Genotyping of HLA-class-I by PCR-SSP of Iraqi Breast Cancer Patients

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Abstract

Background: The aetiology of breast cancer is multifactorial, in which genetic predisposition; environmental factors, hormones and even the infectious agents are thought to interact in the manifestation of disease. In this regard, alleles of HLA are important immunogenetic risk factors, but their associations show different frequencies in different populations.

Objectives: This study was established to shed light on the possible association of HLA class I alleles with BC in Iraqi female patients.

Subjects and Methods: The study included 60 subjects: 30 breast cancer patients, 12 patients with benign breast lesions as first control and 18 apparently healthy subjects as second control. Polymerase chain reaction-specific sequence primers (PCR- SSP) assay was conducted to assess HLA- typing.

Results: Out of 95 HLA class I alleles (A= 24; B= 48; C= 23), one allele (HLA- A*03010101-07, 09-11N, 13-16 allele) showed a significant variation between breast cancer patients and control groups (healthy and disease controls) (50% vs. 16.6%, OR=5, EF= 0.40, P= 0.041), (50% vs. 8.3%, OR=11, EF=O.45, P=0.024) respectively.

Conclusions: The results demonstrated that HLA- A*03010101-07, 09-11N, 13-16 allele may played a role in the etiology of the disease.

Keywords: Breast cancer, HLA, PCR.

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Introduction

With more than 1 million new cases in the world each year, breast cancer (BC) is the commonest malignancy and the leading cause of cancer death in women [(1)]. In Iraq, the BC is the commonest cancer in females; furthermore, currently there is a general trend towards an increase the incidence of disease in females of younger ages [(2)].

Human based studies have suggested that the host genetics predisposition is important in disease pathogenesis and protection [(3)], and considering the importance of immune surveillance during tumorigenesis [(4, 5)], some individuals who inherit specific alleles or haplotypes of the highly polymorphic human leukocyte antigen (HLA) system may be exposed or may resist to specific types of cancers [(6-9)].

Breast cancer occurrence differs among women of different racial and/or ethnic groups, and accordingly, several HLA associations have linked HLA system with susceptibility or protection in the disease. However, the studies have been consistent with respect to the influence of these alleles in immune clearance of tumor cells in a way that could affect BC development. Therefore, HLA genotypes have been suggested to be a biologically based risk factor for BC [(10, 11)]. In the present study, the polymorphism of HLA-A, - B and -Cw alleles was analyzed in a sample of Iraqi females with BC using the PCR – SSP method.

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Subjects and Methods

Subjects:

Thirty breast cancer female patients (invasive ductal carcinoma, invasive lobular carcinoma and in situ ductal carcinoma), with an age range of 28 - 73 years, were eligible for this study. The patients were admitted for surgery at Al-Kadhimia Teaching Hospital and Nursing Home Hospital (Medical City) in Baghdad, for the period March 2006 - March 2007. Pathological data (histologic tumor type grade, tumor stage and lymph node status) were obtained from the medical records of patients and validated by an experienced histopathologist.

Two control groups were included: 12females with benign breast lesions (BBL) (6 cases with fibrocystic disease and 6 with fibroadenoma) and 18 apparently healthy females. The latter subjects had no history or clinical evidence of any breast lesions and matched by age and ethnic backgrounds to BC patients.

Methods:

Blood collection

Two milliliters of venous blood with EDTA as anticoagulant were collected from each subject.

DNA extraction

Extraction of DNA from peripheral blood was done according to the modified method of Miller [12], using the EXTRA-GENE-I kit (BAG-Germany), which is the most suitable method for isolation since pure DNA can be obtained from whole blood in a short time without the use of toxic chemicals or solvents.

PCR amplification

HLA-genotyping was performed by PCR-SSP according to a method presented by Olerup and Zetterquist [13,14], using low resolution typing kits (HISTO TYPE / DNA-SSP Kits-BAG- Germany). Appropriate amounts of DNA and Taq polymerase (Recombinant Taq polymerase from QIAGEN-company) were added to pre-aliquoted primers and PCR conditions were set according to the manufacturer instructions.

Detection of PCR products

PCR products were loaded in 2 % agarose gel containing 0.5 mg/ml ethidium bromide, electrophoresed for 25 min at 12 V/cm, and examined under ultraviolet light. The individual alleles were assigned for the specific pattern of appropriately sized bands.

Statistical analysis

The results were presented in terms of percentage frequencies, and alleles showing variations between patients and controls were further presented in terms of odds ratio (OR), etiological fraction (EF) and preventive fraction (PF). The significance of these differences were assessed by fisher's exact probability (P), which was corrected for the number of alleles at each locus [15,16].

Results

In this study the mean age of BC patients was 48.1 years with a range of 28 - 73 years, while the mean age of BBL was 35.33 years with a range of 21 - 50 years.

In the PCR-SSP method, 24 HLA-A, 48 HLA-B and 23 HLA-Cw specific primer mixes were employed as well as, a negative control and ladder mixes. A successful amplification resulted in the generation of a defined length band as a positive internal control in all lanes except the negative control lane, and when there was no amplification, there was no band. In addition, a positive specific amplification resulted in the generation of a specific band in addition to an internal control band (Figures 1-2).

The observed percentage frequencies of HLA-A,-B and –Cw alleles in the investigated groups are given in tables 1, 2 and 3, respectively, while allele showing a significant

variation is presented in table 4. Out of 95 HLA-class I alleles, one allele (A*03010101-07, 09-11N, 13-16) showed a significant variation between patients and controls. In breast cancer patients, the A*03010101-07,09-11N,13-16 allele accounted to 50% of patients, while its frequency in benign breast lesion patients and healthy controls was 8.3%, 16.6% respectively, such difference was significant (P= 0.024, P=0.041), and associated with OR and EF values of (11,5) and (0.45, O.40) respectively. However, the corrected probability of these differences failed to attend a significant level.

Discussion

The role of genetic factors in the etiology of BC was documented decades ago. As a result, the investigative efforts have focused on the genetic markers of susceptibility to this disease. In particular, HLA system plays a pivotal role in cellular immunity and may be an important genetically determined host trait [17, 18]. This study is the first attempt on the association between HLA class I alleles and BC in Iraqi patients, in which the alleles were characterized by PCR-SSP.

In the present work, there was a significant positive association between HLA- A*03010101-07, 09-11N, 13-16 allele and BC as compared with healthy control. The OR for this allele was 5 and this means that the individuals with A*03010101-07, 09-11N, 13-16 allele have 5 times a greater chance of acquiring BC than those of the same population who lack it. Also, in the present study the comparison of BC patients with a second control (patient control) revealed a significant increased frequency of A*03010101-07, 09-11N, 13-16 allele in BC patients. Therefore, this association of BC with A*03010101-07, 09-11N, 13-16 allele may be considered important in the etiology of BC because it was based in the comparison with two different control groups and unlikely to be attributed to chance factor.

In the context of anti-tumor immunity, HLA- A*03010101-07,09-11N,13-16 allele may differ in its ability to present p53 or other relevant tumor associated antigen derived peptides, thereby contributing to the modulation of risk to develop BC. A similar mechanism has been suggested to operate in case of progression of cervical cancer in HLA-B7 positive patients harboring an HPV variant with uniquely altered peptide sequence of the E6 oncoprotein. The association of HLA- A*03010101-07,09-11N,13-16 allele with breast cancer may also be due to other risk modulating loci that are in linkage disequilibrium with this allele [19].

This result is at variance with some other studies, in serological studies, Bouillenne and Deneufbourg, in (1979), identified HLA - A28 as high risk allele amongst nulliparous pre-menopausal breast cancer patients [20]. A study from India reported a higher frequency of HLA-A2, in breast cancer patients whereas HLA-A11, HLA-Aw19 were suggested to be protective alleles [21]. In addition, Glaser (2005) noticed that the risk increased for whites with A-23 and African-Americans with A-32 after adjustment for age and reproductive risk factors [22].

In the absence of similar findings elsewhere, we must suppose that this association is local, and since, HLA class I proteins are entrusted with the presentation of viral antigens, the A*03010101-07,09-11N,13-16 allele could in fact be related with a viral agent, which in turn is associated with breast cancer in this part of the world. In agreement with this scope, different research groups have recently reported

the involvement of a viral agent in human mammary carcinogenesis (murine mammary tumor virus) [23, 24]. In conclusion, these findings demonstrated that HLA-A*03010101-07, 09-11N, 13-16 allele might play a role in BC susceptibility, suggesting HLA-based different etiopathogenesis.

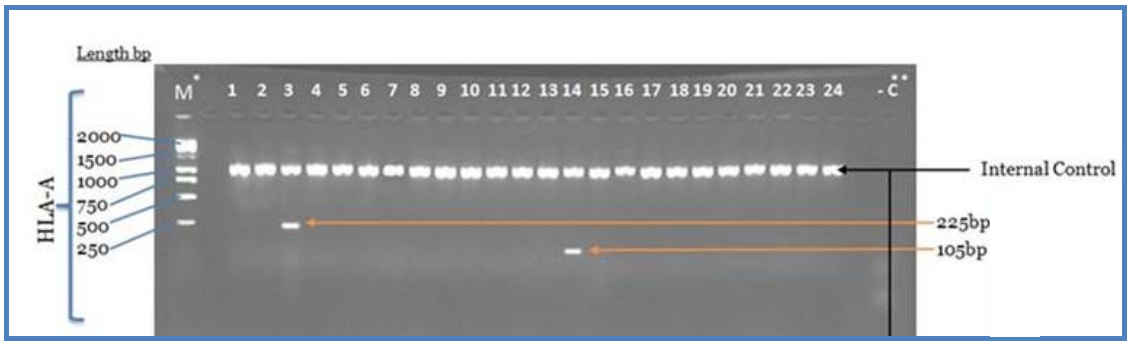

Figure 1: Electrophoresis of HLA-A alleles amplification by PCR-SSP. *Lane M represent 1 Kb DNA Ladder, **Lane-C represent negative control. HLA-A genotyping was obtained by using primers that detect the following alleles as they were present in the numbered wells, respectively:1= A1/ - / Null; 2= A2 /Low A2 / A203 / - / A210 / Null; 3= A2 / - ; 4= A3 / Null /- ; 5= A11 / - / Null ; 6= A23(9) / - / Null ; 7= A24(9) / Low A24/ Null / A2403 / A9 / - ; 8= A24(9) / - ; 9= A25(10) / - ; 10= A26(10),A10,Null ; 11= A26(10) ; 12= A29(19) / Null /- ; 13= A30(19) / - ; 14= A31(19) / - ; 15= - / A31(19) ; 16= A32(19) / - ; 17= A34(10) / - ; 18= A36 / - ; 19= A43 ; 20= A66(10) / - ; 21= A68(28) / A28 / Null / - ; 22= A69(28) ; 23= A74(19) / - ; 24= A80.

Figure 2: Electrophoresis of HLA-A alleles amplification by PCR-SSP. *Lane M represent 1 Kb DNA Ladder, **Lane-C represent negative control. HLA-A genotyping was obtained by using primers that detect the following alleles as they were present in the numbered wells, respectively:1= A1/ - / Null; 2= A2 /Low A2 / A203 / - / A210 / Null; 3= A2 / - ; 4= A3 / Null /- ; 5= A11 / - / Null ; 6= A23(9) / - / Null ; 7= A24(9) / Low A24/ Null / A2403 / A9 / - ; 8= A24(9) / - ; 9= A25(10) / - ; 10= A26(10),A10,Null ; 11= A26(10) ; 12= A29(19) / Null /- ; 13= A30(19) / - ; 14= A31(19) / - ; 15= - / A31(19) ; 16= A32(19) / - ; 17= A34(10) / - ; 18= A36 / - ; 19= A43 ; 20= A66(10) / - ; 21= A68(28) / A28 / Null / - ; 22= A69(28) ; 23= A74(19) / - ; 24= A80.

Table 1: Observed percentage frequencies of HLA-A alleles in healthy controls, breast cancer patients and benign breast lesion.

Specificities	Healthy controls (n=18)		Cases (breast Ca) (n=30)		Benign breast lesion (n=12)	
HLA-A-Allele	N	%	N	%	N	%
A*010101-01010102N,0102-04N,06-11N,12,14,15N	4	22.2	6	20	2	16.7
A*02010101-22,24-33,36-45,47,49-54,57-61,63,64,66-69,71-77,79-86	4	22.2	6	20	3	25
A*0246,48,70	3	16.7	3	10	3	25
A*03010101-07,09-11N,13-16	3	16.7	15	50	1	8.3
A*110101-*1116,20,21N-23	1	5.6	0	0	1	8.3
A*2301,02,04-07N,08N,10,11N,12	1	5.6	3	10	1	8.3
A*2408,21,29,42	2	11.1	0	0	1	8.3
A*260101-0104,03,05,0701-08,10-12,14-18,21-25N,26	2	11.1	3	10	1	8.3
A*300101-*3004,06-14L,15	3	16.7	6	20	2	16.7
A*310102,02,05,06,08,09,11,12	1	5.6	3	10	1	8.3
A*3201-08/B*1595	1	5.6	3	10	1	8.3
A*3301,0301-07	1	5.6	3	10	1	8.3
A*4301	0	0	0	0	1	8.3
A*6601*6604	2	11.1	3	10	1	8.3
A*680101-28	2	11.1	3	10	1	8.3
Blank	6	33.3	3	10	3	25

Table 2: Observed percentage frequencies of HLA-B alleles in healthy controls, breast cancer patients and benign breast lesion.

Specificities	Healthy controls (n=18)		Cases (breast Ca) (n=30)		Benign breast lesion (n=12)	
HLA-B-Allele	N	%	N	%	N	%
B*0714	1	5.6	0	0	1	8.3
B*0809	1	5.6	0	0	1	8.3
B*15170101-1702	2	11.1	3	10	1	8.3
B*1551,52	2	11.1	3	10	1	8.3
B*1820	1	5.6	3	10	1	8.3
B*35010104,03,05,07,08,1401-15,17,23, 24,29,30,32,33,36,38,40N-42,48,50,52, 53N-55-57	1	5.6	3	10	1	8.3
B*350201,0202,0401,06,0901,0902,18	2	11.1	3	10	1	8.3
B*3527,56	1	5.6	3	10	1	8.3
B*3705	1	5.6	3	10	1	8.3

B*4039,51	**1**	**5.6**	**3**	**10**	**1**	**8.3**
B*4101-07	**1**	**5.6**	**3**	**10**	**1**	**8.3**
B*440301,0302,04,07,13,26,28, 30,32,35,36,38,39	**2**	**11.1**	**3**	**10**	**1**	**8.3**
B*4416	**0**	**0**	**0**	**0**	**1**	**8.3**
B*4418	**1**	**5.6**	**3**	**10**	**1**	**8.3**
B*4442	**1**	**5.6**	**3**	**10**	**1**	**8.3**
B*4803	**0**	**0**	**0**	**0**	**1**	**8.3**
B*4901	**1**	**5.6**	**0**	**0**	**0**	**0**
B*5001,04	**3**	**16.7**	**6**	**20**	**2**	**16.7**
B*510102,0202,05	**3**	**16.7**	**3**	**10**	**2**	**16.7**
B*520101,0103,04,05,07	**2**	**11.1**	**6**	**20**	**2**	**16.7**
B*5208	**1**	**5.6**	**3**	**10**	**1**	**8.3**
B*180101-06,08,10-13,17N19	**2**	**11.1**	**3**	**10**	**1**	**8.3**
Blank	**6**	**33.3**	**3**	**10**	**0**	**0**

Table 3: Observed percentage frequencies of HLA-Cw alleles in healthy controls, breast cancer patients and benign breast lesion.

Specificities	**Healthy controls (n=18)**		**Cases(breast Ca) (n=30)**		**Benign breast lesion(n=12)**	
HLA-Cw-Allele	**N**	**%**	**N**	**%**	**N**	**%**
Cw*010201-03,06-09	**1**	**5.6**	**0**	**0**	**1**	**8.3**
Cw*020201-0205,04-08	**1**	**5.6**	**0**	**0**	**1**	**8.3**
Cw*030401-0403,05-10	**1**	**5.6**	**0**	**0**	**1**	**8.3**
Cw*0315	**0**	**0**	**0**	**0**	**1**	**8.3**
Cw*04010101-0102,03-09N,10-13	**4**	**22.2**	**9**	**30**	**3**	**25**
Cw*0501-07N	**1**	**5.6**	**0**	**0**	**1**	**8.3**
Cw*0602,03,07,09,10	**3**	**16.7**	**6**	**20**	**2**	**16.7**
Cw*070101,0102,05,06,08,14,16,18,20-22	**3**	**16.7**	**6**	**20**	**2**	**16.7**
Cw*120201-0203,08	**1**	**5.6**	**3**	**10**	**1**	**8.3**
Cw*120301,06,07,11	**2**	**11.1**	**6**	**20**	**1**	**8.3**
Cw*120401	**2**	**11.1**	**6**	**20**	**2**	**16.7**
Cw*120402,05	**1**	**5.6**	**3**	**10**	**0**	**0**
Cw*1210	**1**	**5.6**	**3**	**10**	**1**	**8.3**
Cw*1701-03	**2**	**11.1**	**6**	**20**	**1**	**8.3**
Cw07020101,020102,10,13,15,19	**1**	**5.6**	**3**	**10**	**1**	**8.3**
Blank	**12**	**66.7**	**9**	**30**	**٥**	**50**

Table 4: HLA- A*03010101-07, 09-11N, 13-16 allele showing significant variations between breast cancer patients and controls.

HLA-A*03010101-07,09-11N,13-16	Patients		Controls		Statistical evaluations		
	N	%	N	%	Odds ratio	EF	P
Breast cancer patients vs. healthy controls	15	50	3	16.6	5	0.40	0.041
Breast cancer patients vs. benign breast lesion patients	15	50	1	8.3	11	0.45	0.024

EF: Etiological fraction; P: Fisher's exact two-sided Probability.

References

1. Ackerman LV, Rosai J. Breast In: Rosai and Ackerman's Surgical Pathology, 9th ed, Ackerman LV and Rosai J (eds), Mosby, St Louis, 2004; pp1764-1824.

2. ICB. Results of Iraqi National Cancer Registry, (1975-1978; 1989-1991; 1995-1997, 1998-2000). Iraqi Cancer Board ICRC, Ministry of Health Baghdad- Iraq; 2000.

3. Shankarkumar U, Ghosh K, Badkere S, Mohanty D. Novel HLA Class I Alleles Associated with Indian leprosy Patients. J Biomed Biotechnol 2003; 3:208-211.

4. Ferguson TA, Green DR, Griffith TS. Cell death and immune privilege. Int Rev Immunol 2002; 21: 153-172.

5. Igney FH, Krammer PH. Death and anti-death: tumor resistance to apoptosis. Nat Rev Cancer 2002; 2:277-288.

6. Pellegris G, Illeni MT, Vaglini M. HLA antigens in malignant melanoma patients. Tumori, 1980; 66: 51-58.

7. Bidwell JL, Soong TW, Raymond PA. HLA genotyping of colorectal carcinoma in the Chinese population. Hum Immunol 1992; 34:19- 23.

8. Wu MS, Hsieh RP, Huang SP. Association of HLA-DQB1*0301 and HLA-DQB1*0602 with different subtypes of gastric cancer in Taiwan. Jpn J Cancer Res, 2002; 93:404-410.

9. Harrath AB, Loueslati BY, Troudi W, Hmida S, Sedkaoui S, Dridi A, Jridi A. HLA class II polymorphism: Protective or risk factors to breast cancer in Tunisia Pathology. Oncology Research 2006; 12, No (2), 79-81.

10. Glaser S. Immune –function genes and racedifferences in breast cancer. NorthernCaliforniaCancerCenter;2002.http://wwwcbcrporg/research/PageGrantasp?grant_id=2275.

11. Glaser S, Join EM, Clarke CA, Erlich HA. Human leukocyte antigen genotype as a contributor to racial/ethnic differences in breast cancer: A population – based, molecular epidemiologic study. Northern california cancer center unioncity 2004; Annual rept 2 June 2003-1 June 2004.

12. Miller SA, Dykes DD, Polesky IF. Asimple salting out procedure for extracting DNA from human nucleated cells. Nucl Acid Res 1988; 16:1215.

13. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with PCR-SSP in two hours. Tissue Antigens 1992; 39:225-235.

14. Olerup O, Zetterquist H.DR low-resolution PCR-SSP typing-a correction and an update. Tissue Antigens 1993; 41:55-56.

15. Emery AEH. Methodology in medical genetics: An introduction to statistical methods. First edition, UK, Churchill Livingstone 1976; 98-102.

16. Sorlie DE. Medical biostatistics and epidemiology: Examination and Board review First ed, Norwalk, Connecticut, Appleton and Lange 1995; 47-88.

17. Fossum B, Breivik J, Meling GI**.** A K-ras 13Gly-->Asp mutation is recognized by HLA-DQ7 restricted T cells in a patient with colorectal cancer Modifying effect of DQ7 on established cancers harboring this mutation. Int J Cancer 1994; 58, 506-11.

18. Bateman A, Howell WM. Human leukocyte antigens and cancer: is it in our genes. J Pathol, 1999; 188: 231-6.

19. Gopalkrishnan L, Patil S, Chhaya S, Badwe R, Joshi N. HLA alleles in pre-

menopausal breast cancer patients from western India. Indian J Med Res, 124, September, 2006; pp 305-312.
20. Bouillenne C, Deneufbourg JM. Positive correlation between breast cancer incidence and HLA antigens. Oncology 1979; 36: 156-9.
21. Biswal BM, Kumar R, Julka PK, Sharma U, Vaidya MC. Human leucocytic antigens (HLA) in breast cancer. Indian J Med Sci 1998; 52: 177-83.
22. Glaser SL. HLA genotype as a contributor to racial/ethnic differences in BC: A population based molecular epidemiologic study. Annual rept June 2005.http://stinetdticmil/oai/oai?verb=getRecord&metadataPrefix=html&identifier=ADA4.
23. Pogo BG-T. A retrovirus similar to MMTV associated with human breast cancer. Breast Cancer Res 2001; 3, p A47.
24. Stewart A. MMTV-related env sequences in human breast tumor. Int J Cancer 2003; 10 (106), p 138.