

Human Leukocyte Antigens (HLA) Typing For Iraqi Vitiligo Patients

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Summary:

Background and objectives: The genes of HLA region controls a variety of functions involved in immune response and influences susceptibility to autoimmune diseases.

Many diseases are associated with the certain HLA alleles, which occurs more frequently among patients compared to the healthy population. This study aims to shed light on the role of HLA alleles in causing vitiligo disease.

Methods: The study was carried out on two groups. The first was of 35 patients (21 males & 14 females) with vitiligo, and the second was of 60 (30 males & 30 females) unrelated healthy controls.

By using of microlymphocytotoxicity test, HLA typing for class I and class II alleles for T and B-lymphocytes were performed.

Some of immunological investigations were carried out, such as interleukin-8 (IL-8) concentration level by using of ELISA technique, and antinuclear antibodies (ANAs) by using of FANA test.

Results: HLA typing for class I (A,B and C) and class II (DR and DQ) alleles revealed statistically that Cw6,Cw7 and DR3 were highly associated with vitiligo patients.

The high levels of IL-8 concentration (for both sexes) associated with inflammatory vitiligo only, especially in the younger ages and females with high association of A2 alleles.

All results of the antinuclear antibodies test were negative.

Conclusion: 1. The genetic predisposition for vitiligo disease increase with the associations of Cw6,Cw7 and DR3 alleles.

2. The level of IL-8 concentration has been raised in the sera of patients especially in cases of inflammatory vitiligo, with high frequently of A2 allele.

Introduction:

Vitiligo is a disease which has been known since immemorial times. The earliest information concerning this disease came from the ancient Egyptian writing at the time of the Pharaohs (1), and the Indian writings which were dated back to 1400 BC (2). The Arabic synonym for vitiligo is (bahak).

Vitiligo is depigmenting disorder characterized by the loss of melanocytes from the cutaneous epidermis (3). Although the exact etiology of vitiligo has not yet been established, the abnormal immune responses frequently observed in vitiligo patients have led to the suggestion that, in some cases, the condition has an autoimmune component. Briefly, circulating autoantibodies and autoreactive T cells that recognize pigment cell antigens have been detected in the sera of

a significant proportion of vitiligo patients compared with healthy individuals (4). In addition, vitiligo is often associated with other disorders that have an autoimmune origin, including hashimoto's thyroiditis, Graves' disease, type 1 insulin-dependent diabetes mellitus (IDDM-1) and Addison's disease. Furthermore, effective use of immunosuppressive therapies to treat vitiligo (5).

The association of vitiligo with certain major histocompatibility complex antigens and evidence from animal models of the disease have all added credence to the hypothesis that immune reactions play a role in vitiligo pathogenesis (6).

Aim of the study:

This study aims to shed light on the role of HLA alleles in causing vitiligo disease throughout the following evaluations:

1. HLA class -I antigens.
2. HLA class-II antigens.

In order to identify the highest frequencies of alleles that formed the risk factors for the disease, Moreover, to identify the protective alleles among healthy normal controls.

3. Antinuclear antibodies (ANAs).

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In order to determine if the patient has an additional autoimmune disease, which are investigated by this test.

4. Quantification of interleukin 8 (IL-8) concentrations by Enzyme linked immunosorbent assay (ELISA) technique in the patients and control groups' sera and their relation with the frequencies of alleles.

Methods:

Two studied groups were investigated in this work, they include:

A. Patients group:

A total of (35) Iraqi Arab vitiligo patients were involved in this study (21 males and 14 females) their ages ranged from 8-61 years. Those patients were attending the consultant clinic for dermatology in Baghdad teaching hospital from January 2004 to may 2004. The committee of dermatology performed the clinical examination of patients.

B. Control group:

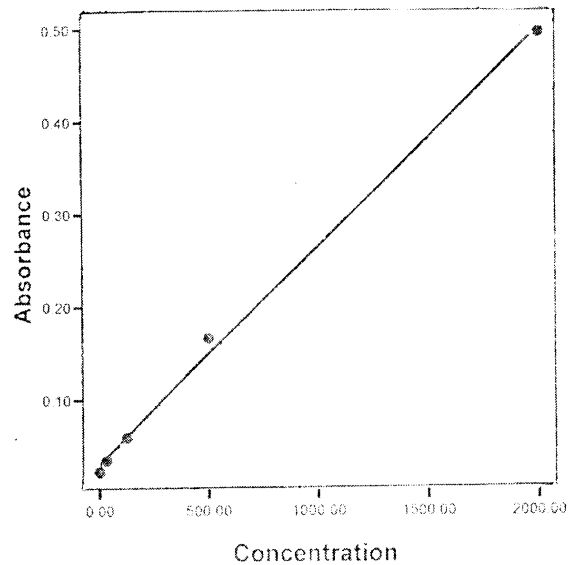
Apparently healthy control group with total number of 60 (30 males and 30 females) their ages ranged from 4-52 years. These healthy controls who have no history or clinical evidence of vitiligo or any other chronic disease, and no obvious abnormalities, i.e., healthy controls were age, sex, and ethnic matched with patients group.

Twenty milliliters (ml) of venous blood were collected from patients as well as controls. The collected blood was immediately transferred into different test tubes, to be readily used in the different tests, as follows:

1. Fifteen ml of the blood in Plastic universal tubes (20 ml) containing lithium heparin (10 IU/ml blood) as anti-coagulant, followed by a gentle mixing, and By using of microlymphocytotoxicity test, HLA typing for class I (A, B, and C) and class II (DR and DQ) alleles were performed, depending on known antisera, for both T and B-lymphocytes respectively
2. Five ml of the blood in Plastic test tubes (10 ml) and by centrifugation, the separated serum was used for detection of ANAs by using of fluorescent ANAs test and for quantification of IL-8 levels by using of ELISA technique.

The sample results of IL-8 concentration were calculated by interpolation from a standard curve that was performed in the same assay as that of the sample. The curve was drawn by plotting on the horizontal axis the IL-8 concentration of the standards and on the vertical axis the corresponding absorbance. The absorbance was located for each sample on vertical axis and read off the corresponding IL-8 concentration on the horizontal axis (Figure 1).

Figure (1): Standard curve for IL-8 concentration and the absorbance



This study was performed in the Histocompatibility and Immunology Laboratories of the Teaching Laboratories/Baghdad Teaching Hospital from January 2004 to June 2004.

Statistical analysis:

Data were collected and analyzed by using statistical package for social sciences (SPSS) version 10 for Windows (SPSS, Chicago, Illinois, and USA). The distribution of variables was not normal and was determined by using Kolmogorov-Smirnov test. Differences between groups were examined by Mann-Whitney U test, spearman correlation

was done to detect the significance of the relation between age and interleukin level. Fisher's exact probability test was used to detect the significance of the relation between the various variables. P value < 0.05 was considered as statistically significant.

Results :

Class-I HLA Molecules:

It was found that the HLA-A2 significantly increased in the vitiligo patients (51.4%) compared to healthy controls (28.3%), (Table 1). Such observation was associated with a Relative risk (RR) value of (2.67) and Etiological fraction (EF) value of (0.32) (P = 0.02), however, the significant increase of HLA-A2 was to be not significant after correction of P value (Pc = 0.22), (Table 2). Similarly the antigen frequency of HLA-B5 significantly increased in vitiligo patients (22.8%) as compared to healthy controls (3.3%), (Table 1). Also such finding was associated with RR value of (8.59) and EF value of (0.2) (P = 0.004), but this

association was not regarded significant after the correction of P value ($P_c=0.08$). (Table 2). The frequencies of HLA-Cw6 and HLA-Cw7 antigens significantly increased in vitiligo patients (71.4% & 40%)

as compared to healthy controls (1.6% & 11.6%), (Table 1). Such observations were associated with RR value of (147.5 & 5), EF value of (0.71 & 0.32) and highly significant P value of (0.00001 & 0.002 respectively). These associations remained highly significant after the correction for HLA-Cw6 ($P_c = 0.00007$), but significant for the HLA-Cw7 ($P_c = 0.014$), (Table 2).

Class-II HLA molecules:

The observed numbers and percentages of HLA-class II (DR, DQ) for vitiligo patients, as compared with healthy controls are shown in table (1), while HLA antigens with significant differences compared to healthy controls are shown in table (2).

The frequency of HLA-DR2 significantly decreased in vitiligo patients (8.5%) compared to healthy controls (31%), (Table 1). Such observation was associated with a relative risk (RR) value of (0.2), and the preventive fraction (PF) value was (0.25) ($P = 0.01$). These associations were not regarded significant after correction of P value ($P_c=0.11$), (Table 2). HLA-DR3 frequency significantly increased in vitiligo patients (71.4%) compared to healthy controls (23.3%), (Table 1). Such observation was association with RR value of (8.21), EF value of (0.62) ($P = 0.00009$) and highly significant corrected P value ($P_c = 0.00099$), (Table 2).

None of the HLA-DQ molecules showed significant differences between patients and controls, though HLA-DQ1 was regarded as a risk factor (EF = 0.35). So from all what had been mentioned previously, it appeared that Cw6, Cw7 and DR3 formed a big significant difference between patients and the normal persons since their: Cw6: RR = 147.5, EF = 0.71, $P = 0.00001$, $P_c = 0.00007$, (Highly significant).

Cw7: RR = 5, EF = 0.32, $P = 0.002$, $P_c = 0.014$, (Significant).

DR3: RR = 8.21, EF = 0.62, $P = 0.00009$, $P_c = 0.00099$, (Highly significant).

Table(1): The observed numbers and percentages of HLA-class I & II alleles for vitiligo patients and healthy controls.

HLA	Patients No. = 35		Controls No. = 60	
	No.	%	No.	%
A1	9	25.7	8	13.3
A2	18	51.4	17	28.3
A9	4	11.4	15	25.0
A11	1	2.8	11	18.3
B5	8	22.8	2	3.3
Cw6	25	71.4	1	1.6
Cw7	14	40	7	11.6
DR1	13	37.1	11	18.3
DR2	3	8.5	19	31.6
DR3	25	71.4	14	23.3
DQ1	17	48.5	12	20

Table (2): Significant variation of HLA-alleles in patients with vitiligo compared to healthy controls.

Alleles	RR	EF	PF	P	P_c
A1	2.25	0.14		NS	
A2	2.67	0.32		0.02	0.22 (NS)
A9	0.38		0.15	NS	
A11	0.13		0.16	NS	
A28	0		0	NS	
B5	8.59	0.2		0.004	0.08 (NS)
Cw2	0		0	NS	
Cw6	147.5	0.71		0.00001	0.00007
Cw7	5	0.32		0.002	0.014
DR1	2.63	0.22		NS	
DR2	0.2		0.25	0.01	0.11 (NS)
DR3	8.21	0.62		0.00009	0.00099
DQ1	3.77	0.35		NS	

RR = Relative risk.

EF = Etiological fraction.

PF = preventive fraction.

P = Fisher's exact probability.

P_c = Corrected Fisher's exact probability.

NS = Not significant variation.

IL-8 concentration:

As showed in table (4), the IL-8 concentrations in patients were not significant or nearly significant ($Z = -1.901$, $P = 0.057$). The relationship between sex and IL-8 level in patients and controls is shown in table (4). In male patients, there was not any significant difference ($Z = -0.805$, $P = 0.421$), while in female patients, there was a significant difference ($Z = -2.009$, $P = 0.045$). So from all what had been mentioned above it appeared that sex had important relationship with IL-8 level.

especially in female patients. The relationship between age and IL-8 level in patients and controls is shown in table (5). There was a significant negative linear correlation between the IL-8 concentration and the age of patients ($r = -0.469$, $P = 0.004$), (Figure 2), but there was not any significant negative linear correlation between the IL-8 concentration and the age of controls ($r = -0.083$, $P = 0.769$), (Figure 3).

Table (5) showed the correlation between IL-8 concentration and the duration of the disease, there appeared no statistically significant correlation ($r = -0.172$, $P = 0.324$), (figure 4), but the IL-8 concentration slightly increased when duration decreased. The relationship between highest IL-8 concentration and frequencies of HLA alleles is shown in table (6). There were increases in alleles frequencies such as HLA-A2 associated with the other alleles causing vitiligo in the high levels of IL-8 concentration, especially when inflammatory vitiligo happens. The percentage of HLA-A2 equals 75% in patients with higher IL-8 levels or inflammatory vitiligo. ANAs detection:

All results of vitiligo patients, as in healthy controls were negative.

Table (3): The sex relationship with the mean, standard deviation and standard error of IL-8 concentration in studied groups.

Sex	Group	No.	Mean	Std. deviation	Std error
	IL-8 Patients	35	92.0000	185.5152	31.3578
	Controls	15	1.2000	2.2424	0.5790
Male	Patients	21	63.5714	163.8492	35.7548
	Controls	7	1.4286	2.6992	1.0202
Female	Patients	14	134.6429	213.1773	56.9740
	Controls	8	1.0000	1.9272	0.6814

Table(4): The sex relationship with Z value and probability of error of IL-8 concentration.

sex	value	IL-8
	Z	-1.901
	P	0.057
Male	Z	-0.805
	P	0.421
Female	Z	-2.009
	P	0.045

Table(5): The correlation between IL-8 concentration, age and the duration of disease.

Group	Value	Age	Duration
	r		-0.172
	p		0.324
Patients' ages	r	-0.469	
	p	0.004	
Controls' ages	r	-0.083	
	p	0.769	

Table (6): The HLA-A2 allele associating with alleles causing vitiligo and its percentage in patients.

Patients No. = 8	IL-8 level pg/ml	HLA-A2	HLA-Cw6	HLA-Cw7	HLA-DR3
1	178.00	+	-	+	+
2	190.00	-	+	-	+
3	220.00	-	+	-	+
4	308.00	+	-	-	+
5	527.00	+	-	+	-
6	554.00	+	+	-	-
7	570.00	+	-	+	+
8	587.00	+	+	-	-
Σ		6+	4+	3+	5+
%		75%	50%	37.5%	62.5%

(+) = Present allele
(-) = Absent allele

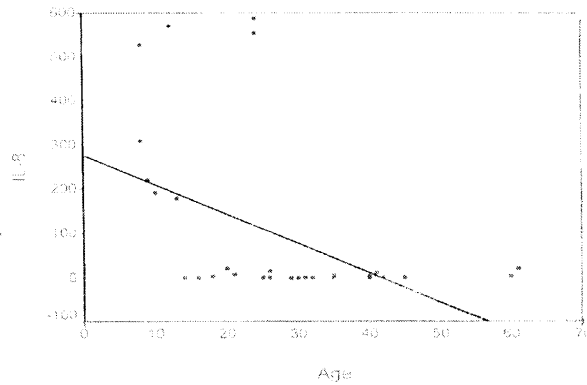


Figure (2): The linear correlation between IL-8 concentration and age of vitiligo patients.

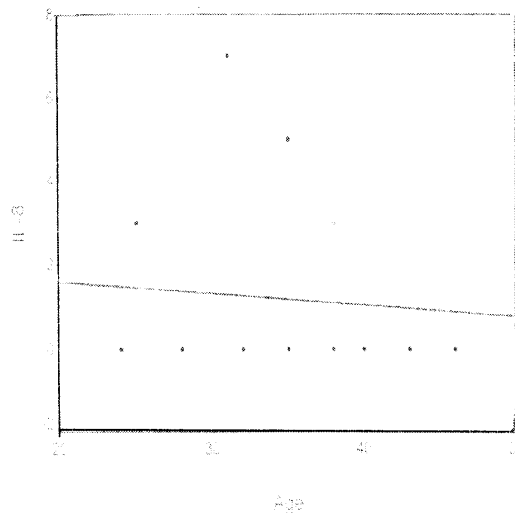


Figure (3): The liner correlation between IL-8 concentration and age of healthy controls.

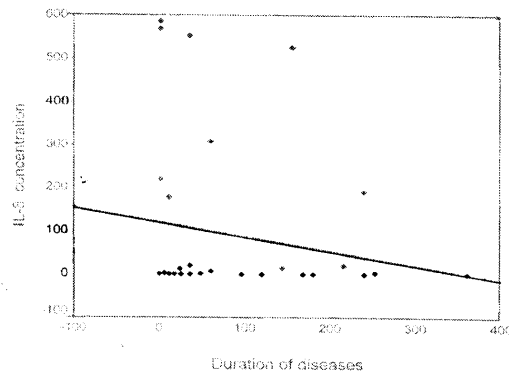


Figure (4): The linear correlation between IL-8 concentration and the duration of disease.

Discussion

Vitiligo or in general autoimmune diseases constitute one of the main problems in human clinical medicine. Moreover, its importance resides not only in its high prevalence but also its victims are usually forming the young and economically active people (the mean of age of onset = 20.5).

In the present study, The percentage of familial clustering of vitiligo patients was equal to 31.5%, i.e. among thirty five patients there were eleven of them having relatives suffering from vitiligo. This explanation is consistent with the ideas of Seung- Kyung Hann (7), in which about 30% of patients have familial clustering of cases. This fact is because that the disease itself is not inherited, but the disposition of having vitiligo is inherited.

The relative risk (RR) and both the etiological fraction (EF) and the preventive fraction (PF) tell something about the biological importance of an association between alleles and the disease, but these estimates of the strength of that association have rather different meanings, while the

RR refers to the risk of a single individual, the EF refers to the involvement of the HLA factor in question in the entire population (8).

The statistical significance of an association can be evaluated by Fisher's exact test or by various approximate tests, e.g. a chi-square test. Fisher's exact test gives the exact probability (P) of finding differences as extreme as or more extreme than those observed.

The RR value indicates how many times, as frequently, the disease is likely to occur in individuals carrying the antigen relative to those lacking it. The RR value of (1) means that the HLA alleles are expressed with the same frequency in the patients and general population, therefore, the allele confers no increased risk for the disease. By contrast the RR value < 1 indicates a protective effect, which is associated with the PF which indicates how much a disease

is prevented by the disease associated factor, and the RR value > 1 indicates increased susceptibility to that specific disease, which is associated with the EF which indicates how much of a disease is due to the disease associated factor (9).

The genes of HLA region controls a variety of functions involved in immune response and influences susceptibility to autoimmune diseases.

In this study, HLA-typing was performed using lymphocytotoxicity method which was the available one. Other methods such as Polymerase chain reaction (PCR) (10), and ELISA (11), though are highly sensitive and specific but not yet used in routine work and not easily obtained.

In the present study, the results of HLA-typing for HLA-class I alleles revealed significant increase frequencies of Cw6 and Cw7, while for HLA-class II alleles, the DR3 antigen was the most frequent one in patients. These findings may suggest their role in increasing the susceptibility to vitiligo.

Furthermore, positive A2, B5 with RR value of > 1 and negative DR2 with RR value of < 1 associations were also observed ($P < 0.05$), but the difference did not reach a significant level after correction of P value to count for chance significance ($P_c > 0.05$).

Many previous studies dealt with the subject of vitiligo disease, they were about HLA-alleles association with vitiligo in different ethnic populations and areas, for example, increased frequency of HLA-DR4 in blacks (12), HLA-B13 in Moroccan Jewish, HLA-B35 in Yemenite Jewish (13), and eventually in present work HLA-Cw6, -Cw7, and -DR3 in Iraqi Arab patients. This indicates that allelic frequency of HLA differs according to geographical locations.

In this study, HLA-DR4 alleles had shown no significant association, while HLA-DR3 alleles had shown a highly significant positive association to act as etiological factor (EF = 0.62, $P_c = 0.00099$), these results were contrary to the findings mentioned in the abstract by Netherlands Institute (14), about HLA association in vitiligo patients which was done on Dutch populations and the results had shown a significant positive associations with DR4 (etiological factor) and a significant negative association with DR3 (preventive factor). This may be due to the differences of races.

For the same reason, the study of Dunston and Halder (12) revealed that HLA-DR4 significantly increased in black patients (America African), 38% vs. 11% for local black controls. HLA-DQw3 also increased in patients 58% vs. 32% for controls. The present work achieved the contrary especially for HLA-DR4 which had not significantly increased in patients, 25.7% vs. 25% for controls, HLA-DQw3 too, had not

significantly increased in patients, 20% vs. 21.6% controls.

The present study is compatible to many researches and studies performed by scientists. Finco (15) suggested by a study done on 87 patients, that Italian population had high frequency of A30, Cw6, DQw3. The current study is in agreement with those observations in respect to the frequency of Cw6, but it is in contrast to the other alleles. In Hungary, by investigating a group of 88 unrelated patients suffering from vitiligo by Poloy (16), it was found that HLA-DR1 and HLA-DR3 alleles significantly increased in patients compared to healthy controls. The significant increase of HLA-DR3 in the present study was applied on these observations.

For the nearest areas, as in Kuwait, AL-Fouzan (17) declared by a study done on 40 Kuwaiti vitiligo patients that there is a significant association with Cw6 and B21. This compatibility to our present study in respect to the increased frequency of Cw6 but not with B21, the frequency was lowered in the present study 8.5% vs. 11% for controls (RR=0.7, P = 0.74). This is due to the fact that Iraq is nearby Kuwait and shared by the geographical region or may be similar regarding the ethnical group.

The current study revealed that the age of onset is 5-40 years, the mean equals 20.5 years and no differences between the age of onset in both sexes in vitiligo patients groups. This result was consistent with the study of Seung-Kyung Hann (7) who observed that the mean age of onset unlikely varies between sexes.

The present work coincides with many studies that mark different age of onset in vitiligo. The study of Finco (15) indicated the pediatric form of vitiligo while others indicated the adult form. These facts emphasize that vitiligo may appear from birth to senescence. But it is rarely seen in infancy or old age, nearly all vitiligo is acquired early in life.

In the present study, the duration was very important in vitiligo patients, as the duration decreased when the activity of the disease increased because the disease in the progressive stage spreads to other parts of the body, while the activity of the disease decreased when the duration increased because in this stage the disease has nearly stabilized or perhaps stopped its spreading. As mentioned previously in the literature review, IL-8 plays a major role in inflammatory responses and wound healing mainly due to its ability to attract neutrophils to sites of tissue damage and inflammation, which could lead to the synthesis of proteolytic enzymes that degrade the extracellular matrix (18).

In this study, among thirty five vitiligo patients only eight of them were with significant increased IL-8 concentration, the mean was (92±31.357 pg/ml) compared to (1.2 ± 0.57 pg/ml) for healthy controls (Table 3). There are two types of vitiligo: Active and inactive vitiligo, the active one means, the disease is in progressive or spreading stage and includes inflammatory reactions. In the case of inflammatory reactions, many different types of cells such as monocytes, macrophages, endothelial cells, fibroblasts and neutrophils are secreting IL-8 (19).

The Z values and their probability of errors are very necessary for the evaluation of the significance of IL-8 level in patients group (Z=-1.901, P = 0.057) compared to healthy controls group. The P value declared that IL-8 concentration is nearly to significant, but when Z value extracted according to sex, it became significant in females (Z = -2.009, P = 0.045) because P values is < 0.05, and not significant in males (Z = -0.805, p = 0.42) compared to healthy controls. The above

results denoted a high concentration of IL-8 among females rather than males, which may be due to the hormonal differences between them.

The correlation between age and concentrations of IL-8 is found to be highly significant in patients (r = -0.469, P = 0.004) compared to healthy controls which was not significant (r = -0.083, P = 0.769). This suggested an inverse relationship between age and IL-8 concentration.

The presence of HLA-A2 alleles with high frequency 75% among the eight patients with high level of IL-8 concentration together with the other alleles which play an important role in causing vitiligo such as Cw6, Cw7, and DR3 may represent an etiological factor to inflammatory vitiligo.

Antinuclear antibodies (ANAs) are unusual antibodies that have the capability of binding to certain structures within the nucleus of the cells. ANAs indicate the possible presence of autoimmunity, therefore, this test can help to determine if the patient has some additional autoimmune disease or not (20).

In this study, all results of the test were negative for all vitiligo patients. This means that there was no additional autoimmune diseases which are investigated by this test, that affected the genetic predisposition to have vitiligo. Moreover, it can be concluded that there is no change in the nucleus and no foreign component or structure in the nucleus of melanocyte cells and it is not targeted by ANAs.

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