligand inhibitor in the binding site of 5-LOX.

**Kinetics and inhibition studies of the peptides by spectrophotometry**

In our previous study, we had shown that YWCS can inhibit 12-LOX with a very good efficacy. Owing to the similarities in the active site between 5-LOX and 12-LOX the ability of YWCS to inhibit 5-LOX was assessed in this study. Enzyme activity was indirectly estimated by measuring the rate of product formation. The graph was plotted between percentage residual activity and concentration of YWCS. It was observed that the residual activity of 5-LOX declines in a dose-dependent manner with YWCS concentration. YWCS was able to inhibit 5-LOX with an IC\textsubscript{50} value of 2.2 µM (Figure 3A).

**Binding study of peptide by SPR**

The SPR signal for immobilization of recombinant 5-LOX was found to be 5251.9 RU. Three different concentrations of pure YWCS peptide in HBS-EP buffer were passed over the immobilized 5-LOX protein and the respective RUs obtained were 981.9, 758.3 and 563.9. The plot (Figure 3B) shows the sensorgram for the binding of the varying concentrations of the peptide. The alteration in RU with varying concentration of peptide showed the change of mass on the 5-LOX immobilized on chips with time. The binding of peptide YWCS with 5-LOX was the strongest due to the faster on (association), K\textsubscript{A} = 2.03 × 10\textsuperscript{7} M as well as slower off rate (dissociation), K\textsubscript{D} = 4.92 × 10\textsuperscript{−8} M.

**Silencing of 5-LOX**

After transfection of the constructs into MDA-MB-231 cells, western blot was performed to check the level of knockdown with each siRNA. As evident from Figure 4A on lane 5, transfection of pU6/328 in MDA-MB-231 cells resulted in maximum reduction in 5-LOX expression. The percentage of surviving cells after transfection of the pU6/siRNA vector was estimated by the MTT assay. As compared to the untreated and empty vector control, the percentage survival of the MDA-MB-231 cells reduced significantly in a dose-dependent manner. The percentage survival of cells transfected with 0.25, 0.5 and 1 µg of pU6/siRNA was 80, 75 and 60, respectively (Figure 4B). Thus, the decline in the viability of MDA-MB-231 cells can be attributed to 5-LOX silencing.

**Discussion**

Human cancer tissues display an enhanced expression of 5-LOX. The inhibitors of 5-LOX, arrest cell cycle progression and induce apoptotic cell death in a number of cancer cells (20). It has also been suggested that insulin like growth factor-1 (IGF-1) is an important growth factor for prognosis of tumor in breast cancer (21–26). Further studies showed that this growth factor could be neutralized by inhibiting 5-LOX pathway of AA (27) and mediate growth arrest and apoptosis in breast cancer (20). There is an evidence that 5-LOX and 12-LOX have potential role in carcinogenesis. Since, we have studied earlier the level of 12-LOX in breast cancer, this preliminary study, for the first time reports the significant elevated level of 5-LOX in serum of breast cancer patients compared to similar age as control group. In the previous study, it was observed that the incidence of cancer was higher in the middle age which might be due to the age associated hormonal change (10). So, this study included the patients only of mean age 49 years and found no significant difference in 5-LOX level in between pre and post-menopausal stage in breast cancer patients. The concentration of 5-LOX protein was found to be elevated in both early and late stage of the disease. Any clinicopathological factor did not correlate with the changes in concentration of the 5-LOX protein. The efficacy of the 5-LOX protein to detect breast cancer was determined by adjusting the other covariates and it was observed that the level of 5-LOX remained significantly high even after adjustment. The ROC analysis also

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**Figure 1.** (A) Scatter graph showing the concentrations of serum 5-LOX in breast cancer patients and controls. (B) ROC analysis showing the area under curve for 5-LOX to differentiate breast cancer patients from the healthy individuals.

**Figure 2.** Docked position of peptide ligand (Tyr-Trp-Cys-Ser) in the binding pocket of 5-LOX.

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**Figure 3.** (A) Docked position of peptide ligand (Tyr-Trp-Cys-Ser) in the binding pocket of 5-LOX. (B) Sensorgram for the binding of the varying concentrations of peptide YWCS to 5-LOX. The change of mass on the 5-LOX immobilized on chips with time is shown. The binding of peptide YWCS with 5-LOX was the strongest due to the faster on (association), K\textsubscript{A} = 2.03 × 10\textsuperscript{7} M as well as slower off rate (dissociation), K\textsubscript{D} = 4.92 × 10\textsuperscript{−8} M.

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**Figure 4.** (A) Western blot showing the level of knockdown with each siRNA. Transfection of pU6/328 in MDA-MB-231 cells resulted in maximum reduction in 5-LOX expression. (B) MTT assay showing the percentage survival of the MDA-MB-231 cells after transfection of the pU6/siRNA vector. The percentage survival of cells transfected with 0.25, 0.5 and 1 µg of pU6/siRNA was 80, 75 and 60, respectively. Thus, the decline in the viability of MDA-MB-231 cells can be attributed to 5-LOX silencing.
directed towards the utilization of 5-LOX as serum marker for breast cancer as reflected by the area under curve, specificity and sensitivity values. On the basis of obtained results, it is stated that the serum level of 5-LOX was elevated in both early and late stage of the breast cancer and high sensitivity and specificity of the proteins may be useful to diagnose the disease with high accuracy by avoiding false positive and false negative results. These findings strongly supports the serum level of 5-LOX can be a good blood-based non-invasive marker for the early detection of the breast cancer in the clinical setup.

Previously, we reported the inhibition of 12-LOX, by YWCS peptide inhibitor, effectively induced cytotoxicity and apoptosis in MCF-7 and MDA-MB-231 breast cancer cells (11). As the active site of 12-LOX and 5-LOX are homologous, the same peptide YWCS were docked with the 5-LOX protein and found to have strong interaction in the binding site. The peptide YWCS occupies the hydrophobic pocket and the Trp and Tyr side chains occupies the non-polar binding region of 5-LOX, which reflects the efficiency of peptide to compete with its substrate, AA.

The kinetic inhibition assay suggests that, as the concentration of YWCS increases, the activity of 5-LOX decreases. The peptide YWCS also inhibited 5-LOX by higher potency with IC50 value 2.2 µM which confirmed competitive inhibition in the presence of its substrate.

The SPR study showed lower K_D (nano molar) of the YWCS for 5-LOX which emphasizes the high affinity of the peptide for enzyme. This finding supports the inhibitory efficiency of YWCS for 5-LOX protein which can have pharmacological importance as a therapeutic molecule in future.

The siRNA mediated 5-LOX gene silencing was performed to check the effect on proliferation of MDA-MB-231 breast cancer cell line. The reduction of expression of 5-LOX was obtained after transfection of siRNA containing vector (pU6/328). The percentage cell survival was also analyzed by MTT assay after transfection with different concentrations of the 5-LOX siRNA. The results revealed that knockdown of 5-LOX gene can increase the cell death and thereby play a key role in reducing the tumor proliferation. Numerous autonomous studies by different groups of researchers now support the correlation between the expression of 5-LOX and cancer cell viability, invasion through extracellular matrix destruction, cell migration, proliferation, metastasis and activation of anti-apoptotic signaling cascades, which makes the 5-LOX effective therapeutic regimen (28).

![Figure 3](https://academic.oup.com/carcin/article-abstract/37/9/912/2450017)

**Figure 3.** (A) Graph showing a decline in the percentage residual activity of 5-LOX with increasing concentration of YWCS. The IC50 of YWCS peptide against 5-LOX was found to be 2.2 µM. (B) Sensogram showing binding of different concentrations of peptide YWCS (0.11, 0.17 and 0.22 mM) with immobilized 5-LOX on the CM5 sensor chip.

![Figure 4](https://academic.oup.com/carcin/article-abstract/37/9/912/2450017)

**Figure 4.** (A) Western blot showing 5-LOX silencing via different siRNA. Lane 1: untreated MDA-MB-231 cells, lane 2: transfected with pSilencer 1-U6, lane 3: transfected with pU6/226, lane 4: transfected with pU6/321, lane 5: transfected with pU6/328. β-actin was used as a loading control. (B) Effect of siRNA treatment on survival of MDA-MB-231 cells. Different concentrations of pU6/328 were transfected for 48 h and MTT assay was performed thereafter.
The earlier evidences interpreted the efficacy of peptide, YWCS, to inhibit 12-LOX and its utilization as an anti-breast cancer molecule by inducing cytotoxicity and apoptosis in MCF-7 and MDA-MB-231 breast cancer cells (11). In this study, the peptide, YWCS was found to be active against 5-LOX also and thus adding further information regarding the mechanism through which it induce cytotoxicity in breast cancer cells. This study can be translated to the clinical practice by further exploring the role of 5-LOX as one of the serum protein marker and a potential therapeutic target for breast cancer. The chemopreventive strategy exploiting inhibition of COX-2 pathway of AA metabolism faced limitation due to its long-term side effect on cardiovascular system, gastrointestinal tract, and kidney (29). Thus, the current scenario presents the opportunity to explore the other inflammatory AA metabolizing pathway like 5-LOX. The use of small peptide inhibitor YWCS can be chemopreventive in future owing to its ability to inhibit both the 12-LOX and 5-LOX.

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