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Haplotype Polymorphisms in Cytokines Genes Using Pcr-Sscp Technique in Iraqi Breast Cancer Patients

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ABSTRACT

The present study was carried out to detect the association of cytokines (IL-2 and IL-4) haplotypes polymorphisms with breast cancer in Iraqi patients, PCR-SSCP technique used in present study, imbedded tissue was used to DNA extraction, the results show that there was strong association between IL-4 and cancer tissue in three haplotype from five, IL-2 show strong association with breast cancer in tow haplotype from five. The present study concluded that there was association between IL-4 and IL-2 polymorphisms with breast cancer, our finding need more investigation to use this polymorphism as early indication of cancer incidence.

Keywords: Cytokines, PCR-SSCP technique, Haplotypes, Polymorphisms

INTRODUCTION

Immune responses have been major role in tumors progressive and pathogenesis of tumors especially in the tumors microenvironment, it contributed in inhibition tumors development and progression. Alternatively, cancer cells can respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis. Thus it is important to understanding cytokine–tumors-cell interactions to provide chance for improving cancer immunotherapy [1].

Interleukin-4 (IL-4) is cytokine has major role in the regulation of the immune system in many levels [2,3]. Also it consider as a growth and survival factor for lymphocytes [4-6] and has role in regulating T cell differentiation during the immune response [4,7,8], now known to provide potent antitumor activity against various tumors including breast cancer. Study improved that IL-4 can induce apoptosis in cultured breast cancer cells, and plays an important role in the regulation of estrogen synthesis enzymes including 17β-HSD and 3beta-HSD. These findings imply that IL-4 is a key enzyme not only for Th2 type immune reactions but also for tumor cell growth itself in human breast cancer [9]. It can regulate proliferation, differentiation, and apoptosis in multiple cell types of hematopoietic and non-hematopoietic origin including myeloid, mast, dendritic, endothelial, muscular, and neuronal cells [10-12].

IL-2 is a 15.5-kDa cytokine secreted predominately by Ag simulated CD41 T cells, and it can also be produced by CD81 cells, NK cells, and activated dendritic cells [13].

Using cytokines of the IL-2 family such as interleukin (IL)-2, IL-7, IL-15, and IL-21 to activate the immune system in cancer patients, the first successful immunotherapy for completely eradicate of cancer proveing by immunotherapy. Other studies improved that used IL-2 family cytokines (IL-4, IL-7, IL-9, IL-15, and IL-21) which had unique biological effects playing important roles in the development, proliferation, and function of specific subsets of lymphocytes at different stages of differentiation with some overlapping effects with IL-2 [14-16].

MATERIALS AND METHODS

Sample collection

About 30 breast cancer embedded tissue was collect from histopathology unit in Al-Saader medical city, these samples was diagnosis by specialist physician as a breast tumor tissue, also all samples were used to diagnosis tumors in females that don't treated with any anticancer therapy, and 30 blood sample of control were collected from healthy female that have age (35-65 years).

DNA extraction

DNA was extracted from embedded tissue according to the leaflet of Geneaid manufacture with modification, in (in DNA lab of biology department) briefly; About 40 mg of tissue was put in eppendorf tube contain 1 ml of xylene, then it mixed and incubate at room temperature for 15 min, then Centrifugation at 14000 for 3 min, and supernatant was removed, Absolute ethanol was added (1 ml) to mixture then Centrifugation at 14000 for 3 min, and supernatant was removed, the mixture was Incubated at 37˚C, GT buffer was added (200 µl) with homogenize by micro pestle, then Proteinase K (40 µl) was added and incubate for 20 min. at 60˚C with inverting every 5 min. GBT buffer was added (200 µl) with mixing, then incubate at 60˚C for 20 min and Absolute ethanol was added (200 µl) with mixing, then transfer mixture to GD column after that it Centrifuged at 14000 for 2 min, the flow-through was discarded and W1 buffer was added to column (400 µl), then Centrifugation at 14000 for 20 s. the flow-through was discarded also. Wash buffer was added (600 µl) Centrifugation at 14000 for 30 s. the flow-through was discarded, then it Centrifuged at 14000 for 3 min again, finally DNA was eluted using dH_2O (100 µl). Healthy DNA was extracted from whole blood using (Genaid extraction kit), in briefly; A 300 µl of frozen blood was transferred to eppendorf tube, then 40 µl of proteinase k was added and incubated it at 60˚C for 20 min, then GB buffer was added (200 µl) and it shaking vigorously, after this absolute ethanol was added (200 µl) and miter was mixed by shaking, then it centrifuged at 15000 rpm for 5 mint. the Supernatant was transferred to GD column, and centrifuged at 15000 rpm for 1 min. the flow-rate was discarded and 400 µl of W1 buffer was added, then centrifuged at 15000 rpm for 1 min, the Fallow rate was discarded also, about 600 µl of wash buffer was added, then centrifuged at 15000 rpm for 1 min. columns were Re-centrifuged after discarded flow ate for 5 min at the same speed to dry column, finally 100 µl of d H_2O was added to column and left 2 min at room temperature to absorb it. DNA eluted in new eppendorf tube by centerfield column for 2 min at 15000 rpm.

1. Primers, IL-2 forward, TCATGTGACATCTGGAGGGTTA reverse, AAAATGAATTTCGTCAATTCGAG, [17], and IL-4 primer was (5'-TAAACTTGGGAGAACATGGT-3' for the upstream primer and 5'-TGGGGAAAGATAGAGTAATA-3' for downstream [18].
2. PCR conditions and size products, PCR experiments performed as a following; for IL-2 per-denaturation for 5 min at 94˚C, then 35 cycles (30 s at 94˚C, 30 s at 58.4˚C, 30 s at 72˚C, and finally 10 min at 72˚C). For IL-4 denaturation for 5 min at 94˚C, then 35 cycles (30 s at 94˚C, 30 s at 37˚C, 30 s at 72˚C, and finally 10 min at 72˚C). PCR products were determined by electrophoresis pattern in agarose gel (1.5% agarose, 70 V, 20 mA for 45 min) with ethidium bromide staining, the PCR size product were (195) bp for IL-4 and (200) pb for IL-2. Statics, the results were statically analysis using odd ratio at CI 95% nd p value <0.05).
3. SSCP technique, PCR products were denaturation using SSCP dye (EDTA, formamid and bromophynol blue) 1/1 V:V in water bath for 5 min at 95˚C then its child in ice for 2 min.
4. SSCP electrophoresis, the products were electrophoresis as a following About 10 µl of the samples (sample+ dye) were loaded into wells of 8% acrylamide/bis gel containing 7% glycerol, and 1X TBE buffer. In more details; for recipe a 20 × 20 × 0.1 cm gel format. 8 ml of 40% acrylamide/bis (stoke solution 37.5:1) mixed with 8 ml of 5X TBE, 2.8 ml,100% glycerol, then 40 µl TEMED and 400 µl of 10% ammonium per sulfate were added with 20.8 ml of dH_2O After gel was casting sample were loaded and Run under the following conditions. Buffer 5.5 X TBE, Buffer temperature 10˚C, Run time 1.5 h and 100 V. Then gel was staining using ethedium bromide for 15 min.
5. Haplotype frequency were determination by variety of bands between patients and control.

RESULTS

The results of present study included gene polymorphism of IL-2 and IL-4 in cancer tissue comparison with healthy women, in the first of all the DNA extraction was low concentration in cancer tissue it was less than 50 ng/µl while in control it was more than 150 ng/µl, the DNA concentration in embedded tissue have been the most important problem in biotechnology laboratory, thus in present study we used modification methods for overcome this problem we used replicated column for every sample with incredible tissue Wight (50 µg) with discarded paraffin before adding xylene in the first step of extraction, all this modifications led to get 40-50 ng of DNA.

The PCR products of IL-4 was 195 bp as show in Figure 1 and PCR products of IL-2 was bp as show in Figure 2.

Gene polymorphisms were detected using PCR-SSCP technique because it can be give indicators about haplotype secondary structure of the amplified segments of the gene, other studies used PCR- RFLP technique although of its fidelity but its detect point mutation only while PCR-SSCP detect stereo state of single strand of DNA which dependent on chemical structure of nucleotide and stationary of chemical bonds of DNA components that dependent in gene expression and predisposition to mutagenesis, also it detected insertion, deletion and duplication of DNA sequence in single strand which it need high cost long time in another technique.

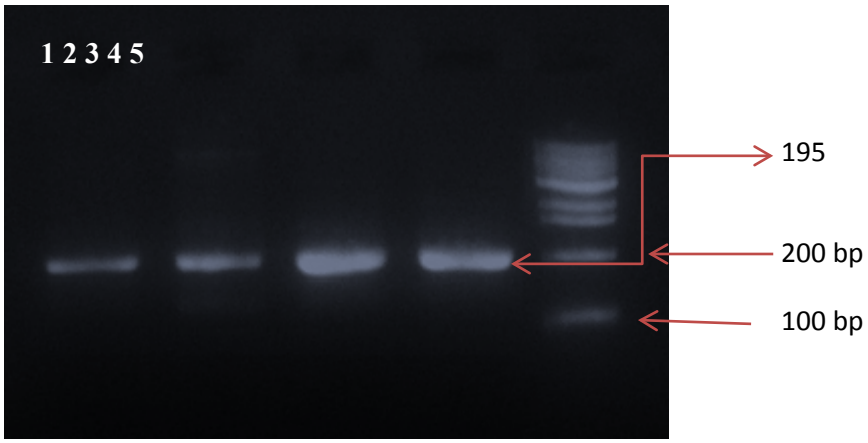

Figure 1: Electrophoresis pattern of PCR product of IL-4 and IL-2 gene, lane 1, 2 IL-4, lane 3,4 for IL-2, lane 5 DNA marker (100 bp)

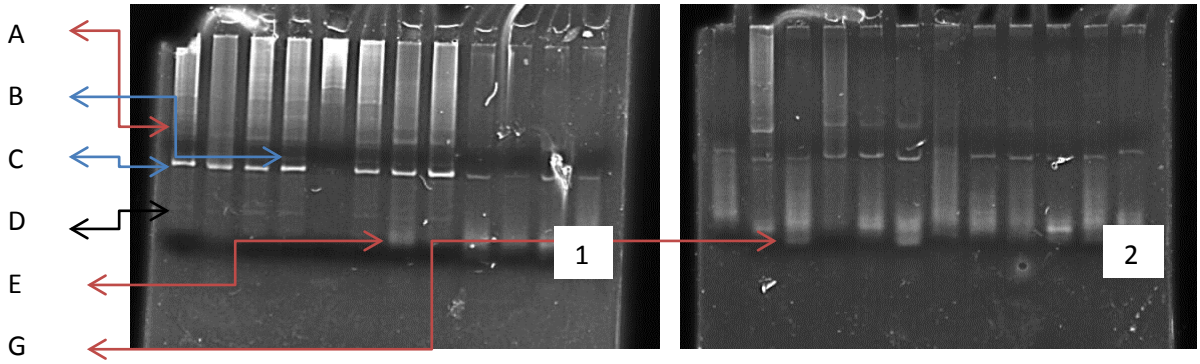

Figure 2: Electrophoresis pattern of PCR-SSCP of IL-4 gene polymorphism for patients and control 1, control; 2 patients

The results of haplotype polymorphism in IL-4 gene show six pattern of haplotype labeled as (A, B, C, D, E, and G) (Table 1, Figure 3), the haplotype B and D were present in control (30, 50%) respectively while haplotype G was presence in patients only (15%), other haplotype A, E were present in high percentage in patients (72.22%) for every one while in control were (40 and 15)% respectively. Haplotype C was appeared in all patients and control. The significant variation in D and E haplotypes, other studies improved association of IL-4 gene polymorphism with different cancer type, Lou et al., show that IL-4 rs2243250 carriers had an increased risk of developing multiple bladder carcinomas. IL-4 polymorphisms rs2243228, rs2243250, rs2227284, and rs2070874 were associated with prostate cancer risk and mortality, in meta–analysis study [19] in present study five haplotypes associated with breast cancer (A, B, D, E and G) although of the little of studies which deal with immunogenic of breast cancer in Iraqi populations, in other populations, *IL4* was significantly associated with no estrogen receptor – no progesterone receptor tumors [20] *IL4* rs3024543 has been associated with shorter breast cancer survival in a group of African-American and Hispanic women, but not among Caucasian women [21].

Table 1: Haplotypes frequency of IL-4 gene in patients and control

Haplotype	Control (%)	Patients (%)	Odd ratio	CI (95)%	P-value
A	40%	72.22%	0.2564	0.0655-1.0044	P=0.0507
B	30%	0%	16.5862	0.8616-319.2810	P=0.0627
C	100	100%	1.1081	0.0209-58.7166	P=0.9596
D	50%	0%	37.0000	1.9630-697.3882	P=0.0159
E	15%	72.22%	0.0679	0.0137-0.3373	P=0.0010
G	0%	15%	0.1080	0.0052-2.2486	P=0.1508

The results of IL-2 of gene polymorphism which show in Table 2 and Figure 3 clarified the variation of haplotypes in patients and control, there were five pattern (A, B, C, D and E), polymorphisms show no differences between patient and control unless in C and E, C was appeared in control only and E appeared in patients only in significant variation at ($p>0.05$). Zhang and others improved in meta-analysis that the association between IL-2 and cancer susceptibility dependent on the site of the point mutation, they discovered that rs2069762 polymorphism of *IL-2* contributed to an increased susceptibility to cancer, whereas no association was identified between rs2069763 polymorphism and cancer susceptibility [22] also meta-analysis suggests that IL-2 -330T/G polymorphism has an increased risk of cancer in Asians [23]. In present study tow haplotype association with Brest cancer in Iraqi population (C, E).

The variation of cytokines gene polymorphisms between Iraqi patient and other population resulted from many factors, the more

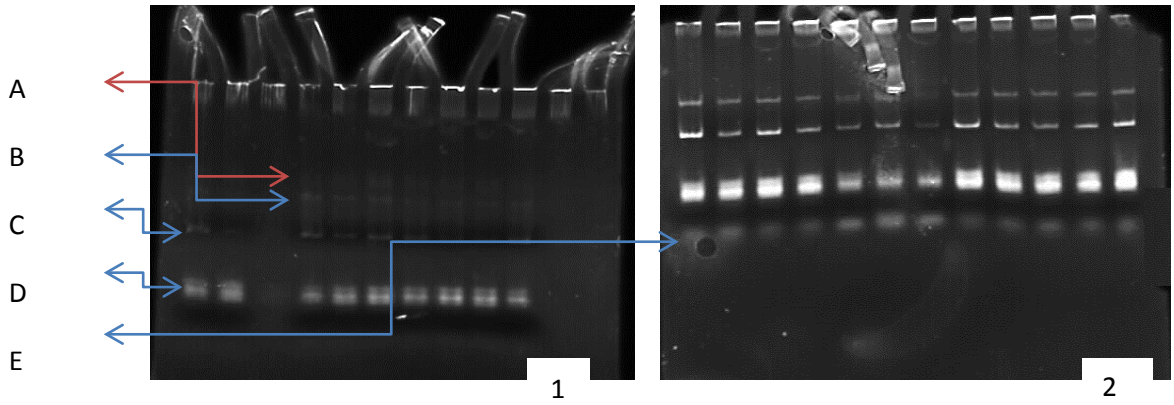

Figure 3: Electrophoresis pattern of PCR-SSCP of IL-2 gene polymorphism for patients and control 1, control; 2 patients

Table 2: Haplotypes frequency of IL-2 gene in patients and control

Haplotype	Control(%)	Patients (%)	Odd ratio	CI (95)%	P-value
A	100%	100%	1.1081	0.0209-58.7166	P=0.9596
B	100%	100	1.1081	0.0209-58.7166	P=0.9596
C	100	0	1517.000	28.6290 -80383.0928	P=0.0003
D	100%	100	1.1081	0.0209-58.7166	P=0.9596
E	0%	100	0.0007	0.0000-0.0349	P=0.0003

important is variation in the genetic of population which dependent on the environmental adverbs, pollution of the environment which improved by studies deal with pollution in Iraqi environments led to increment some heavy metals in tumor tissue and in others like lactating mother mike causes accumulation effect in DNA [24,25].

The other reasons of variation are cancer type, causes and stages that association with type and site of mutation, cancer which caused by inflammation that need to overexpression of cytokines resulted from varied in polymorphism, in Iraqi patient Al-Ghurabi found that There was association between elevated serum level of IL-4 and breast cancer and it was correlated with advanced stage of disease. In addition, there was no association between the statistical significant decrease of IL-2 serum level and the advanced stage of breast cancer [26]. Also oxidative stress have been improved that has major roles in carcinogenesis, in this patient we study oxidative stress related gene, we found that antioxidants enzyme genes polymorphisms (GSTT, GSTM) strongly linked with breast cancer in Iraqi women [27] Al-Sayigh found that the deletion in VNTR sequence of endothelial nitric oxide synthase (eNOS) gene associated with breast cancer in Iraqi patients [28,29]. Our finding needs more investigation to estimate other mutation in cytokines related gene in breast cancer of Iraqi population.

Conclusions: the present study concluded that there were five haplotype associations with breast cancer in IL-4 gene three of these were strong association. Il-2 results show tow haplotype strong association with cancer tissue.

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