Research Article

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Genotyping of HLA-DRB1 by PCR-SSP in Javanese patients with recurrent aphthous stomatitis.

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Recurrent Aphthous Stomatitis (RAS) is the most common inflammatory ulcerative condition of the oral mucosa. Association of genetic factor and recurrent aphthous stomatitis has been implicated by numerous studies and this can be determined by HLA subtypes. The present study was conducted to determine the possible association of Class II alleles with recurrent aphthous stomatitis in Javanese patients. The study included 63 subjects i.e. 42 subjects were suffering from recurrent aphthous stomatitis and 21 healthy subjects were included as control. Polymerase chain reaction-specific sequence primers (PCR-SSP-Morgan TM) assay was conducted to assess HLA-typing. Genotyping of subjects for HLA class II revealed that HLA-DRB1*15:01:01:01; DRB1*15:01:01:01-6; HLA-DRB1*3:01:01:01 and DRB1*07:01:01:01 genes were predominant with high frequency alleles in RAS patients. The present study showed a significant association of DRB1*16:01:01:05/DR16 alleles with RAS as compared with control subjects. In conclusion, this study demonstrated that gene HLA-DRB1 has predominant allele and has important role in etiology of the disease especially in the Javanese people.

Key words: RAS, HLA alleles, PCR-SSP, Aphthous stomatitis.

Recurrent aphthous stomatitis (RAS) was oral soft tissue disorder with round or oval ulcer shaped, severe pain and of recurrent without any systemic disease. Various therapeutic strategies or treatment efforts have been conducted to prevent the relapse of the disease, but it still cannot be optimally solved. It is because the definitive factors of RAS were still unclear (Sonis, 2005; Preeti L et al. 2011). The prevalence of RAS was increased, as evidenced from the results of the study showed the number varies between 17 % - 66 % of population (Scully, 2008, Barros et al. 2010). Various therapies have been made to reduce the recurrence by improving the early diagnosis or treatment, prevention and treatment but the problem cannot be solved satisfactorily because the pathogenesis of RAS is still not fully understood (Mimura et al. 2009). The interaction of various internal factors such as a genetic system, the immune system and environmental factors make a major contribution to the understanding of the pathogenesis of this disease through research that has been done (Wilbelmsen et al. 2009).

HLA system can be a good genetic marker (genetic sign) because it can be found from birth until the individual dies and composition of HLA type system has not changed. Examination of the HLA system gives enormous possibilities to predict the tendency of a person suffering from an illness (Gallinaa et al. 2005). The focus of this research was to explore the types of molecules HLA class I and HLA class II as a susceptibility gene expression or association which has a significant correlation with the occurrence of RAS and get immunogenic HLA receptor - specific RAS populations of people in Indonesia, especially Java.

MATERIALS AND METHODS

This study used exploratory observational and

analytical methods within the scope of genetic research, because HLA antigen was the expression of gene on chromosome number 6, so it needs characteristic approach in genetic field. Observed subjects were the people who have carried out the examination for the clinical diagnosis of RAS and normal subjects. Thus the study individuals can be classified into two groups: the RAS (major, minor) and the control group.

Population, samples and sampling technique

Study Population: All patient came to the Hospital (Oral Dentistry Hospital) and Oral Medicine Department of Dentistry Faculty Airlangga University with a clinical diagnosis of Recurrent Aphthous Stomatitis (RAS). Sample: The samples were RAS-type major, minor, and non-RAS patients as controls. People with control samples taken from the one who have never suffers the RAS. Sampling technique, sampling was carried out in a total population of over 5 months from April 2013 to August 2013 and must meet the criteria for inclusion or exclusion

Inclusion criteria

- Patients with RAS cases with good general health (not suffering from diabetes mellitus and other systemic diseases)

- Patients who ever had ulcer at least three times or more a year on a periodic

- Oral mucosa without other abnormalities or inflammation except RAS lesions.

- Ulcer diameter between 3-10 mm.

- Non-RAS patient with good general health (no systemic disease). Healthy oral mucosa, absence of inflammation or other lesions. Patients have been willing to participate in the study after receiving an explanation of the objectives and procedures of detailed study by signing a letter of approval (informed consent).

- Patients born in Indonesia, Indonesian parent (Java)

Exclusion criteria

- Patients who have ulcers in the oral cavity caused by manifestations of systemic disease.

- Patients who do not accord the criteria for RAS

- Criteria group normal population / Non-RAS (Control) : The subject were healthy individuals, never suffered from RAS. Subjects were Indonesian willing to participate in the study after receiving an explanation of the objectives and procedures of detailed study by signing a letter of approval (Informed consent).

Research procedures

Determining clinical characteristic of RAS samples: RAS Patients who come to the Hospital and Clinic of Oral Medicine, Faculty of Dentistry Airlangga University were examined and subsequently recorded based on their history of the disease, the phase of the lesion, lesion diameter, lesion number, gender, frequency of recurrence, as well as triggering factors. Patients who have the inclusion and exclusion criteria were asked to sign a consent form (informed consent). This consent form was made specifically for research on the family or the patient related to this research, after that patient was informed about the research.

Collecting blood samples

Blood sampling in RAS patients and non-RAS subjects was done by taking 5 ml of venous blood. The blood was then transferred in test tube having 1 drop of heparin 5000 IU / ml. For collection of serum proteins with RAS and non-RAS, serum separation process was done by putting the 5 ml blood at room temperature (25 °C). The samples were then centrifuged at 1600 rpm for 10 minutes at a temperature of 4°C. Finally, each sample was separated in a sterile eppendorf and then stored at -70 °C until further processing.

Examination stage of HLA - DRB Isolating blood

Peripheral blood leukocytes was the primary sample for DNA extraction therefore it's easier and less invasive than other tissue cells in the human body. A material used in this study was the blood of patients with RAS and normal subjects (non-RAS). Blood sample was taken from cubital vein (3 cc), and accommodated in vacutainer having EDTA.

DNA extraction procedure (Method of High Pure PCR Template Preparation Kit (RocheCat.11796828001).

Reagents for DNA extraction: sucrose, triton X-100, $MgCl_2$, Tris HCL, Guanidine thiocyanate.

DNA Extraction Procedure: Take 200 ml of whole blood, add 200 ml of PBS, and then incubated for 5 minutes. Add 40 ml of protease / proteinase K, and incubation in an incubator at 70°C for 10 minutes. Then added 100 ml of isopropanol vortex entered in spin coloum and centrifuge at a speed of 8000 g for 1 minute. Then added to 500 mL inhibitor remove buffer and then centrifuged 8000 g for 1 minute, , then add another 500 mL wash buffer and then centrifuged at a speed of 8000 g for 1 minute. Supernatant was discarded and then added 500 ml washing buffer and then centrifuged at a speed of 13000 g. and finally eluted with 200 mL of DNA entering 70°C with buffer AE, after 1 min centrifuged at a speed of 8000 g. Replace a new tube and add 200 mL elution buffer, and then incubated at least 1-2 minutes and centrifuged at a speed of 8000 g for 1 minute. DNA extraction was then used as the basis of determining the amount of DNA in the HLA-DRB1 locus amplification and HLA-DQB1 by PCR.

Characterization of degree of DNA purity was tested with spectrophotometer at 260 nm and 280 nm wavelength. The DNA molecule was pure if both ratios of these values were more than one (range from 1.8 to 2.0).

DNA / PCR amplification (The Morgan ^{MT} SSP HLA - DRB Typing Kit).

Materials for PCR : Taq DNA Polymerase (5U/ mL); Distilled water or DNA diluents; adjustable pipets; pipets disposable tips; vortex mixer; micro centrifuge; centrifuge table top for 96 well PCR plates; cover with heated thermal cycle (Applied Biosystems Gene Amp 9600 and 9700); pressure pad.

Procedure: Prepare samples for PCR buffer dropped to 24. Components for the final reaction volume 12 mL ; Take a 0.2 ml PCR tube and enter the master buffer 260 mL 1.6 mL polymerase add tags , submit to contamination control (10 µl) plus 2 µl of distilled water / nuclear Free water , then the rest of the master mix and add 50 µl of DNA samples were then distributed 12 µl into each well (23 well i.e. B2 H24 till then vortex , spin down and insert the tray (24 well) and then insert it into a thermal cycler with the following amplification conditions : Initial denaturation : 95° C for 2.5 min ; denaturation : 95 °C for 15 seconds. Annealing: 60°C for 60 seconds, do as many as 10 cycles. denaturation: 95°C for 15 seconds. Annealing: 62° C for 50 seconds. extension: 72° C for 30 seconds. Do as many as 22 cycle. PCR products were stored in 4° C

Gel electrophoresis

Preparation of 2% agarose gel: Weigh as much as 0.8 g of agarose and dissolved in 40 ml of 0.5 x TBE buffer (2% concentration). Preheat in the microwave until the solution got clear, let it cool, and then add 2 mL ethidium bromide. Pour the agarose in into a mold and then allow it to solidify.

Procedure: Prepare paraffin, pipettes 1 µl, stop blue (loading dye) and put it on para film. Pipette 2 ml sample and mix with loading dye by pipetting. Load the samples in the agarose gel, along with molecular weight marker. Perform electrophoresis until the sample line move closer to the end of the gel. Document the gel using documentation system.

Analysis with software from The Morgan ^{TM} Typing Kit: Gel documentation interpretation was to examine the positive line, specific primers used between 70-275 bp, then mark the positive line on the paper work (DRB Locus Worksheet). The final analysis to determine the specificity of the HLA alleles using software from The Morgan ^{TM} Typing Kit.

RESULTS

Recurrent Aphthous Stomatitis (RAS) Clinical Characteristics: In this research there were 63 subjects including RAS patients and non-RAS. The results of the clinical examination described that 42 subjects suffer from RAS (30 female patients and 12 male) and other 21 subject were included in control group (non-RAS). It is clear from table 1 that patients with RAS ranged between 10-59 years of age. RAS was more prevalent in women (40 subjects or 80.41 %) as in male patients (12 patients or 10.59 %). Patients aged 20-29 years were 22 or 50.17% of the cases of RAS and was the group with the highest frequency in both women and men.

Table 1: Profile of RAS patients and control subjects

	RAS cases						Kontrol (Non-RAS)		
Age / years	**Mayor**			**Minor**					
	M	F	T	M	F	T	M	F	T
10-19	2	3	5	1	3	4	1	1	2
20-29	4	5	9	1	12	13	6	9	15
30-39	2	2	4	1	2	3	2	2	4
40-49		1	1	1	1	2	-	-	-
50-59		1	1	-	-	-			
Total	8	22	**20**	4	18	**22**	9	12	**21**

The results of the interview with 42 subjects with RAS revealed that they were having RAS since 2-10 years. The frequency of recurrence can occur every year between 3 times to 12 times, so some of them can be expressed as never free of ulcers. An illustration can be seen in figure 1 below.

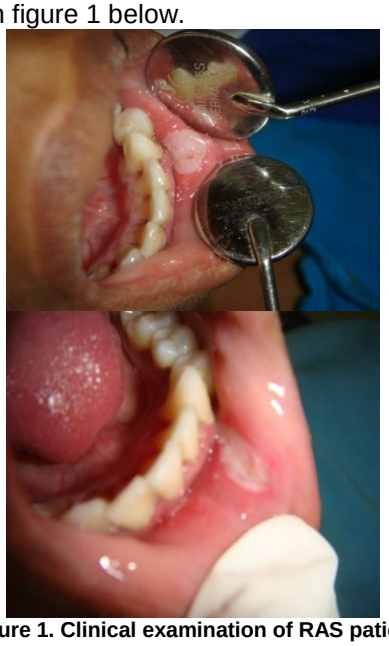


Figure 1. Clinical examination of RAS patient

Results purity detection of DNA can be seen in attachment

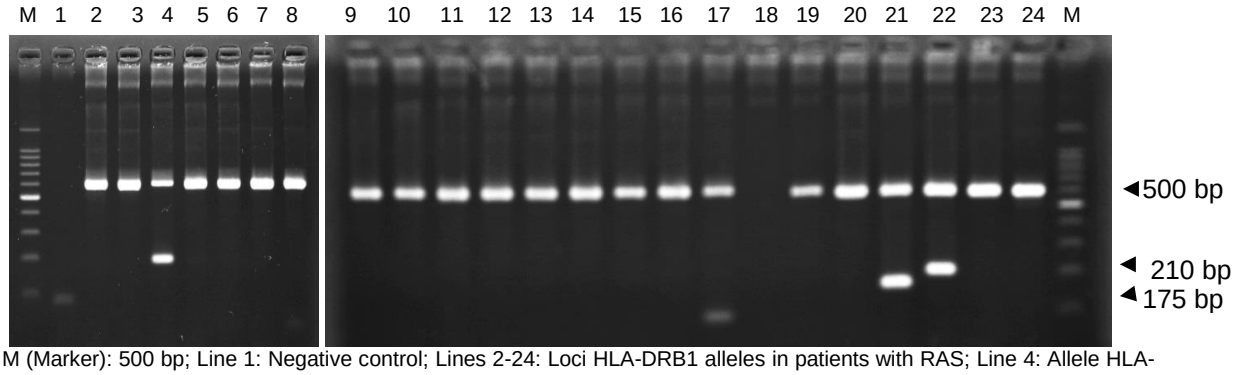
The DNA isolate result detect its allele type by drop it in the special tray from The Morgan MT HLA - SSP on each well (96 well) have been exposed to this type of allele - specific primers. Then performed by PCR processing tool using Applied Biosystems Gene Amp 9600 and 9700. Results of PCR of each sample was expressed in bp using 100 bp ladder as marker (Promega No. G2101).

Examination of HLA - DRB1 locus was carried through the isolation of DNA from blood samples of patients with RAS and non-RAS. DNA isolation result with the High Pure PCR Template Preparation Kit from Roche Cat.1179682801 with DNA purity approximately 1.7 to 2.00 (results attached) , This examination was used as a reference for determining the amount of DNA used in DNA amplification by PCR . PCR results of each sample are expressed through the stages Gel electrophoresis and exposure to visible image can be assessed with a marker Ladder 500 bp, as shown in Figure 2.

Electrophoretic result of HLA-DRB locus in figure 2 indicates that the Human Leukocyte Antigen expression in RAS patients on DRB loci appear to be expressed more (positive) than HLA-DRB gene in RAS patient as seen in Figure 3.

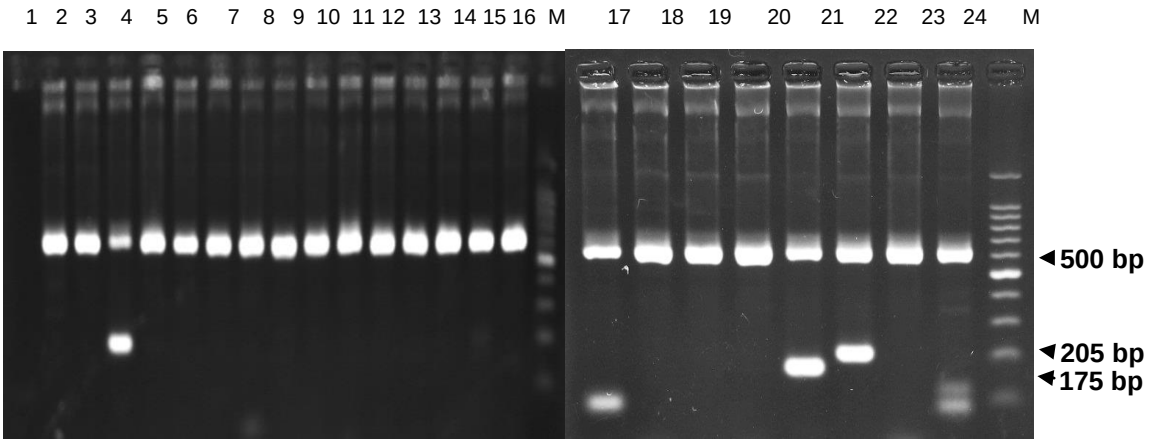
Electrophoresis results of PCR-SSP products indicate that there is no Human Leukocyte Antigen expression on Non RAS patients (figure 4). But therewere 2 strongly expressed (positive) band at the HLA-DQB significant difference with some gene alel in patients with RAS, whereas the HLA-DRB alleles showed significant difference with the absence of some positive band of the HLA-DRB

Figure 2. Electrophoresis of HLA-DRB1 locus in patients with RAS (Ahm).



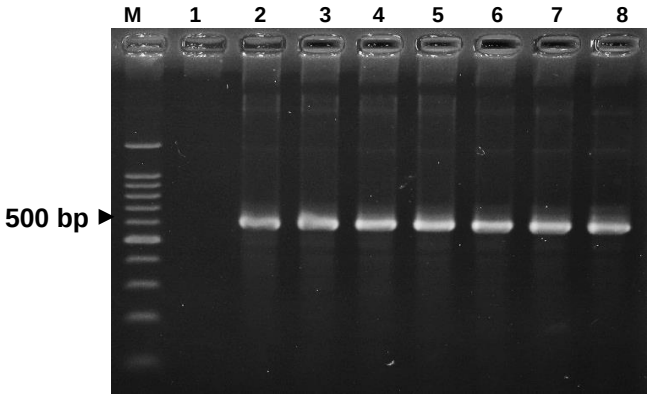
M (Marker): 500 bp; Line 1: Negative control; Lines 2-24: Loci HLA-DRB1 alleles in patients with RAS; Line 4: Allele HLA-DRB1 * 15:01:01:01 (200 bp); Line 21: Allele HLA-DRB1 * 15:01:01 (175 bp); Line 22: Allele HLA-DRB1 * 03:01

Figure 3. Electrophoresis results of HLA-DRB1 locus in patients with RAS.



M (Marker): 500 bp; Line 1 : Control negative; Line 2-24: Loci HLA-DRB1 alleles in patients with RAS; Line 4 : Allele HLA-DRB1 *15:01:01:01 (200 bp); Line 21 : Allele HLA-DRB1 *15:01:01 (175 bp); Line 22 : Allele HLA-DRB1 *03:01 (205 bp).

Figure 3. Electrophoresis Results of the HLA-DRB1 locus in non-RAS patient.



M (Marker): 500 bp; Line 1: Negative control; Rows 2-8 showed no positive bands on HLA-DRB1 gene loci. This indicates that some genes HLA DRB alleles did not show.

DISCUSSION

Susceptibility to some diseases (RAS) is known to be associated with the HLA locus, because the loci HLA (human MHC) has important role in the immune system, and greatly influence the genetic susceptibility of various diseases mediated immune response. HLA (Human leukocyte Antigen/ MHC in humans) has highly polymorphic in different individuals that cause differences in the immune response to the same agent (Abbas et al.2008;. Wilbelmsen et al. 2009). Although it was generally known that a genetic factor is one of the predisposing factors of RAS factors other than bacterial infection, viral or immune system disorders (immune disorders).This research focus in association between HLA serotypes and RAS, but the results were very variable and the differences of the results may

occur because the study subjects taken from a different race or ethnicity so its cause the different distribution of HLA type that will lead to different result of the association of HLA and RAS will be different on every ethnic group / race. Secondly, HLA phenotype can be classified into several subtypes that were phenotypically cannot be differ with serological examination. Examination method of HLA- DRB system use DNA typing - PCR test sequence specific primers test (Morgan TM HLA SSP DRB Typing Kit) and the results will be analyzed using software from Morgan TM HLA SSP Typing, then detection of gene alleles will be analyzed with fisher 's exact probability (P). Using PCR - SSP test (Morgan HLA SSP DRB Typing Kit) has found several bands in patients with clearly expressed RAS / positive with length 175 bp , 200 bp and 205bp. HLA - DRB1* Gene have some alleles that were dominant and high frequency in patients with HLA - DRB1 RAS * 15:01:01:01 ; DRB1 * 15:01:01:01-6 , HLA - DRB1 * 3:01:01:01 and as well as DRB1 * 07:01:01:01 gene . In patients with non- RAS there were some alleles HLA DRB1* 08:20; HLA-DRB1*11:01:01 (DR8; DR11;DR13); DRB1* 03:01 (DR17;DR1, the one that has significant differences were DRB1 * allele gene 16:01:01:05 / DR16 (2).

PCR examination on HLA-DRB1 locus on RAS and non-RAS patient was a method or an accurate diagnostic method with high accuracy to determine the susceptibility gene locus that contributes to a disease. In addition, most documented factor in research was hereditary factor. In the study involving 1303 children from 530 families, found the presence of increased susceptibility to the RAS in children whose parents were people with RAS. Patients who had parents with RAS up to 90% risk of developing RAS, whereas patients whose parents were never exposed to RAS only risk 20%. Furthermore, human leukocyte antigen (HLA)-specific genetically turns identified in RAS patients, especially in certain ethnic groups. (Wilbelmsen et al, 2009). There were also studies linking the RAS minor genetic factors related to immune function, especially genes that control the release of interleukin (IL)-1B and IL-6. It is supported on the sample characterization in patients with type RAS patients who have a major or minor genetic background (Albanidou et al. 2004; Gallinaa et al. 2005; Sun et al. 2000). Susceptibility to several diseases known to be associated with the HLA locus, since this locus was polymorphic and associated with changes in the immune response. This study proves that patients with RAS (major or minor) was derived and related to HLA. An increase and a decrease in the frequency of HLA alleles of RAS signaling that the disease was closely relation to genetic factors

In conclusion, Gene HLA-DRB1 * have some dominant and high frequency aleles in RAS patients with HLA-DRB1 * 15:01:01:01; DRB1 * 15:01:01:01-6, HLA-DRB1 * 3:01:01:01 and as well as DRB1 * 07:01:01:01 gene. In non-RAS patients obtained with the HLA-DRB1 * 8:20; HLA-DRB1 * 11:01:01 (DR8; DR11; DR13); DRB1 * 03:01 (DR17; DR1, while that has significant differences were alel gene DRB1 * 16:01:01:05 / DR16 (2). HLA-DRB1 gene characteristic have dominant alel frequencies involved in RAS patients especially Javanese population.

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