Original Article

Genotyping of HLA-class-I and II by PCR-SSP of Patients with Recurrent Aphthous Stomatitis

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Summary:

Background: Recurrent aphthous stomatitis is a common condition characterised by recurrent episodes of oral ulceration. Genetic factors have been implicated by numerous studies on the association of recurrent aphthous stomatitis and the genetically determined HLA subtypes.

Objectives: Current study was established to shed light on the possible association of HLA class I and II alleles with recurrent aphthous stomatitis in Iraqi patients.

Subjects and Methods: The study included 55 subjects: 30 recurrent aphthous stomatitis patients and 25 apparently healthy subjects as control. Polymerase chain reaction-specific sequence primers (PCR- SSP) assay was conducted to assess HLA- typing.

Results: The present study showed a significant association of HLA-Cw*12:02:01-and HLA-DQB1*02:01:01- alleles with recurrent aphthous stomatitis as compared with healthy control (P= 0.041and P=0.028 respectively). Another interesting finding in this study was the significant low frequency of HLA-DQB1*05:01:01- allele in patients when compared with healthy control (P=0.036).

Conclusion: These findings demonstrated that HLA-Cw*12:02:01- and HLA-DQB1*02:01:01-alleles may play a role in the etiology of the disease. Whereas HLA-DQB1*05:01:01-05 may confer protective effects against recurrent aphthous stomatitis.

Keywords: RAS, HLA allele, PCR.

Fac Med Baghdad 2012; Vol. 54, No. 1
Received Mar. 2011
Accepted Dec. 2011

Introduction:

Recurrent aphthous stomatitis (RAS) is a common disease that causes the appearance of recurrent aphtha in the oral mucosa. Its incidence is of about 20% in the world population. It is more frequent in females. Despite the high incidence, many studies dedicated to unveiling its causes, it still is very controversial regarding etiology (1). Recurrent aphthous stomatitis is characterized by intermittent episodes (ranging from days to months) of 1 or more painful ulcers in areas of nonkeratinized mucosa, such as the buccal mucosa, floor of the mouth, and ventral surface of the tongue. The disease usually has three clinical forms, based on the aspect and size of the ulcerations: minor, major and herpetiform (2, 3). Minor RAS affects 80% of the patients with RAS, and it is characterized by a shallow painful, oval or round-shaped ulcer, smaller than 5mm in diameter. The lesions heal within 7 to 10 days. Major RAS is the most severe form; aphtha diameter can get bigger than 1cm, remaining in the oral cavity for a period longer than two weeks, often times leaving a scar. The third form of the disease, and the less common one is herpetiform RAS, characterized by multiple and painful 1 to 3mm ulcers, which may coalesce, forming larger and irregular lesions (4, 5). Family history seems to be relevant in the genesis of RAS, and reports of cases in the same family are found in 24% to 46% of the times (6). Moreover, patients with family history of RAS can develop ulcers earlier on and have more severe manifestations than those without family history (7). Numerous associations and non-associations of HLA and RAS antigen have been reported in the medical literature. The association between the disease and HLAB12 was described by Lehner and colleagues (8) and Malmstr□m *et al.*,(9), however it was not confirmed by other authors (10,11). In groups of patients of different ethnical origin, a significant association between HLA-DR2 and RAS was noticed (8, 12). RAS is common oral cavity disease in Iraq, yet no available data on the association of HLA-alleles with RAS. This prompted us to carry out this study in order to shed light on the association between HLA-class I (A and C) and -class II (DR and DQ) alleles with RAS, as well as to provide information about genotypes that confer susceptibility or resistance to develop the disease.

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Subjects and Methods:

Thirty Iraqi Arab patients with RAS (16 females and 14 males) were included in this study. They were among patients attending to the teaching hospital dentistry college in Baghdad, during the period between September2009 till March 2010. Their age ranged from 18-40 years. Diagnosis was made by specialized dentists in the hospital. All the cases had received no treatment with no complain of chronic or systemic diseases. Apparently healthy volunteers their ethnic, ages, and gender were matched, consisted of 25 individuals who were considered as control. All of them have no history or clinic evidence of RAS lesions. Their age ranged from 18-31years. Two milliliters of venous blood with EDTA as anticoagulant were collected from each subject. Extraction of DNA from peripheral blood was done according to the modified method of Miller (13), using the EXTRA-GENE-I kit (BAG-Germany). HLA-genotyping was performed in HLA-laboratory of Forensic Medicine Institute/ Baghdad by PCR-SSP according to a method presented by Olerup and Zetterquist (14, 15), using low resolution typing kits (HISTO TYPE / DNA-SSP Kits-BAG- Germany). In the PCR-SSP method, HLA-A, HLA-Cw, HLA-DR and HLA-DQ specific primer mixes were employed as well as, a negative control and ladder [DNA-lenth standard (x174-HaeIII)]mixes. Appropriate amounts of DNA (50-100 ng/µl) and Taq polymerase (Recombinant Taq polymerase (recombinant taq polymerase 5u/µl from QIAGEN-company) were added to pre-aliquoted primers, (table 1) and PCR conditions were set according to the manufacturer instructions. PCR products were loaded in 2 % agarose gel containing 0.5 µg/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 were presented in terms of percentage frequencies, and alleles showing variations between patients and controls were further presented in terms of odds ratio (OR) and etiological fraction (EF). The significance of these differences was assessed by fisher's exact probability (P) (16).

Results:

In this work the age of RAS patients ranged between 16-40 years with a mean age of 23.1 years. 43% (13) of patients had positive family history of RAS, while 57% (17) showed negative family history. In the PCR-SSP method, a successful amplification resulted in the generation of a defined length band as a positive internal control (specific for the human G3PDH gene 1070 bp) 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 frequency of distribution of various HLA- alleles for thirty patients with RAS were typed for HLA-genotyping class I (A & C) and class II (DR&DQ). These frequencies for two studied groups were presented in tables (2, 3, 4 and 5). Comparison between patients and control group showed several alleles deviations in their frequencies. Regarding HLA-A locus, the statistical analysis revealed no significant association in patients as compared with healthy control as shown in table (2), meanwhile, HLA-C locus: the C*12:02:01-alleles revealed higher frequencies (17.2%) in RAS patients than healthy control with OR of (11.00), table (3). Among DQB alleles that have significant risk effect in a disease, there was DQB1*02:01:0- which was noticed in high frequency among patients (48.3% vs. 20.8%, OR: 3.55, EF: 0.347) with significant differences in comparison with healthy control ($P<0.05$), table (5). On the contrary, there was significant decrease in the frequency of DQB1*05:01:0 among patients ($P=0.036$) as compared with healthy control.

Table.1 Composition of the Master-Mix for different numbers of reaction mixes.

Number of mixes	DNA-solution (50-100 ng/µl)	Aqua dest	10 x PCR buffer	*Taq*-polymerase (5u/ µl)	Whole volume
1	1	8	1	0.08	10 µl
8	10	79	10	0.8	100 µl
24	28	222	28	2.2	280 µl
30	34	269	34	2.7	340 µl
32	36	285	36	2.9	360 µl
48	52	412	52	4.2	520 µl
54	58	459	58	4.6	580 µl
56	60	475	60	4.8	600 µl
72	78	618	78	6.2	780 µl
80	86	681	86	6.9	860 µl
96	102	808	102	8.2	1020 µl

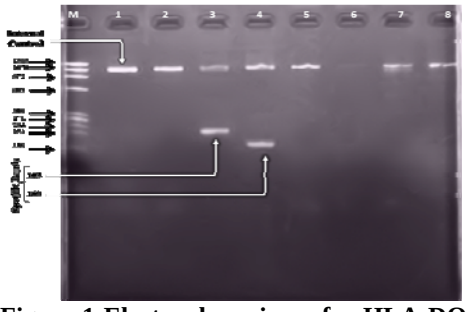

Figure-1:Electrophoresis of HLA-DQ alleles amplified by PCR-SSP of RAS patient.

Lane M represent [DNA-lenth standard (x174-HaeIII)] DNA ladar, lanes (1-8) represent class-II HLA- DQB alleles. Lanes (3,4) represent positive bands. This genotyping was obtained by using primers that detect the following alleles as they were present in the numbered wells,respectively:-wellNO.1=40GCC(5primer)220CGA(3primer)DQB1*

05allele;NO.2=75CGT(5primer)259GTA(3primer) DQB1*06 allele;NO.3=63ACA(5primer)198ATG(3primer) DQB1*02 allele; NO.4=77TTA(5primer)198ACT(3primer) DQB1*03:01 allele;NO.5=170TGC(5primer)250CTG(3primer) DQB1*03:02allele; NO.6=77TTA(5primer)141CGA(3primer) DQB1*03:10 allele; NO.7=77TCT(5primer)164TCG(3primer) DQB1*03:03 allele; NO.8=63ACC(5primer)198ATG(3primer) DQB1*04 allele.

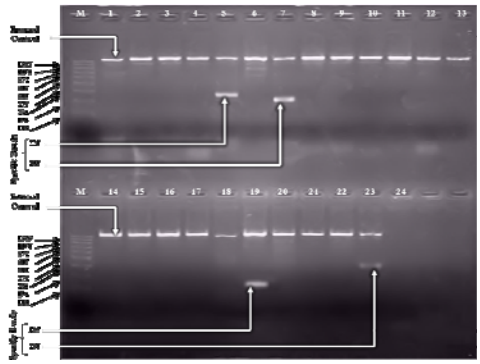

Figure -2:Electrophoresis of HLA-DR alleles amplified by PCR-SSP of RAS patient.

Lane M represent [DNA-lenth standard (x174-HaeIII)] DNA ladar, lanes (1-24) represent class-II HLA- DR alleles. Lanes (5,7,19,23) represent positive bands. This genotyping was obtained by using primers that detect the following alleles as they were present in the numbered wells,respectively:- well NO.1=42GAA(5primer)199GAG(3primer)DRB1*01:01 allele; NO.2=42GAA(5primer)199GAT(3primer)DRB1*01:03 allele;NO.3=39AGG(5primer)207TCT(3primer)DRB1*15 allele; NO.4=39AGG(5primer)199GAG(3primer)DRB1*16 allele; NO.5=38GTC(5primer)218GGC(3primer)DRB1*0301 allele; NO.6=48GAG(5p)218GGC(3P)DR*03:02; no.7=97GTC(5P)257CAC(3P)DR*04;NO.8=40ATA(5P)257CAC(3P)DR*07;NO.9=46GTT(5P)220CAG(3P)DR*08;NO.10=82TGC(5P)180CAG(3P)DR*09;NO.11=91GCG(5P)257CAC(3P)DR*10;NO.1232GCT(5P)173CCT(3P)DR*03:08;NO.13=174GAG(5P)211CTC(3P)03:08;NO.14=86GAC(5P)210CTG(3P)DR*12; NO.15=29GCT(5P)199GAT(3)DR*11;NO.16=38GTC(5P)169GCT(3P)DR*03:12;NO.17=38GCT(5P)209TCT(3P)DR*08;NO.18=174GCC(5P)211CTC(3P)DR01:01;NO.19=38GCT(5P)140GGA(3P)DR*03:01;NO.20=35TAC(5P)209TCC(3P)DR*04:24;NO.21=38GTC(5P)173CCG(3P)DR*03:01;NO.22=109AGA(5P)173CCG(3P)DR*03:01;NO.23=29GCT(5P)230AAT(3P)DRB3*01:01;NO.24=38GTA(5P)174TCA(3P)DRB4*01:01.

Table 2: HLA-A alleles genotyping in RAS cases in comparison to healthy control group.

	Controls		Cases(RAS)				P (Fisher's exact)
	N	%	N	%	OR	EF	
A*01:13,17:	**2**	**8.3**	2	**6.9**	**0.81**	******	**NS**
A*02:01:01:	1	4.2	1	3.4	0.82	**	NS
A*03:01:01:	2	8.3	0	0	0.15	**	NS
A*03:30	2	8.3	1	3.4	0.39	**	NS
A*11:43	1	4.2	1	3.4	0.82	**	NS
A*23:03:01	2	8.3	3	10.3	1.27	0.022	NS
A*24:02:01:	4	16.7	3	10.3	0.58	**	NS
A*24:77	0	0	1	3.4	2.58	0.021	NS
A*24:94	0	0	2	6.9	4.45	0.054	NS
A*25:01:01,01	0	0	1	3.4	2.58	0.021	NS
A*25:05	0	0	1	3.4	2.58	0.021	NS
A*26:09	1	4.2	0	0	0.27	**	NS
A*29:01:01:01	0	0	1	3.4	2.58	0.021	NS
A*31:03,04	2	8.3	0	0	0.15	**	NS
A*31:07,10	0	0	1	3.4	2.58	0.021	NS
A*32:17	0	0	1	3.4	2.58	0.021	NS
A*33:01:01-01	2	8.3	1	3.4	0.39	**	NS
A*33:19	1	4.2	0	0	0.27	**	NS
A*34:07	1	4.2	0	0	0.27	**	NS
A*36:01-05	1	4.2	0	0	0.27	**	NS
A*66:01,04,06	0	0	1	3.4	2.58	0.021	NS
A*66:03	0	0	2	6.9	4.45	0.054	NS
A*68:29	1	4.2	0	0	0.27	**	NS
Total	25	30	29	100			

Table 3: HLA-Cw alleles genotyping in RAS cases in comparison to healthy control group.

	Controls		Cases (RAS)				P(Fisher's exact)
	N	%	N	%	OR	EF	
C*01:17	1	4.2	1	3.4	0.82	**	NS
C*02:27	2	8.3	0	0	0.15	**	NS
C*03:03:04,13,18	1	4.2	0	0	0.27	**	NS
C*03:04:02,04:06	1	4.2	0	0	0.27	**	NS
C*03:27,38:01	1	4.2	0	0	0.27	**	NS
C*03:38:02	2	8.3	1	3.4	0.39	**	NS
C*04:01:01:01	0	0	1	3.4	2.58	0.021	NS
C*05:10 C*05:23	3	12.5	5	13.8	1.12	0.015	NS
C*05:26	2	8.3	2	6.9	0.81	**	NS
C*06:02:01:0	1	4.2	1	3.4	0.82	**	NS
C*06:17/*12:09	1	4.2	1	3.4	0.82	**	NS
C*07:02:01:01-02	0	0	1	3.4	2.58	0.021	NS
C*07:05,08,14,	0	0	1	3.4	2.58	0.021	NS
C*07:07	1	4.2	2	6.9	1.70	0.029	NS
C*07:09	0	0	1	3.4	2.58	0.021	NS
C*07:12,41	1	4.2	1	3.4	0.82	**	NS
C*08:01:0102:2	1	4.2	0	0	0.27	**	NS
C*08:05,21	0	0	1	3.4	2.58	0.021	NS
C*08:28	0	0	1	3.4	2.58	0.021	NS
C*12:02:01-	0	0	5	17.2	11.00	0.156	0.041
C*12:14:02	1	4.2	0	0	0.27	**	NS
C*12:21,28	1	4.2	1	3.4	0.82	**	NS
C*15:02:01-0	1	4.2	1	3.4	0.82	**	NS
C*16:02:01,02:0	1	4.2	0	0	0.27	**	NS
B*40:76	0	0	1	3.4	2.58	0.021	NS
C 87	2	8.3	1	3.4	0.39	**	NS
Total	25	100	30	100			

Table 4: HLA-DR alleles genotyping in RAS cases in comparison to healthy control group.

	Controls		Cases (R AS)				P(Fisher's exact)
	N	%	N	%	OR	EF	
DRB1*01:03	4	16.7	6	20.7	1.30	0.048	NS
DRB1*01:23w,	0	0	1	3.4	2.58	0.021	NS
DRB1*16:01:	1	4.2	2	6.9	1.70	0.029	NS
DRB1*04:01:0	0	0	1	3.4	2.58	0.021	NS
DRB1*04:02,1	2	8.3	2	6.9	0.81	**	NS
DRB1*08:01:0	2	8.3	3	10.3	1.27	0.022	NS
DRB1*09:01:0	3	12.5	2	6.9	0.52	**	NS
DRB1*11:01:0	1	4.2	1	3.4	0.82	**	NS
DRB1*11:45	3	12.5	1	3.4	0.25	**	NS
DRB1*13:01:0	0	0	1	3.4	2.58	0.021	NS
DRB1*13:01:0	0	0	1	3.4	2.58	0.021	NS
DRB1*13:05:0	1	4.2	1	3.4	0.82	**	NS
DRB1*13:37,7	1	4.2	1	3.4	0.82	**	NS
DRB1*13:55	2	8.3	0	0	0.15	**	NS
DRB1*13:60,8	1	4.2	3	10.3	2.65	0.064	NS
DRB1*14:01:0	1	4.2	1	3.4	0.82	**	NS
DRB1*14:19	1	4.2	1	3.4	0.82	**	NS
DRB1*14:24.3	1	4.2	1	3.4	0.82	**	NS
Total	25	100	30	100			

Table 5: HLA-DQ alleles genotyping in RAS cases in comparison to healthy control group.

	Controls		Cases (RAS)				P (Fisher's exact)
	N	%	N	%	OR	EF	
DQB1*05:01:0	4	16.7	0	0	0.08	**	0.036
DQB1*06:01:0	2	8.3	3	10.3	1.27	0.022	NS
DQB1*06:02:0	3	12.5	2	6.9	0.52	**	NS
DQB1*02:01:0	5	20.8	14	48.3	3.55	0.347	0.028
DQB1*03:01:0	3	12.5	2	6.9	0.52	**	NS
DQB1*03:02:0	3	12.5	2	6.9	0.52	**	NS
DQB1*03:03:0	2	8.3	3	10.3	1.27	0.022	NS
DQB1*03:04,1	0	0	1	3.4	2.58	0.021	NS
DQB1*03:10	2	8.3	0	0	0.15	**	NS
DQB1*04:01:0	0	0	2	6.9	4.45	0.054	NS
Total	25	100	30	100			

Discussion:

The role of genetic factors in the etiology of RAS was documented many decades ago. As a result, the investigative efforts were focused on the genetic markers of susceptibility to this disease. Moreover, the high familial incidence of RAS suggests the possibility of a linkage or an association of disease with HLA. In the current study positive family history was observed in 43% of patients with RAS. The high rate of a positive family history in the present study raises the possibility of a genetic basis for RAS transmission (17). The present work revealed a significant association of HLA- Cw*12:02:01-alleles with RAS patients (p= 0.041), as compared with healthy control. Different results regarding this association was reported, results differ in different population. In Brazilian study conducted by Wilhelmsen and colleagues (18), observed that high frequencies of HLA-A33, HLA-B35 and HLA-B8 were found in patients with RAS as compared to healthy control. Correspondingly Malmstr◌m and co-workers also noticed that HLA- HLA-B12 frequency was higher when they studied 14 Finish patients with RAS (9). In 2001, twenty two Israeli Arab patients with RAS were studied, a statistically significant increase in HLA-B52 and B44 molecules in patients with this disease was observed when compared to the control group (19). The HLA-B51 frequency has been found in some reports associated with RAS in countries like Israel and Korea (20 and 21). In this study no differences in frequencies of HLA-DR alleles with RAS patients were shown as compared with control group, this result was in contrast to that reported by other authors (12 and 22) in which there were a positive significant association between this disease and HLA-DR.An interesting finding in this study was the higher expression of HLA- DQB1*02:01:0- in RAS patients. This results is at variance with some other studies (10, 12 and 23) which lack such association between HLA- DQ and this disease, while reporting positive association with other antigens of HLA- class II. For instance Gallina and colleagues, (10) studied a sample of 26 Sicilian patients with RAS, they found a statistically significant increased value for HLA-DR7 frequency. Meanwhile another study revealed an increased frequency of HLA-DR5 and decreased HLA-DR4 in Greeks patients with RAS (12). Similarly Sun *et al.,* stated that the HLA-DRw9 frequency can be considered a genetic marker for RAS in the Chinese population. Their study was made up of 80 Chinese patients with RAS. It is very important to point out that, as mentioned by Louzada-Junior and associates (23) the reduced frequency of HLA typing could be considered as a protective factor for RAS. The current study found decrease frequency of HLA-DQB1*05:01:0 allele in RAS patients when compared to controls.

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