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Analysis of Fra-X Gene Using Hair Root DNA

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Abstract: Extract of DNA for analysis of fragile X syndrome is usually performed by blood, the researches using hair root as specimen have been gradually spread. In this study, analyze fra X gene of the patients in mentally retarded children facilities was conducted using hair root DNA with molecular biologic test (PCR). The number of total subjects was 24, boys were 12, the average age was 17(±3), and girls were 12, the average age was 18(±2). In girls, normal size of band of 222 bp appeared in all lanes. Also, in all lanes except control in 517 bp, micro band appeared. Moreover, with appearance of band of 1198bp in lanes 2, 3, 4, 5, it is estimated that it is the band of full mutation whose CGG repeated sequences are more than 200. But it showed the peculiarity that it appeared with normal band in all the same lanes, thus it is not reasonable to judge it is the band of full mutation and further studies are needed. These results appeared in 50%, 6 of 12 mentally retarded girls. As the result of mentally retarded boys, normal band appeared in about 222 bp in control, however in experiment group, normal band did not appear. In 43%, 7 out of 12 boys, band did not either appeared in 1198bp, which showed different patterns from that of girls.

Keywords: fra X gene, hair root, DNA, PCR

Introduction

The research using human's hair follicle has been used in biochemical studies since 1970s. That is, it has been used to diagnose inborn errors of metabolism, female carriers of X-linked disorders, deficiency of glucose-6-phosphate dehydrogenase and Fabry Disease etc.[1-3]

Fragile X syndrome features the impediment in speech, motion and social ability in growth phase with stoppage or imperfection of psychogenesis.

This has been known as one of the most common causes of mental retardation with Down syndrome, which has the frequency of 1 out of about 4×10^{-4} ~6×10^{-4} boys or girls.

The diagnosis of fragile X syndrome has depended on chromosome test,[4] however, (FMR1) gene region linked to this disease was found at the same year. Therefore, recently, the expression mechanism of fragile X syndrome has been known in the level of DNA.[5,6]

It has been found that this disease is caused from abnormal amplification of CGG repeated sequence sited at FMR1 (FRAXA) gene located at Xq 27.3 of X chromosome.[4]

The FMR-1 (CGG) microsatellite normally varies in size from 6 to 54 repeats. Affected patients have expanded alleles (full mutations) with more than 200 reiterations. Individuals with between 54 and 200 repeats are considered to carry a permutation.[7,8]

While, as the reasons of mental retardation, not only genetic factor but also hypoxia, head injury after birth, central nervous system infection, acquired child disease and surrounding environmental pollution and social and cultural effects have been known.[9]

However, the extraction of DNA to analyze fragile X syndrome usually uses blood, recently, the studies using hair follicle as specimen have been spread.

Rob Willemsen *et al.*[10] manufactured special mouse monoclonal antibodies to analyze fra X gene by taking hair root of experiment subjects.

They developed the technique to analyze mental retarded children by making red color appear by treating these antibodies to the hair root.

Also, in human's DNA finger printing and diagnosis of livestock's hereditary disease, hair root, hair DNA and hair color are used.

For example, the discrimination of gene, Melanocortin 1 Receptor (MC1R) involved in hair color is applied in industry by identifying the breeds of beef by using the difference of hair color between

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milk cattle and Korean native cattle.[11,12] Hair root is more easy to take, move and store than blood. In the our further study,[13] mentally retarded persons are not searched excessively Pb that known caused mental retardation. Therefore, this study extracted the DNA of hair root and conducted molecular biologic test to analyze fra X gene of the patients in mental retarded children facilities.

Materials and Methods

Subjects of Study

The subjects are mental retarded teenagers in mentally retarded children facilities where I used to go for hair styling service once a month from September 2003.

The number of total subjects was 24, boys were 12, average age was 17(±3) and girls were 12, and the average age was 18(±2).

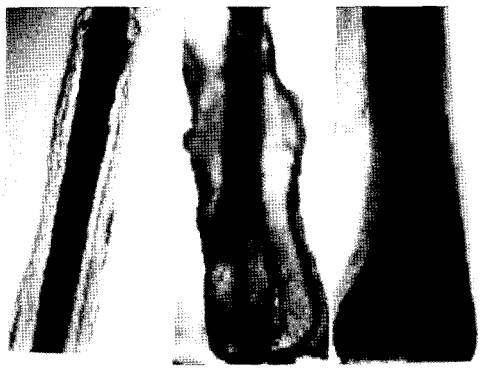

Fig. 1. Hair root of anagen phase.

Fig. 2. Hair root of telogen phase.

Picking of Hair Root

Hair root was plucked by fixing 2~3 hairs with pincette. By investigating picked hair root with naked eye, telogen phase (Fig. 2) was excepted and only anagen phase (Fig. 1) that hair root is about 3~5 mm was used. Over 10 per one person were taken. Subsequently, hair root of about 0.5 cm including hair shaft was taken and submerged in PBS solution and stored in refrigerator.

Genomic DNA Isolation

Plucked 10 hair roots were performed washing, that is, put in microcentrifuge tubes respectively, and the addition of 1 m*l* of distilled water, vortexing and centrifugation were performed.

From washed hair root, DNA was extracted by genomic DNA purification system (Promega, USA). That is, digestion solution was manufactured as Table 1.

This digestion solution features the addition of DDT in case of hair, and 1~2 sec vortexing was performed. Next, it was cultured in 63°C water bath for 3~4 hours. At this time, by stirring at every 30 minutes, the digestion was accelerated. By centrifuging the specimen in digestion for about 1 minute, it was settled, and with the addition of 250 μ*l* Lysis buffer, foreign matters were removed by centrifugation for about 1 minute after digestion for about 5 minutes. Subsequently, it was moved to 1.5 m*l* tube after centrifugation for 3 minutes (13000 × G), subphase was discarded and with addition of wash liquid (including 95% ethanol), it was washed about 4 times (for 1 minute at 13000 × G).

For dry, it was centrifuged (13000 × G) for 2 minutes, then it was moved to Wizard SV Mini-column of new 1.5 m*l* tube, with the addition of 45 μ*l* of Nuclease free water, it was set aside for about 10 mins. at room temperature, next, after the

Table 1. Component of digestion solution

Nuclei Lysis Buffe	200 μ*l*
0.5M of EDTA	50 μ*l*
Proteinase K 200 mg/m*l*	20 μ*l*
RNase solution 4 mg/m*l*	5 μ*l*
DDT	20 μ*l*
Total volume	295 μ*l*

Table 2. Primers of PCR for Fra(X) syndrome

Primer name	Primer sequence	Annealing site within FMR-1gene[A]	Effective priming temperature °C[B]
F[C]	5'agccccgcacttccaccaccagctcctcca3'	13963-13992	95
Eag-U	5'cgacctgtcaccgcccttcagccttcc3'	13463-13489	88
Eag-L	5'cgctgcgggtgtaaacactgaaaccacgtc3'	13656-13685	88

[A]Numbers correspond to nucleotides of the FMR-1 sequence from the GenBank(L 29074).
[B]According to Fu *et al.* (1991).
[C]Primer f is the same used by Fu *et al.* (1991).

centrifugation (13000 × G) for 2 minutes, DNA was extracted.

Specification of DNA was performed by comparing 1 OD value (1 m*l*/50 μg) using UV-spectrometer and control of agarose gel electrophoresis.

PCR Analysis

Composition of Primer

The base sequence of primer for PCR was manufactured in accordance with the primer of LA, Haddad *et al.*[14] (Table 2), and PCR method was also performed in accordance with the similar method.

PCR Reactions

The final volume of PCR reactions was controlled as 25 μ*l*. That is, the compound except genomic DNA solution and Taq DNA polymerase was controlled as 21 μ*l*, the ingredients are as followings:

50 m*l* KCL, 10 mM TRIS-HCL (pH8), 0.1% TritonX-100, 2.0 mM $Mgcl_2$, 10% dimethylsulfoxide, 200 μm of dATP, dCTP, TTP, respectively, 50 μm dGTP, 150 μm 7-deaza-d GTP(Pharmacia, Sweden), 0.08 μm primer Eag-L, 0.3 μm primer Eag-U, 0.2 μm primer f. As well, 2 μ*l* of genomic DNA solution 40 ng/μ*l* and 2 μ*l* of Taq DNA polymerase were mixed. Besides, a drop of sterilizing oil was added so as to prevent specimen from drying at high temperature. The temperature conditions of PCR are as followings:

It was heated at 98°C for about 10 minutes and cooled down at 72°C for about 3 minutes, and then 2 μ*l* of compound (Taq DNA Polymerase (Promega corp, Madison, wis., USA) 50 mM Kcl, 10 mM TRIS-HCL, 0.1% Triton X-100) was added to each tube.

Then, it was amplified to 35 cycles at the following conditions (Table 3).

Table 3. Consisted condition of 35 cycles

90 sec. of denutrition at 94°C
60 sec. of annealing at 65°C
2 min. of extension at 72°C
Final extension for 10 min.

By mixing 5 μ*l* of amplification product amplified as the above to 5 μ*l* of specimen buffer (2 × TBE, 0.1% bromphenol blue, 0.1% xylene cyanol, 10% Ficoll 400), until it was drawn enough to 6% of non-denaturing poly acrylamide gel, eletrophoresis was performed for over 30 minutes.

After that, gel was silver-stained and photographed.

DNA Ladder Marker

PGEM® DNA Markers cat# G1741 Load 1 μ*l*/ lane of Promega was used as DNA ladder marker (Fig. 3-1).

Results

The process of PCR is performed because CGG as mutant of fra (X) gene exists in homologous chromosomes as excessive satellite. Consequently, primer is important, so already used primer Eag-u and f were used by considering it. The sizes of these primers are situated in about 557-bp of the products of CGG homologous chromosomes.

Accordingly, single upper primer (Eag-u) used two different lower primers (Eag-L and f) for competitive good amplification.

This competition can make amplification easy because the sizes of DNA segments of healthy people are different from those of patients with fra

(x) gene. Therefore, primers Eag-u and f are very suitable for PCR and they detect polymorphic CGG segment very strongly and certainly.

Fig. 3 shows the mosaic size of fra-X gene using hair root of mental retarded girls.

M is DNA ladder and lane 1 is control. Lanes 2~7 are analyses of DNA of mentally retarded children. They feature that band of 200 bp of normal size appeared in all lanes. 222 bp (arrow)~517 bp (arrow and mid micro band at arrow head) band can be interpreted as normal group whose CGG repeated sequences are less than 50. As well, in all lanes except control, lane 1 on 517 bp, micro band appeared. Besides, with appearance of band of 1198bp in lane 2, 3, 4, 5 (head of arrow), it is assumed as band of full mutation whose CGG repeated sequences are more than 200. However, it showed the peculiarity that normal band appeared together in all lanes 2, 3, 4, 5, thus it is not reasonable to judge that it is the band of full mutation. It is considered that future researches are needed.

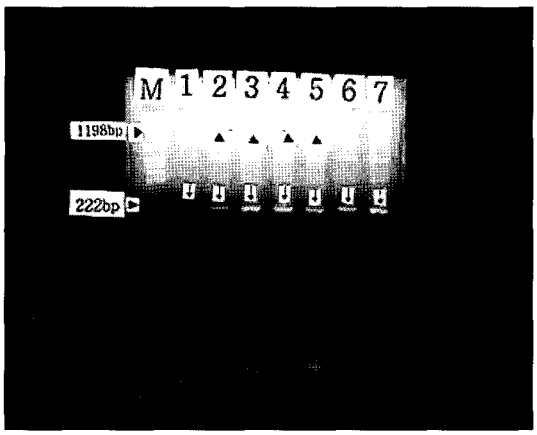

Fig. 3. PCR analysis of FMR-1 gene (Female). M is ladder marker. 1 lane is Nomal female. 2~7 lane is Patient.

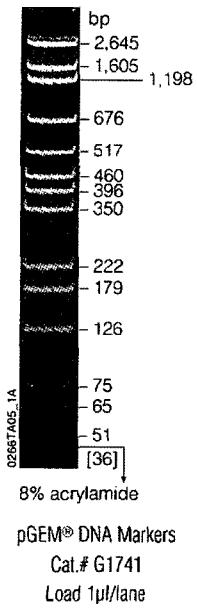

Fig. 3-1. DNA ladder marker.

These results corresponded to bands of 222 bp and 517 bp, the results of Luciana A Haddad *et al.* (1996), however they did not agree with the results that band did not appear above 1198 bp. The same results with those of lane 2, 3, 4, 5 appeared in 47%, 8 of 17 mental retarded girls.

Fig. 4 is the result of mentally retarded boys, M is DNA ladder, lane 1 is control, normal band appeared in 222 bp, and other bands did not appear. Therefore, it can be analyzed as normal. The rest lanes 2~7 were to analyze DNA sizes of mental retarded boys. In lanes 3, 6, 7, normal band did not appear and micro band appeared in 1198 bp finely, thus it is considered as full mutation by molecular genetic analyses.

In lanes 2, 4, 5, normal band of 222 bp appeared with control, and in 1198 bp, band did not appear. These results were same in 222 bp with those of Luciana A Haddad *et al.*[14], however, different in

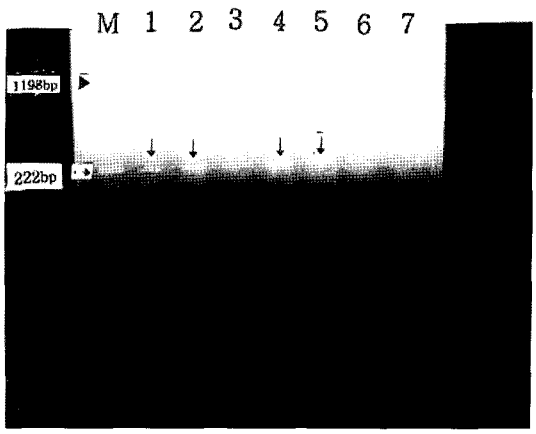

Fig. 4. PCR analysis of FMR-1 gene (Male). M. ladder marker. 1lane. Nomal Male. 2~7.lane. Patient.

1198 bp. In 43%, 5 of 12 boys, band did not either appeared in 1198 bp, which was different from those of girls.

Discussions

CpG island, the region CpG is clustered exists in base sequence of mammal.

Most existing CpG island is not methylated, thus methylation of CpG island has a significant meaning. There are tens of kinds of the gene groups causing heredity abnormality when all genes of each chromosome inherited from parents display functions.

Thus, only genes of either part should be expressed. This process is called genomic imprinting, and the genes controlled by it are called imprinting genes. It is possible because that the CpG island of corresponding gene is selectively methylated and suppresses the expression in early stage of genesis of embryo. The expression of only allele not methylated controls gene dosage.

In case of normal females, cytosine is methylated in inactivated X chromosome among 2 X chromosomes, but methylation cannot be found in activated X chromosome.

Even in fragile X syndrome patients, methylation of FMR-1 gene region in activated X chromosome is observed, which is estimated because of the expansion of CGG base repeated sequences in CpG island, and the expression of FMR-1 gene has been reported to be controlled by this.[15]

Therefore, in case of mentally retarded children, abnormal methylation of CpG island occurs even in activated X chromosome, so mRNA of FMR-1 is not produced, which causes diseases. Methylation of DNA is caused by DNMT (DNA methyltransferase). Methylation obstructs recognition of transcription factor and inhibits the transcription process.

Meanwhile, the diagnoses of fra (x) syndrome differ in researchers. In the past, fragile X syndrome was investigated by using cytogenetic diagnostic methods. For example, Thake *et al.*[16] reported that fragile X syndrome was found in 6.5% of male patients and 9.6% of female patients by using cytogenetic diagnostic methods, and Kahnonen *et al.*[17] reported that it was found in 6.1% of male patients and 4.0% of female patients. However recently, PCR, molecular genetic examination method has been introduced, thus the diagnosis of fragile X syndrome can be achieved more accurately.

For instance, Tumer *et al.*[18] reported it was found in 2.1% of English people and in 2.7% of Australians and Slaney *et al.*[19] and Murray *et al.*[20] reported 2.5% was found.

While, in domestic, Moon *et al.*[21] reported that fragile X chromosome was observed in 6.4% of mental retarded males, Kim *et al.*[22] reported that fragile X chromosome was not found although they performed chromosome test for fragile X syndrome.

Also, Choi *et al.*[23] reported that fragile X chromosome was found in 2.8% after investigating 212 mentally retarded patients by using PCR and Southern blot analysis.

In this research, as the result of PCR with more advanced method, it showed good results that peculiar band of 1198 bp was detected from 43% of male patients diagnosed as mentally retarded children by doctors. These results are considered due to 3 specialized primers used in this study.

However, female patients showed the peculiarity that 2 bands of normal and patients' bands appear together, it seems worthy to study more about the differences between males and females.

It is generally similar to the results that exact fra X gene was detected from males than females from the above researchers' studies.

This study used hair follicle instead of blood to extract DNA, which is a very different material from existing researchers. However, 4~5 hair follicles were taken and used as DNA sample without discrimination of anagen, catagen and telogen phase, so sometimes PCR failed due to small DNA quantity.

Therefore, it is considered that it is appropriate to use over 10 hairs in anagen phase for experiment. This experiment proved that hair root could be a very useful experiment material due to its more easiness to take than blood and the simplicity of storage and transfer. I expect hair root to be used widely as experiment material by readying the chance various body regions can be used.

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