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A Preliminary Study on the Use of Unaffected Siblings for the Marker Allele Frequency of Sib-Pair Linkage Analysis

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We have searched the disease-susceptibility genes of rheumatoid arthritis (RA) using fluorescence-based microsatellite marker analysis and affected sib-pair linkage method. One unaffected sibling was typed in addition to affected sib-pairs, since most parents were not typed due to late onset nature of the disease. In order to verify appropriateness of using unaffected siblings for the calculation of marker allele frequency of the population, we have compared the calculation of marker allele frequency based on unaffected siblings of patient families with that based on unrelated healthy control. Chi-square test showed no statistically significant difference between them. It thus appears that unaffected siblings can be used for the calculation of marker allele frequency especially when unaffected siblings are selected carefully by paying attention to the penetrance of disease.

Key Words

Sib-pair analysis,
Marker allele frequency,
Disease susceptidility gene,
Rheumatoid arthritis,
Microsatellite marker analysis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic symmetric polyarthritis of unknown etiology that affects -1% of the population worldwide.[1)] The risk of the disease in the siblings of affected individuals is, however, much higher at 8%,[2)] suggesting that genetic factors may be important as a cause of familial clustering.[2,3)] We have searched the human genome for genes that predispose to RA using fluorescence-based microsatellite marker analysis and affected sib-pair linkage study.[4)]

We have typed one unaffected sibling in addition to the corresponding affected sib-pair, since parents were not available for typing in most of the RA families at the time of the diagnosis of RA because of late onset nature of the disease. Since there exist a little concern about the appropriateness in typing unaffected siblings of patients with RA and using their data for the calculation of the marker allele frequency in the population, we have compared the calculation of the marker allele frequency based on unrelated siblings of patient families with that based on unrelated healthy controls. Improved practical conditions for polymerase chain reaction (PCR) for the family-based large scale micro-

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satellite marker analysis is also introduced.

METHOD

Study Design Microsatellite markers were amplified by the polymerase chain reaction (PCR) using fluorescence-tagged primers and sized based on the difference of CA repeats on DNA fragments using ABI 377 sequencer operated under the software GENESCAN™. The DNA fragments were properly sized by lane-to-lane gel comparison and automatic allele calling using GENOTYPER™. Linkage analysis was made using the computer program SIB-ADONE which was developed by us[5] according to the method of Holmans & Clayton.[6] This approach was based on the IBD (identity-by-decent) sharing states of the affected pair only, using the unaffected siblings to infer parental genotypes, and was expected to yield a close approximation to the full likelihood especially when penetrance was low.

Family studies Japanese 41 families including affected sib-pairs who met the diagnostic criteria of the American College of Rheumatology (formerly, the American Rheumatism Association[7]) with clear and identifiable joint destruction of more than stage 2 of Steinbroker's X- ray classification[8] were studied. We paid attention to that unaffected siblings in a family should be older than at least one of the affected sib-pair within the family and if unaffected siblings were still younger than corresponding sib-pairs, such families were excluded unless the age of the unaffected siblings was older enough than the age of disease onset of corresponding affected sib-pair. Seven out of 41 families were of this type and the youngest unaffected sibling in these families was 50 years old. Peripheral blood (10ml) was withdrawn using EDTA and gently mixed with 20ml of buffer I (0.32M sucrose, 1%v/v Triton X-100, 5mM $MgCl_2$, 12mM Tris HCl (pH 7.6)) to lyse cell membrane. After centrifugation, the precipitate of nuclei was reacted with buffer II (4M guanidine thiocyanate, 12mM EDTA, 375mM NaCl, 0.5% sodium N-lauroyl sarcosinate, 0.1M β-mercaptoethanol, 12mM Tris HCl (pH7.6)) at 55 ℃ for 10min to lyse nuclear membrane. Genomic DNA was then extracted by ethanol precipitation.

DNA sizing[9] Fluorescence-tagged primers for 358 microsatellite markers that define a 10.8cM resolution human index map were purchased from PE Applied Biosystems-Roche Molecular Systems, Inc. (Branchburg, New Jersey). However, the markers D1S502 and D6S344 were not used because of technical difficulty in amplifying their DNA and the marker D6S276 was substituted to D6S299, D6S265 and D6S273 to specifically examine the HLA-D region. Other microsatellite markers were newly synthesized according to the published primer sequences[10] (Biologica Co., Nagoya, Japan).

PCR was performed in 96-well microtiter plates (# 6511, Corning Coster Corp., Cambridge, MA) in a 15μl volume containing 30ng of DNA, 0.2μM of primer mixture, 0.2mM of each dNTP, 1 unit of AmpliTaq Gold DNA polymerase (PE Applied Biosys-

tems), 2.5mM $MgCl_2$ and 1x PCR buffer II (PE Applied Biosystems). PCR products labeled with 6-FAM, TET or HEX were mixed together with TAMURA-labeled size standards and applied on a 36cm-well-to-read gel (4% acrylamide/ 6M urea) plate. Electrophoresis was carried out in a ABI 377 sequencer (PE Applied Biosystems) at 3,000V for 2hr. The peak, height and area of DNA fragments were identified using the GENESCAN (Ver.2.0.0) computer analysis software (PE Applied Biosystems) with reference to TAMURA-labeled size standards. The DNA fragments were properly sized by lane-to-lane gel comparison and automatic allele calling using the data analysis and transformation software GENOTYPER™ (Ver.1.1) (PE Applied Biosystems).

RESULTS AND DISCUSSION

Amplification conditions appropriate for the PCR in a MJ Research PTC-100 thermocycler using hot bonnet was found to be 94℃ for 10 min followed by 27 cycles of 94℃ for 30 sec, 55℃ for 1 min, and 72℃ for 2 min, and 72℃ for 5 min after the last cycle. $MgCl_2$ at 2.5mM was most efficient. Using this amplification condition, we could amplify total 358 microsatellite markers except D1S502 and D6S344. Since these two markers were highly resistant to any amplification conditions used in this laboratory, we had an impression that the primers for these two markers might be defective. However, except for the two markers, amplified DNA was efficiently sized. Figure 1 shows a representative elec-

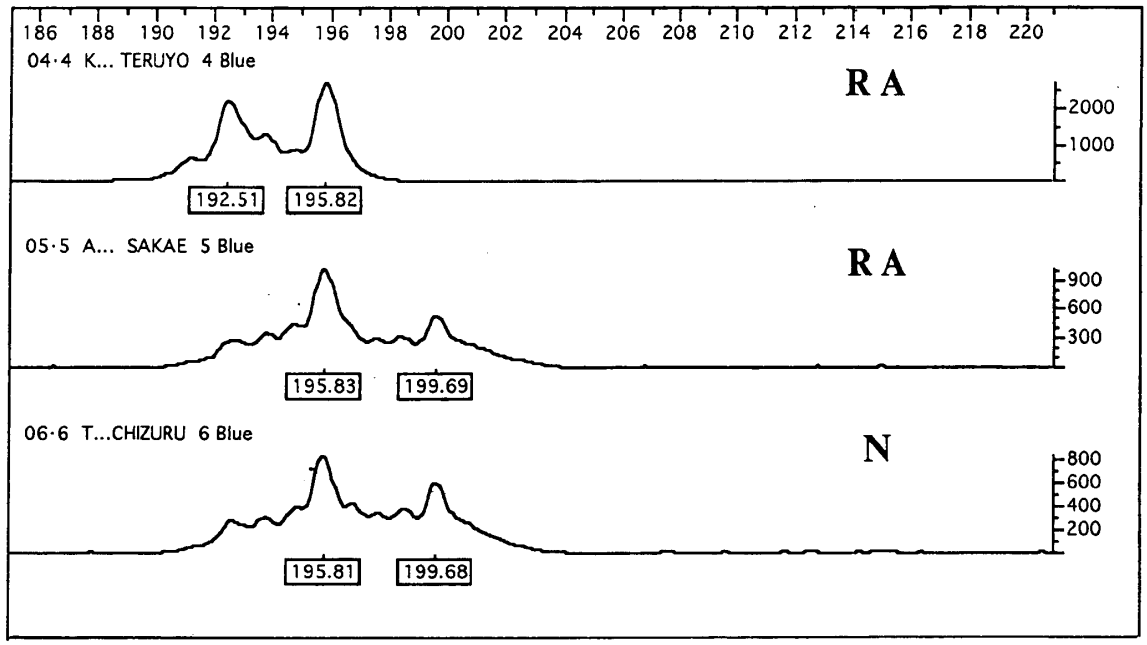


Figure 1. Data obtained by electrophoresis operated under the control of computer program GENESCAN are properly sized using GENOTYPER software. Sharing of DNA by three siblings are clearly identifiable using this technique.

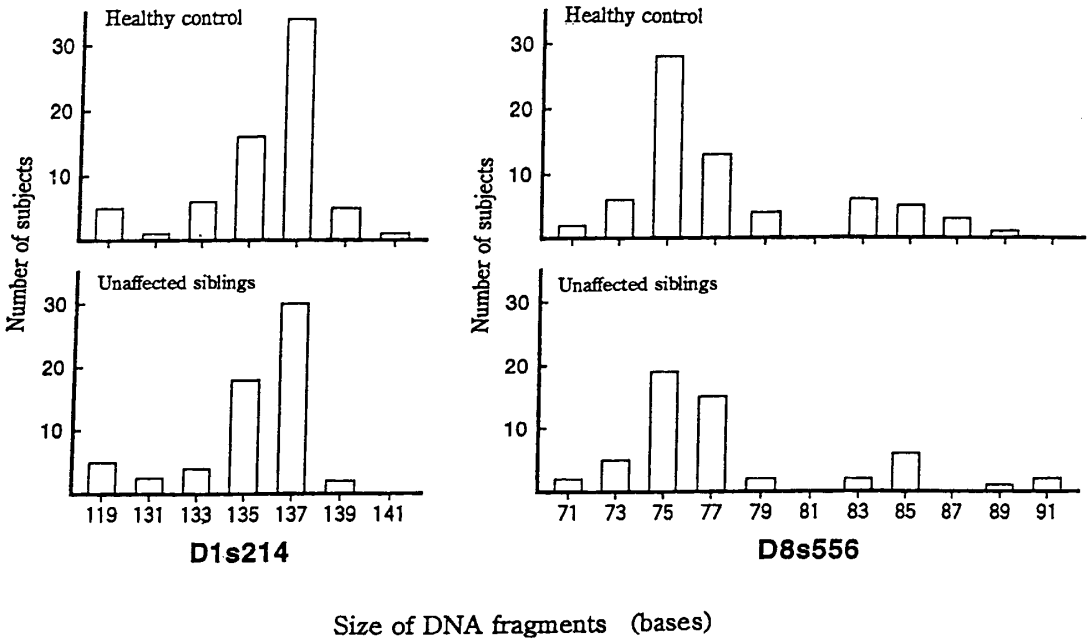


Figure 2. Marker allele frequency for microsatellite analysis. Comparison between unrelated healthy control and healthy siblings of patients with rheumatoid arthritis is shown for chromosome loci D1S214 and D8S556.

trophoresis illustrating that separation has been carried out efficiently to facilitate following process of proper DNA sizing.

Previous studies expressed some concern about the appropriateness of typing unaffected siblings of patient families and using their data for the calculation of the marker allele frequency in the population.[11] We therefore wished to verify whether or not the use of healthy siblings of the patient families with RA was appropriate for the calculation of the marker allele frequency in the population. We have also paid a very careful attention in collecting unaffected siblings from the families of RA patients by using the criteria that unaffected siblings should be older than at least one of the affected sib-pair within the family and if unaffected siblings were still younger than corresponding sib-pairs, such families were excluded unless the age of the unaffected siblings was older enough than the age of disease onset of corresponding affected sibpair.

In order to compare the result of calculation, we have examined three principal regions of linkage that we have shown to contain three candidate genes that predispose to RA,[4] chromosome loci D1S214, D8S556, DXS1047 and DXS1227. Chi-square test showed that there were no statistically significant differences between the marker allele frequencies calculated based on healthy siblings of patient families and the marker allele frequencies calculated based on unrelated healthy controls in the chromosome loci D1S214, D8S556, DXS1047 and DXS1227. Figure 2 shows the result for D1S214 and D8S556. The result therefore supports our current calculation[5] for the disease-susceptibility genes of RA and

consequently substantiates our previous identification of three gene loci at D1S214, D8S556 and DXS1047-1227 as the gene loci that predispose to RA[4] under the criteria of the lod score being more than the prescribed level 3, which was generally acceptable for the linkage analysis. One of the major reasons that unaffected siblings of patient families showed the distribution identical to those of unrelated healthy control would be that we have chosen unaffected siblings by paying a very careful attention to their age: the problem of penetrance has been ruled out by the method used in the present studies. The present result therefore supports the appropriateness of incorporating data of unaffected siblings to the sib-pair linkage method.

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