RESEARCH ARTICLE

Evaluation of C-reactive protein in breast cancer by enzyme linked immunoassay technique

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Abstract

Objective: To explore the serum levels of C-reactive protein in breast cancer patients, and to investigate the relationship between inflammation and progression of breast cancer.

Methods: The case-control study was conducted at Bahria University Medical and Dental College, Karachi, from September 2015 to December 2018, and comprised breast cancer patients in group A and and an equal number of age-matched healthy women in control group B. C-reactive protein levels were evaluated in serum samples using enzyme-linked immunosorbent assay in both the groups and micro ribonucleic acid levels in serum were quantified using real time polymerase chain reaction. Data was analysed using SPSS 16.

Results: Of the 170 subjects, 85(50%) were in each of the two groups. C-reactive protein and micro ribonucleic acid expression were significantly different in group A ($p<0.001$). There was no correlation ($r = 0.162$, $p>0.01$) between the tumour markers in group B ($p>0.05$).

Conclusion: Significantly raised C-reactive protein levels showed there was a link between inflammation and breast cancer.

Keywords: Breast cancer, C-reactive protein, MicroRNA 16. (JPMA 71: 424; 2021) **DOI: https://doi.org/10.47391/JPMA.297**

Introduction

Breast cancer is the most predominant kind of cancer in the world and is a major health risk for women. One million women are diagnosed with breast cancer per year and this leads to 0.5 million deaths on a yearly basis.[1] Breast cancer is basically a tumour which originates from the breast tissue and ascends from milk ducts' internal lining or the lobules supplying the ducts with milk.[2] The prevalence of breast cancer is increasing at an alarming rate with various factors involved like gender, age, genetics, lack of childbearing, breastfeeding, higher hormonal levels and individual lifestyle. In Asia, Pakistan has the highest rate of breast cancer.[3] Therefore, there is need to plan and implement strategies for the prevention and control of breast cancer in clinical research.

C-reactive protein (CRP) is a classical acute phase reactant protein released as a result of acute inflammation, infection and tissue damage.[4,5] It is synthesised by hepatocytes against inflammation, trauma and tissue damage, and is raised in chronic inflammatory states. CRP is a cyclic pentameric structure with ligand binding site. Five identical noncovalently associated protomers are situated symmetrically around a central pore. Each promoter is composed of 206 amino acid residues with ligand binding site having a pocket with two ions of calcium. Calcium ions are required for ligand binding and stability of the molecule CRP.[6]

CRP is known to be a highly sensitive marker showing progression of inflammation in various diseases, like Crohn's disease and inflammatory bowel disease. Higher CRP levels are found to be in different types of cancer, such as gastro-oesophageal cancer, lung cancer and prostate cancer.[7-11] Several studies have explored the association between chronic inflammation and carcinogenesis and it may be suggested that tumour develops and extends at the site of chronic inflammation. However, tumour cells attract immune cells and increase the production of cytokines and chemokine, showing a tumour microenvironment. It indicates that cancer is associated with persistent inflammatory condition.[12] Inflammatory pathways also play a significant part in the causation of breast cancer. Some studies performed with the diagnosis of breast cancer have shown an association between elevated CRP and poor prognosis while some have shown no relation between inflammation and breast cancer prognosis[13,15] and stillothers have shown no relationship at all.[16,17]

The current study was planned to explore the role of CRP in breast cancer patients, and to determine the association between inflammation and breast cancer.

Patients and Methods

The case-control study was conducted at Bahria University Medical and Dental College, Karachi, from September 2015 to December 2018. After approval from the institutional ethics review committee, the sample size was calculated

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using the Epi statistical programme from the Open Source Statistics for Public Health and results were presented using methods of Kelsey, Fleiss, and Fleiss (2010).[18] Patients suffering from breast cancer of invasive ductal category in stage III were selected from Ziauddin Cancer Hospital, Karachi. These were newly diagnosed cases who had not undergone any chemotherapy or surgery. Age-matched healthy females comprised the control group. They had no disease history, and were randomly selected from the community. Those identified with any metabolic disorder or endocrinological problem, like diabetes mellitus or thyroid disorders, were excluded.

After taking written consent, a questionnaire was handed to all the subjects. Data obtained included dietary pattern and disease history for the cases. Weight, height, waist and hip circumference were measured, and body mass index (BMI) was computed. Breast cancer information about diagnoses and prognostic tumour characteristics, like tumour size and grade, presence of distant metastases, lymph node status, of index breast cancers were obtained from the relevant breast cancer clinics.

A 5ml blood sample was drawn from each subject, and, after centrifugation, serum was kept at –70ºC for further analyses regarding CRP using enzyme-linked immunosorbent assay (ELISA) kit (DIA source ImmunoAssays, Belgium). The assay was sandwich type,with two types of specific monoclonal antibodies.

One monoclonal antibody which was immobilised on to the microwell plate was specific for CRP while other was bound to horse radish peroxidase (HRP) and specified particular region of CRP. When the samples were loaded to the plate, CRP from the samples were bound to the plate. After washing to remove unbound contents, it was further incubated with HRP conjugate. It was then again washed and enzyme substrate was added. The reaction was stopped by adding the stopping solution. The absorbance was recorded on a microtiter plate reader. The darker the colour of reaction mixture, the more was the concentration of CRP in the sample. The concentration of CRP was determined by plotting standard curve of known calibrators.

For micro ribonucleic acid-16 (miRNA-16) extraction, RNeasy® Plus Mini Kit SN R-060953 (Hilden, Germany) was used. After miRNA-16 extraction, complementary deoxyribonucleic acid (cDNA) was synthesised using miScript RT II kits as per the manufacturer's instructions. The cycling conditions were set at 40 cycles and real-time PCR (RT-PCT) were adjusted for 15s at 94ºC for denaturation, 30s at 55°C for annealing, and 30s at 70ºC for extension. RT-PCR reaction was performed using miScript SYBR Green PCR kit (Synergy Brands Inc). In this protocol, 10 pmol Caenorhabditis (C.) elegans miR-39 miRNA mimic spike-in control was used as positive control (PC) and reference for miRNA-16 primer was used along with melting curve in a Light Cycler 480 System (Roche, Switzerland) . The fold change was then calculated to determine the gene expression for all the samples. The sequence of miRNA-16 primer used was 5 'tagcagcacgtaaatattggc3' (Forward primer: TAGCAGCACG TAAATATTGGCG (included in the Quanti Mir RT kit, cat no RA420A-1; Reverse primer: QuantiMir Universal Reverse primer http://microrna.sanger.ac.uk/ http://microrna.sanger.ac.uk/sequences/search.shtml.[19] The efficiency test of primer was performed by making serial dilution of template mixture of cDNA in a ratio of 1:5 and used all the dilutions from 1:1, 1:2, 1:3, 1:4, 1:5 as a template in each reaction with forward and reverse primers. Quantitative RT-PCR (qRT-PCR) was performed for 40 cycles along with melting curve in triplicates and a semi-log was plotted with Ct (cycle threshold i.e number of cycles required for the fluorescence signal to reach a threshold where it can be detected as it exeeds background level) versus copy number/dilution and finally, the slope was determined using the formula e = 10^ (-1/slope) to calculate the efficiency of the primer.[20,21]

Serum miRNA-16 relative quantification was calculated using the equation; amount of target=2^^ct-((Ct pc - Ct miRNA-16)-(Ct mean pc - Ct mean miRNA-16)) and results were expressed as fold change and calculated to determine the gne expression in all the groups.[22,23] Data was evaluated using Roche LC480 software, and results were analysed using SPSS 16. Student's t-test and Pearson's correlation coefficient were employed for statistical analysis. The standard curve of high sensitivity CRP (hs-CRP) was plotted between CRP solution in protein-based buffer concentration ranging 0-10,000 ng/ml.

Results

Of the 170 subjects, 85(50%) were in each of the two groups. The standard curve of (hs-CRP showed R2=0.998, indicating accuracy of values (Figure 1).

Serum CRP levels were significantly higher in cases compared to controls (Table 1, Figure 2).

Table-1: Serum C-reactive protein (CRP) Levels In Breast Cancer Patients.

	CRP (ng/ml) Mean±SD	Sample Size n (%) Total subjects n=170
Normal (Control)	1025 ± 2.65	85 (50%)
Breast Cancer Patients	3010 ± 2.89*	85 (50%)

*$p < 0.001$ using student t test with respect to normal group

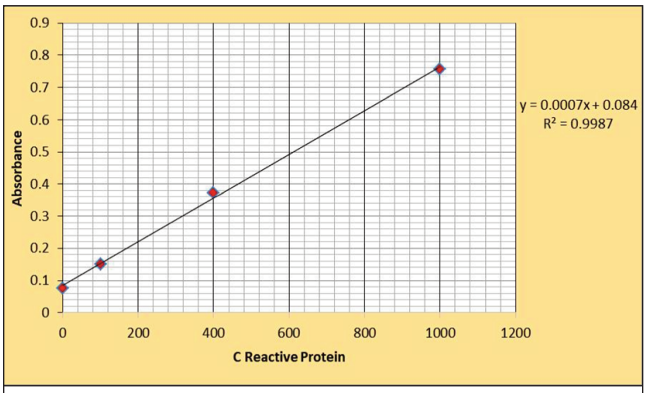

Figure-1: Standard curve of C-reactive Protein (CRP).

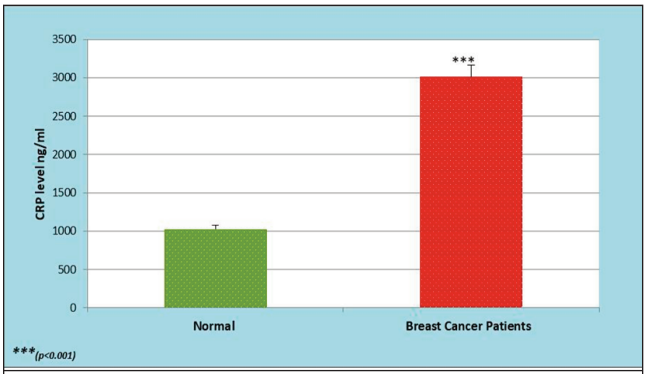

Figure-2: Comparison of CRP levels in Breast Cancer Patients and Normal subjects.

Table-2: Expression Profile OF micro ribonucleic acid (miRNA) 16 In Breast Cancer Patients And Normal Subjects.

	CT (Mean ± SD)	PQ fold change
Normal Subjects	33.8 ± 7.19	0.47
Breast Cancer Patients	31.37± 7.61	1.06 *
Positive Control	28.35 ± 1.78	1.23
Negative Control	30.77± 6.63	0.36

*p value < 0.001 using student t test with respect to normal group

Table-3: Correlation of Serum C-reactive protein (CRP) Levels with micro ribonucleic acid (miRNA) 16 Expression.

	r-values
Normal (Control)	0.05
Breast Cancer Patients	0.162

r-values are not significantly correlated at the level of significance $p < 0.001$

The expression of miRNA-16 in patients was significantly high (Table 2) but was not significantly correlated with CRP levels (Table 3).

Discussion

The present study used two-step sandwich-type ELISA method for serum estimation of hsCRP. The advantage of using this method over conventional methods is that it has better precision and sensitivity to detect sub-clinical inflammation even at low concentrations of CRP in serum. On the other hand, conventional automated methods for CRP measurement typically cannot analyse within the very low range of CRP concentrations in peripheral blood and, hence, have very limited sensitivity.[24]

In the current study, serum CRP levels were significantly higher in patients of breast cancer compared to normal subjects (p<0.001), showing that inflammation exists in persistent and predominant state in breast cancer cases. Similar results have been reported in various prospective and case-control studies.[25] According to one study, serum hsCRP levels were associated with advanced stages of breast cancer and CRP values were significantly high in stage II and III than stage I.[26] It seems that the inflammatory constituent contributes more in almost all stages of tumourigenesis from the beginning with local and systemic invasion. One study found no correlation of hsCRP with breast cancer risk.[27] This finding is inconsistent with the majority of studies showing higher CRP levels in breast cancer patients compared to healthy subjects, and extremely increased levels in advanced stages as a sign of increased tumour burden.[28] Hence, it can be concluded that inflammation plays an active role in the progression of tumour. It has been suggested that CRP, which is released in tumour environment as a result of ilterleukin-6 (IL-6), holds tumour cells by phospholipids which stimulates C1q complement pathway and results in tumor cell lysis. It means that activated inflammatory responses in tumour cell results in DNA damage and finally the metastasis.[29]

In the present study, correlation between miRNA-16 and CRP levels was also determined. MiRNA-16 is involved in the promotion of apoptosis of cancer cells via influencing faint expression in normal tissues, aberrant over-expression in tumours (FEAT).[30,31] It is found that there is no significant correlation found between miRNA-16 and CRP, indicating that there is no direct involvement of CRP with progression of tumour through expression of miRNA-16. It has been suggested that some other molecular pathway is involved between inflammation and cancer progression. According to one study, Sphingosine-1-phosphate (S1P) upregulate expression of CRP which then stimulates metalloproteinase-9 to be transcriptionally active via oxygen species, calcium ions, and c-fos (proto-oncogene , function as transcription factor subunit within cells and regulate downstream target genes), leading to breast cell invasion and CRP.[32] Further studies are required to explore the correlation of different other markers of breast cancer and CRP regulation mechanism to explore the molecular pathways between breast cancer and inflammation. It is evident from the studies mentioned above that the basic constituent of carcinogenesis involves inflammatory cells which are filled in microenvironment of tumour. Cancer-

related inflammation has evolved to be an essential component of cancer and chronic inflammation is involved in recurrence of the disease by the distribution and multiplication of metastatic seeds. It has been reported in multiple clinical trials that non-steroidal anti-inflammatory drugs (NSAIDs) arrest the cell cycle and suppress the growth of the tumour by blocking angiogenesis, neovascularisation, and minimising the prostaglandin synthesis, showing its role in reducing the risk of breast cancer.[33] However, CRP is a nonspecific marker of inflammation and is also found increased in other acute and chronic infections. It cannot be used alone, but with other breast cancer markers it can assess the risk. Similarly, the use of antinflammatory drugs can be effective if used in combination with chemotherapeutic drugs to improve the survival of breast cancer patients.

Conclusion

C-reactive protein was found to have an important role in progression of cancer via inflammation, but miRNA-16 marker of breast cancer is not directly involved in this cascade.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: None.

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