WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 5, Issue 02, 1444-1455. Research Article ISSN 2277– 7105

STUDIES ON INFLAMMATORY MARKERS (IL-1, IL-4) IN BREAST CANCER DISEASE PROGRESSION

Safoora Habeeb,[1*] Dr. Kaiser Jamil,[2] Javeed Ahmed[2] and Dr. M. Uma Devi[1]

[1]Department of Biotechnology, Aurora's Technological Research Institute, Hyderabad, India.

[2]Bhagwan Mahavir Medical Research Centre, Hyderabad, India.

Article Received on
12 Dec 2015,

Revised on 02 Jan 2016,
Accepted on 26 Jan 2016

***Correspondence for Author**
Safoora Habeeb
Department of Biotechnology, Aurora's Technological Research Institute, Hyderabad, India

ABSTRACT

Awareness and understanding of cancer disease will help in making correct decisions. The aim of the present studies involves identification and confirmation of role of interleukins as inflammatory markers in breast cancer disease progression. Cytokines play a very important role in nearly all aspects of inflammation and immunity. 'Interleukins' (ILs) are a group of cytokines with complex immunomodulatory functions. They also play an important role in immune cell differentiation and activation. ILs have pro- and anti-inflammatory effects. In the present work the patient's complete history, clinical symptoms and pathology of the disease were studied. Blood was drawn from 60 individuals (30 cases and 30 controls), DNA was isolated from the blood samples of cases and controls. The genes that code for IL-1β and IL-4(VNTR intron 3) were amplified by PCR using gene specific primers followed by RFLP, its product was then resolved on agarose gel electrophoresis and based on the RFLP and PCR patterns observed, the role of interleukins as inflammatory markers in breast cancer disease progression was identified and confirmed. Knowledge of this investigation will be useful for helping breast cancer patients to be diagnosed at early stages using IL markers. This will also help in identifying the correlation between existing risk factors and genetics.

KEYWORDS: Breast cancer, Interleukins, risk factors.

INTRODUCTION

BREAST CANCER: Breast cancer is the most common cancer in the world originating from the breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Breast cancer is predominantly a disease of aging, with only 5 to 7% of

patients diagnosed below the age of 40 years in the developed world. [1] Genetic background, dietary practices and environmental factors are likely to contribute to the incidence of DNA damage and breast cancer risk.

Risk factors of breast cancer

The risk factors include age, gender, number of first degree relatives suffering from breast cancer, menstrual history, age at menarche and age at menopause, BMI, duration of breast feeding, alcohol consumption, mutations or modifications in DNA.[2,3] The primary risk factors for breast cancer are female sex and older age. Other potential risk factors may include: lack of childbearing or lack of breast feeding, higher levels of estrogen and testosterone hormones, Tobacco appears to increase the risk of breast cancer with the greater the amount of smoked and the earlier in life smoking began the higher the risk, history of breast cancer in a first-degree relative, menstrual and reproductive factors, use of hormone therapy, a relatively high body mass index (BMI) in postmenopausal women, alcohol consumption, Dietary factors (e.g., a relatively high fat intake and relatively low fruit and vegetable intake, iodine deficiency etc;), age at menopause and menstrual history have also been postulated to play a role in the etiology of breast cancer. oral contraceptives are also associated with the development of premenopausal breast cancer. [4]

INTERLEUKINS

Interleukins are a group of cytokines, they are also called as secreted proteins or signal molecules that were first seen to be expressed by white blood cells. ILs were also described as originating from lymphocytes as a result, they are sometimes referred to as lymphokines.

Functions of interleukins

Interleukins (IL) are a group of cytokines with complex immune modulatory functions including cell proliferation, maturation, migration and adhesion. These cytokines also play an important role in immune cell differentiation and activation. To determine the exact function of a particular type of cytokine is complicated because of the influence of the cell type producing these cytokines, and the phase of the immune response.

Interleukins can cause both inflammatory and anti- inflammatory actions. ILs are very important mediators of the physiological response to infection and also contribute significantly to the pathophysiology of a wide range of disorders. As such, interleukins can also function as potential therapeutic targets.

Inflammation is a well-known risk factor for tumor development in a variety of cancers. Breast cancer provides a typical example of an inflammation-linked malignant disease. Breast tumors are enriched with inflammatory constituents, including cells that are polarized to the tumor-promoting phenotype, and soluble factors. Understanding the mechanisms by which intrinsic factors, such as oncogenes induce inflammation is quite critical. Cumulative findings of a large number of studies indicate that inflammatory components present in the tumor microenvironment actively support breast cancer progression and development.

Cytokines, the signaling molecules that mediate and regulate immunity, inflammation, and hematopoiesis, are an important component of the biological milieu associated with breast cancer. Cytokines have been used as biomarkers in research for prognosis and have been associated with symptoms and adverse outcomes in multiple conditions, including breast cancer. [5, 6] Cytokines, which are low molecular weight pleiotropic glycoproteins, are secreted primarily by immune cells and affect many different adjacent target cells: they alter target-cell function and modulate cell death, growth, and differentiation at very low concentrations.[7] Cytokines produced by cancer tissue, [8,9] and the expression of interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12, and IL-19, monocyte-chemoattractant-protein (MCP-) 1, and macrophage-inflammatory-protein- (MIP-) 1β are upregulated in breast cancer. [10, 11] Inflammatory cytokines, such as IL-1β, IL-4 and other mediators of inflammation have been linked to breast cancer formation and recurrence. Several cytokines regulate the inflammatory tumor microenvironment. Interleukin (IL)-1, IL-6, IL-11, and transforming growth factor-b (TGF-b) stimulate cancer cell proliferation and invasion, [12] and cytokine receptor activation and intracellular signaling by NF-kB accelerate tumor progression.[13,14,15] The need for more and accurate diagnostic markers that predict responses to breast cancer therapy is the need of the hour. In present paper we discuss the biological features of breast cancer, role of interleukins as inflammatory markers in breast cancer disease progression and their statistical implications.

MATERIALS AND METHODS

BLOOD SAMPLE COLLECTION: Two millilitres of venous blood was collected in K_2-EDTA (Vacuette®) from 30 unrelated adult breast cancer patients and 30 age matched controls. The Ethical Committee approval was obtained for the study.

DATA COLLECTION: The patient's case details were obtained in case proforma. The clinical profile consisted of age, CBP, initial diagnosis, social habits, life style, symptoms and medications used were collected.

GENOMIC DNA ISOLATION: Genomic DNA was isolated from blood samples by DNA by Salting out procedure. [16]

DETECTION OF DNA BY AGAROSE GEL ELECTROPHORESIS

The presence of genomic DNA was detected by running the isolated samples on 2 % Agarose gel electrophoresis at 65 V.

POLYMERASE CHAIN REACTION (PCR): The DNA isolated was amplified by PCR in a DNA thermal cycler (Mastercycler, Eppendorf) by using oligonucleotide primers.

The primers used for PCR amplification are the following

Primer used for IL-1βgene

FORWARD PRIMER: 5′ TGGCATTGATCTGGTTCATC 3′

REVERSE PRIMER: 5′ GTTTAGGAATCTTCCCACTT 3′

Primer used for IL-4 gene

FORWARD PRIMER:5′-AGGCTGAAAGGGGGAAAGC-3′

REVERSE PRIMER: 5′- CTGTTCACCTCAACTGCTCC-3′

CONDITIONS FOR INTERLEUKINS: The PCR temperatures are the following:

		IL-1β		IL-4	
Sl No	**Step**	**Temperature (°C)**	**Time**	**Temperature (°C)**	**Time**
1	Initial denaturation	95	5 Min	94	5 Min
2	Denaturation	95	30 Sec	94	20 sec
3	Annealing	55	30 Sec	58	20 sec
4	Extension	72	30 Sec	72	20 sec
5	Final extension	72	5 Min	72	10 min
6	Final hold	4	10 Min	4	10 Min

For the amplification of IL-1βand IL-4 genes steps 2 to 4 were carried out for 35 cycles followed by final extension totaling to 36 cycles.

AGAROSE GEL ELECTROPHORESIS

The PCR products were resolved in 2% agarose gel by electrophoresis.

RESTRICTION FRAGMENT LENGTH POLYMORHISM (RFLP)

Restriction fragment length polymorphism is a molecular biological technique used to compare DNA from samples. Restriction enzymes *AvaI* that cleave the DNA in specific locations were used to digest strands of IL-1βgene. Mutations within the gene result in strands of different lengths. Electrophoresis was then carried out to separate the strands according to their length.

The components are incubated at 37 °C for 12 hours. The RFLP products were then resolved by 2% agarose gel electrophoresis.

RESULTS AND DISCUSSION

DEMOGRAPHIC RESULTS

Age groups

There were total 30 cases and 30 controls involved in our study. As shown in the figure-1 the age group 30-40yrs(cases-0,controls-14),41-50yrs(cases-13,controls-8),51-60yrs(cases-10,controls-4),61-70yrs(cases-4,controls-3),71-80yrs(cases-3,controls-1).According to our study Breast cancer mostly falls under the age group of 41 to 50.

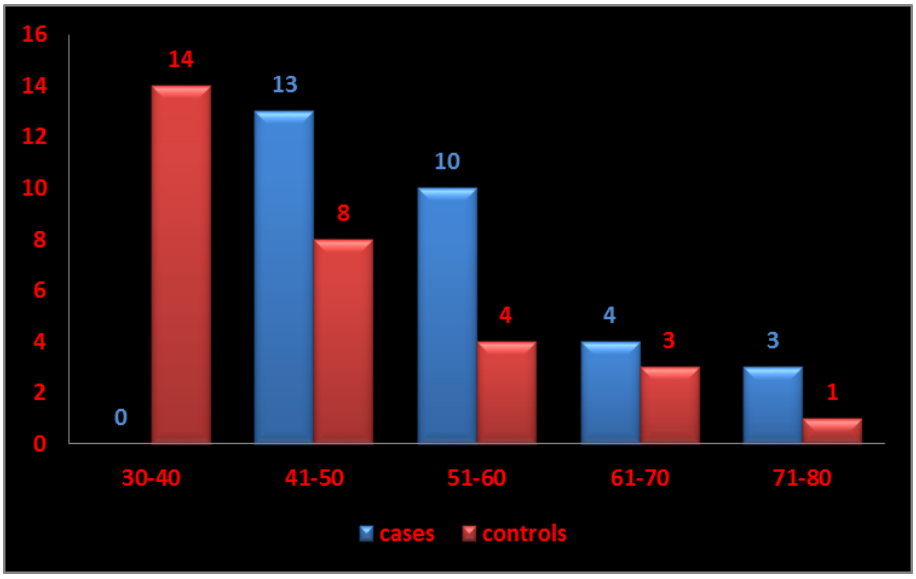

Fig 1: The different Age groups in cases and controls

Smoking

The most significant risk factor for breast cancer is long-term cigarette smoking. The more years you smoke and the more packs you smoke, the greater your risk you have. Pipe smokers, cigar smokers and people exposed to large amounts of second hand smoke also are

at risk. Among the 30 cases we analyzed that number of smokers were 4(13%) and non-smokers were 26 (87%), and among the 30 controls there were 2 smokers (7%) and 28 non-smokers (93%).

Alcohol

Among the 30 cases 10(33%) were found to be alcoholic, 20(67%) were found to be non-alcoholic and among all the 30 controls 3 (10%) are alcoholic and 27 (90%) are non-alcoholic. More number of alcoholic people were found in cases when compared to the controls.

History

Family history plays a major role in breast cancer disease progression and in present study among the 30 patients 3(10%) of cases were found having family history with breast cancer and 27(90%) were not having any family history. There was no history in controls.

Tobacco Pan Chewers

Chewing pan especially with zarda may lead to cancer and in our study we found 14 (47%) of the patients were pan chewers and 16 (53%) were non pan chewers and among the 30 controls 5(17%) were found to be tobacco pan chewers and 25(83%) were non pan chewers. The no of pan chewers were found more in cases than in controls.

GENOTYPING

In this present study we have evaluated the Polymorphism of IL-1 and IL-4 obtained from the Genomic DNA of patients and controls.

I. Genomic DNA extraction and purity: Genomic DNA was extracted by the salting out method as described in the methods and materials section. The extracted DNA from all the Breast cancer samples and normal subjects appeared to be pure and intact as judged by the electrophoresis, as shown in fig 2.

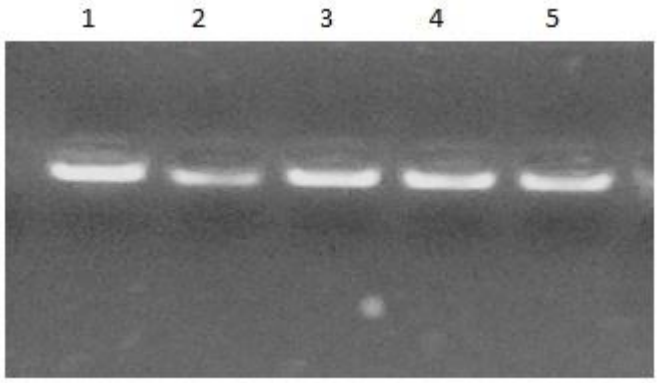

Fig 2: A representative picture depicting purity and intactness of extracted genomic DNA.

1. Mutational analysis for IL-1βgene

The PCR conditions were optimized for the specific amplification of IL-1β gene. The isolated DNA was subjected to PCR to amplify the primer sequence (Fig 3).

Primer used for IL-1 βgene

FORWARD PRIMER: 5 'TGGCATTGATCTGGTTCATC' 3

REVERSE PRIMER: 5 'GTTTAGGAATCTTCCCACTT' 3

The above primer were used to amplify 304 bp fragment

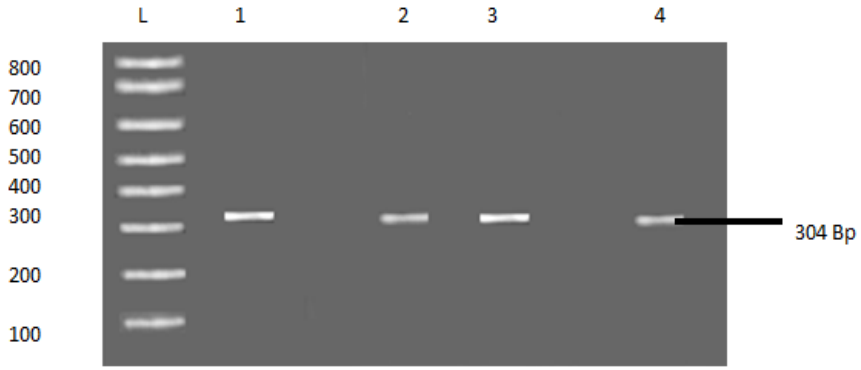

Fig 3. Agarose gel electrophoresis showing PCR products of IL-1βgene promoter region after amplification using100 bp marker Lanes 1 to 4 shows 304bp PCR product.

Restriction Fragment Length Polymorphism (RFLP)

PCR for theIL-1βgene gave a 304 bp fragment following enzymatic digestion of the PCR product using restriction endonuclease enzyme *AvaI* and incubated at 37°C for 12h. Then analyzed by gel electrophoresis, and visualized in UV trans-illuminator. IL-1βgenotypes were

coded as follows: 304 bp for T/T; 190 and 114 bp for C/C; 304+ 190 and 114 bp for C/T genotype (Fig 4).

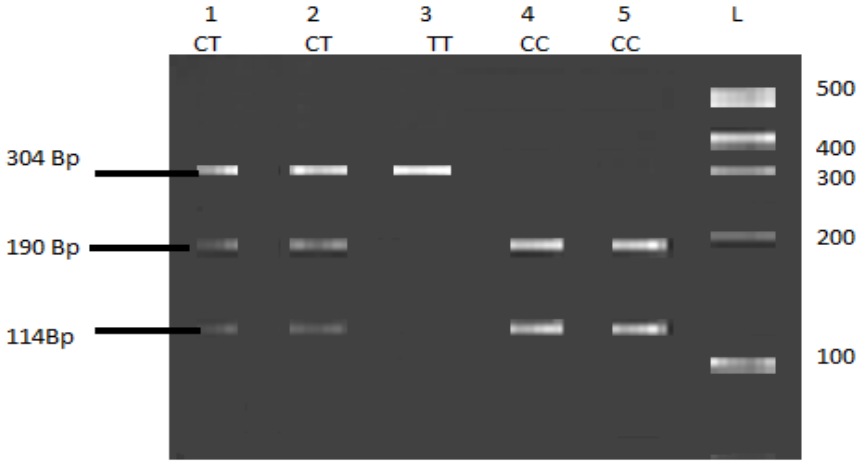

Fig 4: Electrophoresis depicting restriction digestion of IL-1βgene polymorphism with *AvaI* enzyme

Lane 1,2represents heterozygous H+/-CT(304,190,114bp),

lane 3 represents homozygous H+/+ TT(304bp),

lane 4,5 represents wild type homozygous H+/-CC(190,114bp).

2. Mutational analysis for IL-4 gene

PCR.

The genotyping for IL-4 -70bp (VNTR) polymorphism was conducted. The IL4 variable number of tandem repeat (VNTR) has been described as a 70-bp repeat in intron 3.25 region. The PCR conditions were optimized for the specific amplification of IL-4 gene the primers used are.

Primer used for IL-4 gene

FORWARD PRIMER: 5′-AGGCTGAAAGGGGGAAAGC-3′

REVERSE PRIMER: 5′- CTGTTCACCTCAACTGCTCC-3′

The amplification products were then separated using gel electrophoresis on a 4% agarose gel and visualized with a UV transilluminator to detect the presence of DNA bands. In the IL4 (VNTR intron 3). In individuals lacking the IL4 gene polymorphism, a 183-bp band was detected and was designated as B1/ B1 genotype (ie, wild type). But if 253-bp band was detected due to a 70-bp insertion, it was designated as B2/B2 (ie, homozygous B2 allele). If 2

bands, 183 bp and 253 bp, were detected (Fig 5), it was designated as B1/B2 (ie, heterozygous for B2 allele).

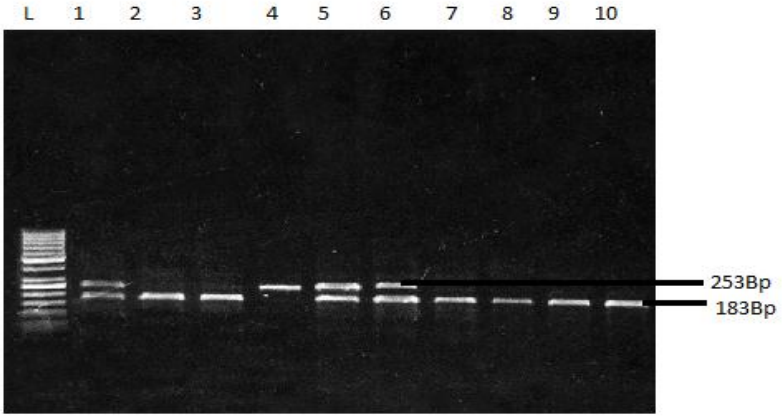

Fig 5: Electrophoresis depicting PCR product

lane 1,5 and 6 shows two bands of 183 bp and 253 bp

lane 2,3,7,8,9 and 10 shows the alleles of 253 bp and

lane 4 shows the wild type one band of 183 bp

Statistical analysis

In this present study the Hematological parameters such as haeglobin, RBC count, WBC count and Platelet count were compared for cases and controls (Table 1).

The pattern of *AvaI* restriction enzyme for IL-1β showed different banding pattern in cases and controls. Out of 30 cases used for IL-1β, CC genotype was 23.3%, CT genotype was 50%, TT genotype was 27% and out of 30 controls genotyped, CC genotype was 27%, CT genotype was 43.3%, TT genotype was 30%. And the banding pattern for IL-4 gene showed the following banding patterns out of total 30 cases, B1B1 genotype was 60%, B1B2 genotype was 33.3% and B2B2 genotype was 6.6% and out of 30 controls genotyped, B1B1 genotype was 67%, B1B2 genotype was 26.6% and B2B2 genotype was 6.6% (Table-2).

Hematological Parameters

Table1: Comparison of Hematological parameters of cases and controls

	Cases	Controls	
	Mean±SD	**Mean±SD**	**P value**
Haemoglobin	10.87± 1.32	9.01± 2.00	0.0001
RBC count	3.75±0.51	4.06±0.94	0.1178
WBC count	8793.33±3101.72	10050±3008.24	P < 0.0001
Platelet count	310166.67±153800.61	267800±99246.61	0.2100

Table 2: Distribution of genotype and allele frequencies of IL-1βand IL-4(70bp VNTR) by the case control status and association with risk in breast cancer Patients.

Genotype	Cases N=30(%)	Controls N=30(%)
IL-1		
Wild type CC	7(23.3%)	8(27%)
T allele	23(77%)	22(73%)
Heterozygous CT	15(50%)	13(43.3%)
Homozygous TT	8(27%)	9(30%)
IL-4		
Wild type B1B1	18(60%)	22(67%)
B2 allele	12(40%)	10(33.3%)
Heterozygous B1B2	10(33.3%)	8(26.6%)
Homozygous B2B2	2(6.6%)	2(6.6%)

Table 3: Comparision of IL-1 and IL-4 gene polymorphism in between cases and controls

Polymorphism	Cases	Controls	P value
IL-1			
Wild type CC	7(23.3%)	8(27%)	0.7657
T allele	23(77%)	22(73%)	
IL-4			
Wild type B1B1	18(60%)	22(67%)	0.4726
B2 allele	12(40%)	10(33.3%)	

Cytokines play a very important role in nearly all aspects of inflammation and immunity and alterations in IL-1β and IL-4 have been suggested to play an important role in carcinogenesis. Interleukin are a group of cytokines with complex immunomodulatory functions and play an important role in immune cell differentiation and activation. IL's have pro- and anti-inflammatory effects and act as inflammatory markers in breast cancer disease progression as breast cancer is one among the malignant inflammatory cancers. IL'S have their role in cancer growth and metastasis. Polymorphism in these genes may serve as potential sensors for cellular DNA damage and a marker for cancer development. Three common banding patterns were observed in our study population.

Demographic findings suggest that breast cancer mostly falls under the age group of 41 to 50. More no of smokers, alcoholic individuals, and pan chewers were found in cases than in controls and 10% of cases showed family history with breast cancer.

Statistical analysis of Hematological Parameters: By comparing the haemoglobin count in cases and controls we got the *p* value as 0.0001, RBC count gave the *p* value as 0.1178, WBC

count gave the *p* value as $P < 0.0001$, platelet count gave the *p* value as 0.2100. This shows that blood parameters are altered in breast cancer cases.

In the Genotypic analysis we found 3 patterns of IL-1β and IL-4 as shown in table 2. The overall mutation frequency of IL-1β gave the *p* value as 0.7657 and IL-4 gave the *p* value as 0.4726 shown in table 3. Despite massive investment and studies in biomarker discovery, only a few have been recognized as potential biomarkers and tested in clinical practice.[10] In the present study we did not find any significant role of inflammatory markers (IL-1β, IL-4) in breast cancer disease progression.

CONCLUSION

Most studies examining inflammatory cytokines, such as IL-1β, IL-4 and other mediators of inflammation have been linked to breast cancer and recurrence as interleukins have their role in inflammation which is one of the risk factor for breast cancer**.** It is concluded from our study that hematological parameters are altered in breast cancer cases. Further there are more number of cases between the age group of 41-50 years. Many of the patients were also alcoholic and tobacco chewers and smokers. The role of inflammatory cytokines in the etiology of breast cancer in our study has shown to be insignificant, while a few other studies have shown a potentially promising area of study as these interleukins are responsible for the cause of inflammation and breast cancer is the malignant inflammatory cancer. Our study confirmed that there is no significant role of inflammatory markers (IL-1β, IL-4) in breast cancer disease progression.

REFERENCES

1. Hatem A Azim and Ann H Partridge. Biology of breast cancer in young women, Breast Cancer Research, 2014; 16: 427
2. Bruunsgaard H, Pedersen M, Pedersen BK. Aging and proinflammatory cytokines. Curr Opin Hematol, 2001; 8(3): 131–136
3. Pfeilschifter J, Koditz R, Pfohl M, Schatz H. Changes in proinflammatory cytokine activity after menopause. Endocrine Rev, 2002; 23(1): 90–119.
4. Merethe Kumle, Elisabete Weiderpass, Tonje Braaten, Ingemar Persson,Hans-Olov Adami, and Eiliv Lund.Use of Oral Contraceptives and Breast Cancer Risk. Cancer Epidemiology, Biomarkers & Prevention, 2002; 11: 1375–1381.

5. Debra E. Lyon, Nancy L. McCain, Jeanne Walter, and Christine Schuber. Cytokine Comparisons between Women with Breast Cancer and Women with a Negative Breast. Biopsy Nurs Res., 2008; 57(1): 51–58.
7. Ying-Yin Chen, Chien-Feng Li Ching-Hua Yeh, Ming-Shi Chang, andChung-Hsi Hsing Interleukin-19 in Breast Cancer, Clinical and Developmental Immunology, 2013; Article ID 294320, 9 pages
8. Rao VS, Dyer CE, Jameel JK, Drew PJ and Greenman J. "Potential prognostic and therapeutic roles for cytokines in breast cancer (Review)." Oncology Reports, 2006; 15(1): 179–185.
9. Boon T and Vanden Eynde B. "Tumour immunology." Current Opinion in Immunology, 2003; 15(2): 129–130.
10. Wilson J and Balkwill F. "The role of cytokines in the epithelial cancer microenvironment." Seminars in Cancer Biology, 2002; 12(2): 113–120.
11. C. Chavey, F. Bibeau, S. Gourgou-Bourgade."Oestrogen receptor negative breast cancers exhibit high cytokine content," Breast Cancer Research, 2007; 9(1): article R15.
12. Sullivan NJ, Sasser AK, Axel AE. "Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells," Oncogene, 2009; 28(33): 2940–2947.
13. Nicolini A, Carpi A, Rossi G. Cytokines in breast cancer. Cytokine Growth Factor Rev, 2006; 17(5): 325–337.
14. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol, 2005; 5(10): 749–759.
15. D. G. DeNardo and L. M. Coussens, "Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression," Breast Cancer Research, 2007; 9(4): 212.
16. Hsing C-H, Cheng H-C, Hsu Y-H, Chan C-H, Yeh C-H, Li C-F, Chang M-S. Upregulated IL-19 in breast cancer promotes tumor progression and affects clinical outcome. Clin Cancer Res, 2012; 18(3): 713–725.
17. Miller SA, Dykes DD and Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research, 1988; 16(3): 1215.