Original Article

Bisulfite Treatment of CG-Rich Track of Trinucleotide Repeat Expansion Disorder: Make the Sequence Less CG Rich

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Abstract

Background: Trinucleotide repeat (TNR) expansion is a kind of mutation with instability in the number of microsatellite repeats. This nature of mutation leads to the different kinds of neurological and neuromuscular disorders; among them, fragile-X syndrome is the main cause of intellectual disability in which the increasing number of CGG TNR in 5' untranslated region is the main reason for epigenetic silencing of Fragile X mental retardation 1 gene. The aim of this study is to decrease the CG content of the candidate region to facilitate amplification by conventional polymerase chain reaction (PCR). Bisulfite treatment of the genomic DNA results in conversion of unmethylated cytosine to uridine and may overcome the diagnostic pitfalls. **Materials and Methods:** The whole blood DNA was extracted and bisulfite treated. Then any simplification in PCR process of desire sequence were assayed through following conventional PCR using specifically designed primers for converted sequence. Bisulfite-treated PCR product of a nearby sequence confirmed our results as a conversion control. **Results:** Both the control and the candidate sequences undergoing bisulfite treatment were successfully amplified by PCR. **Conclusions:** Decreasing the GC content of the sequence by bisulfite treating could be a new approach to overcome difficulties in amplifying GC-rich sequences.

Keywords: *Bisulfite, treatment, fragile-X syndrome, GC Rich Sequences, polymerase chain reaction*

Introduction

Trinucleotide repeat (TNR) expansion is a category of mutation that occurs with instability in microsatellites' repeat number. The unstable length of repeats is the main cause of neurological and neuromuscular disorders.[1] The expanding nature of mutation leads to the various demonstrations of disorder in different affected members of a single family.[2] Normal variants are confirmed via a defined threshold of sequence repeat; hence, exceeded repeat numbers would be considered as a pathogenic allele.[1] The mutant allele's repeats could expand even more while passing down the generations through the pedigrees. More than twenty human disorders are in the form of expanding mutation.[3]

TNRs are divided into two categories based on their expansion location. TNR expansion could affect either noncoding or coding regions of genome.[4] Affected coding region is divided into a bi-part category: polyglutamine-coding tracks or polyalanine-coding ones.[5] In an overview, most of the disorders are due to an expansion of GC-rich repetitive units. The exceeded quantity of repeats could influence gene function and/or its expression which leads to a pathogenic phenotype.[3]

Fragile-X syndrome is the main cause of intellectual disability with TNR expansion in 5' untranslated region (5'-UTR) of Fragile X mental retardation 1 (*FMR1)* gene. *FMR1* is located on chromosome Xq27.3 and the related repetitive track consists of CGG units. In a normal allele, the expansion number is below 54 of repeats, but in a mutant allele, the number of repetition exceeds the threshold of 200 repeats. Expanding repetitions in the range of 55–200 refer as permutation allele.[6,7] As the expansion overpasses the threshold, *FMR-1* would be silenced because of hardly known hypermethylation process[8] and results in silencing the gene and depletion of fragile x mental retardation protein. There is a variety of symptoms in abnormal cases. The symptoms are ranging from slight difficulty in learning and normal IQ level in mild cases to mental retardation,

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autistic behaviors and a considerable decline of IQ level in severe ones.[7-9]

As the number of repeats increases, it becomes more difficult to amplify the region due to repetitions and GC-rich content of the sequence. Hence, conventional polymerase chain reaction (PCR) faces various difficulties to amplify candidate sequence.[10] Various techniques are invented to achieve success in truly sizing the repeated tracks. Enhanced PCRs are applied to overcome amplification problems in PCR extension cycles.[11] Betaine, formamide, $MgCl_2$, DMSO, etc., are additives which are used in gradient concentration for optimizing polymerase activity.[10,12,13] Triplet-primed PCR following by capillary electrophoresis,[14] high-resolution methylation PCR,[15] methyl-specific PCR,[16] and southern blotting[6] are other techniques applied for sizing numbers of repeats in TNR disorders. Considering all existing methods have pitfalls, different modifying approaches are used to obtain more accurate and effective diagnostic procedure for investigating mutant alleles.

Recently, bisulfite treatment of DNA is used to evaluate methylation statues of sequences.[17] In sodium bisulfite DNA treated, all cytosine residues will convert to uracil except those which are in methylated form.[18] It is a chemical reaction with the estimated accuracy rate of 99.5%–99.7%, which specifically eliminates unmethylated cytosine in single-strand DNA and leads to the elimination of complementary base-pairing dogma. Afterward, conventional PCR on bisulfite-converted DNA could alternate thymine instead of uracil.[19,20]

In conclusion, the results of the current study introduce bisulfite treatment (converting repeating cytosine to uracil), as a new method of amplifying GC rich expanding mutation sequences with a conventional PCR.

Materials and Methods

Blood sample collection

Peripheral blood was collected with a sterile syringe from two male adult volunteers, including a normal/healthy individual and a verified case of fragile X who was tested by fragmented analysis before and fragile-X mutation was confirmed in him. Moreover, a whole blood sample of a normal female was also collected for genomic DNA extraction. All genomic DNAs were isolated via the DNA extraction kit (Genetbio-Korea) based on the kit's protocol.

Primer designing

First, two pairs of bisulfite primers were designed by gene runner version 6.5.51 (Hastings Software, Inc., New York, USA,) for CT-converted sequence to amplify converted CCG repeat strand and the conventional PCR. Sequences of primers are listed in Table 1.

As all PCR products are generally unmethylated sequences, a conversion positive control, which went under bisulfite treatment, was considered as a conversion positive control.

Table 1: List of primers

CGG bisulfite primer	sequence
Forward	CRTAACRTAATTTCAATATTTACACC
Reverse	GAAGATGGGGTTTGTTTTAGAG
Positive control primer	
Forward	AAATGAGAGACCAGCGAGGA
Reverse	CACTGCAACACCACAAATTCA
Positive control bisulfite primer	
Forward	GTAGTAAGAAGTGGTAGTT
Reverse	ATATACTCTCAATTAATTAC
Repeat sizing primer	
Forward	TCAGGCGCTCAGCTCCGTTTCGGTTTCA
Reverse	AAGCGCCATTGGAGCCCCGCACTTCC

Conducting conversion control PCR reaction, pair of primers was designed for an upstream sequence of the repetitive track. The repetitive control PCR was considered as a sequence in which all cytosine would be converted to uracil. Thereafter, bisulfite treatment of positive control amplicon based on the protocol of the kit (Zymo-Research-USA) was done. The rate of CT conversion was assayed through following PCR, using positive control bisulfite primers. CCG bisulfite primers were designed for amplifying CCG track of bisulfite-treated *FMR1* of the human genome.

Polymerase chain reaction

The PCR reaction was performed via positive control primers, amplifying the positive control sequence. The total volume of 20 µl PCR reaction, constituting 30 ng genomic DNA with 10 pmol of each forward and reverse primer and Taq DNA polymerase master mix (RED amplicon-Denmark), was used for the amplification reaction. Genomic DNA denaturation at 94°C for 5 min, following 30 cycles of 94°C for 2 min, 63°C for 30 s, and 72°C for 45 s was conducted. The reaction was terminated by a final extension 10 min at 72°C. Analysis of PCR product was managed with electrophoresis on 1% agarose gel staining by red safe and demonstrating under gel documentation (Vilber Lourmat-France). DNA concentration was checked by NanoDrop. The sufficient amount of PCR product was bisulfite treated, based on the instruction of Zymo-Research kit. Subsequently, the second reaction of PCR, using control positive bisulfite primers, on treated amplicon template was performed to detect whether conversion of cytosine to uracil was implemented in the PCR product of positive control sequence.

The normal and affected individuals' genomic DNAs were bisulfite treated according to the instruction mentioned in the kit. Genomic DNA was analyzed in two forms of a double

and a single strand to verify whether bisulfite treatment is different in double- and single-strand DNA. Sufficient amount of DNA was boiled for 10 min, then placed on ice immediately for 2 min to make single strand genomic DNA. To evaluate the alteration of double-strand to single-strand DNA, a pattern of gel electrophoresis of single-strand comparing to double-strand DNA was assessed. Evaluating the quantity of single-strand DNA was also performed by a NanoDrop spectrophotometer and was compared with its origin double-strand DNA.

300 ng of both double- and single-strand bisulfite-treated DNA was eluted in 30 µl of elution buffer. Amplification of the repetitive track was implemented by PCR using CGG bisulfite primers and 5 µl of eluted bisulfite-treated genomic DNA in the total volume of 20 µl reaction, including 10 pmole of each forward and reverse primer and 1× Taq DNA polymerase master mix (Amplicon-Denmark).

The extracted DNA of the confirmed fragile X case was bisulfite treated and then analyzed as the same, with positive control bisulfite and CGG bisulfite primers. To assay efficacy of bisulfite treatment, as well as its simplification of repetitive sequences' amplifying by PCR, comparing gel electrophoresis pattern of normal and mutant alleles showed GC content reduction of the target sequence.

CGG repeat size of normal allele was determined by CG-rich PCR system using 1× GC-rich Taq DNA polymerase and 15 pmol of each forward and reverse primer, named repeat sizing primer [the sequence is shown in Table 1] in the presence of betaine (amplicon-Denmark).

Results

Figure 1 shows positive control PCR product positive control. The positive control-treated sequence was successfully amplified with control bisulfite primers, demonstrates in Figure 2. Hence, bisulfite converting was performed efficiently. Amplification of converted sequence with positive control bisulfite primers is depicted in Figure 2.

The results of PCR product of CGG repeat sizing in two normal and one affected individuals are demonstrated in Figure 3.

Polymerizing reaction result in bisulfite-treated genomes of normal and affected individuals is shown in Figure 4. For the full mutant, a smear of a double strand was detected after the treatment. In the single-strand form of its treated DNA, we noticed more distinct bands with a size of around 900 bp. For both normal individuals, the amplified PCR product of treated double strand DNA was in the expected size range.

Discussion

Our research team aimed to overcome the hardness in amplifying the repetitive track in 5' UTR of the *FMR1* gene. As the sequence is highly GC rich and all available techniques encounter obstacles to amplify it, we intended to decrease GC content to dominate the formation of secondary structures and polymerase detachment to conquer the hardship of amplification.

The strategy was based on the conversion of cytosine to uracil and consequently to thymine in PCR reaction, as the result of bisulfite treatment. When double-strand DNA is treated by bisulfite, there is no complimentary pairing anymore. The status is because of converted uracil or finally, thymidine instead of cytosine in one strand which could not be the complement of guanine in the opposite strand, based on the base-pairing dogma. As a result of *in vitro* polymerizing of the converted strand, adenine would be set as an opposite site of thymine which is instead of cytosine. Hence, in repetitive track, we supposed to have CTT in 5' to 3' leading strand and AAG in the lagging strand. After polymerizing converted sequence, GC

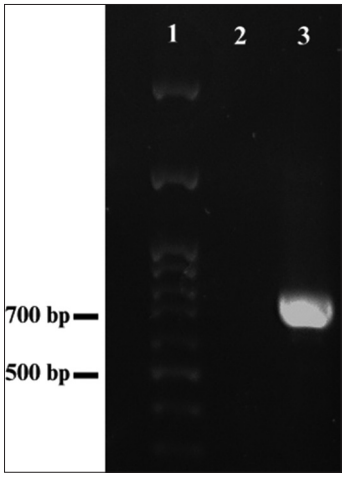

Figure 1: Amplification of positive control sequence: In the first well, a DNA marker was loaded. Second: Negative control. Third: Control polymerase chain reaction product

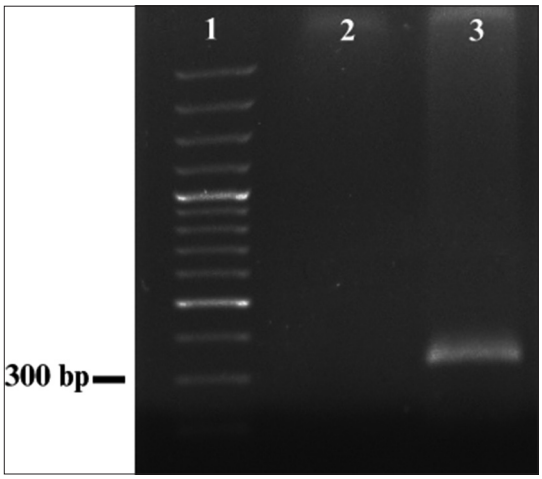

Figure 2: Bisulfite specific polymerase chain reaction following bisulfite treatment of control sequence: In the first well, a DNA marker was loaded. Second: Negative control. Third: Bisulfite polymerase chain reaction of positive control

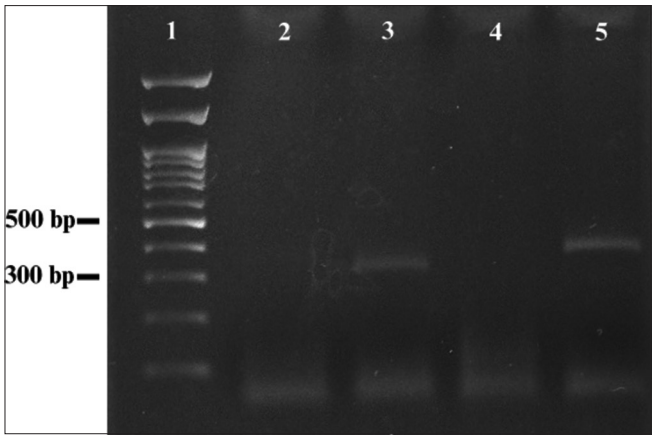

Figure 3: Repeat sizing of two normal and one affected individuals: In the first well, a DNA marker was loaded. Second: Negative control. Third: Repeat sizing product of normal female. Fourth: Repeat sizing of confirmed fragile X. Fifth: Repeat sizing of normal male

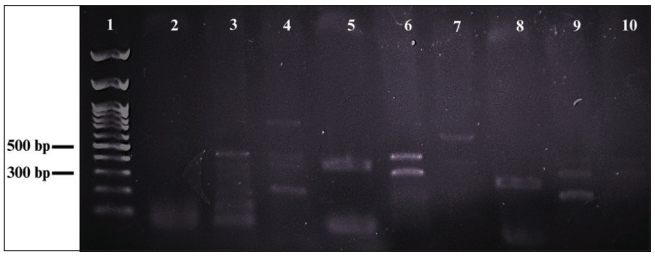

Figure 4: Bisulfite specific polymerase chain reaction following bisulfite treatment of genomic DNA: In the first well, a DNA marker was loaded. Second: Repeat sizing of mutant allele. Third: Bisulfite-treated polymerase chain reaction of double strand of mutant allele, Fourth: On its single-strand DNA. Fifth: Repeat sizing of normal male allele, Sixth: Bisulfite-treated polymerase chain reaction of double strand of normal male allele, Seventh: On its single-strand DNA. Eighth: Repeat sizing of normal female allele, Ninth: Bisulfite-treated polymerase chain reaction of double strand of normal female allele, Tenth: On its single-strand DNA

contents in 5' to 3' sequence and its complement strand decrease from 100% to ~33%. As a consequence result of this reduction, DNA secondary structures would scale down and amplification of DNA would be simpler.

Thanks to the high re-annealation tendency of repetitive sequence, conversion of C to U is ceased or rarely happened;[20] therefor, bisulfite conversion, in the repetitive sequence, has an efficient limitation. As the C-G hydrogen bonds are more vigorous, comparing with A-T, efficient reduction at GC-rich regions is an expectation. Moreover, considering the optimum proportion of DNA and bisulfite is essential and the insufficiency of available bisulfite or high DNA concentration would be the reason for declined converted DNA.[20]

Of note, mammalian somatic tissues are in hypermethylation status.[21] Most of all this chemical modification occurs on C5 position of cytosine at CpG sites. The level of methylated CpGs is about 70%.[22] Hypermethylation modification of bases is not randomly distributed and is more prevalent at CpG island in the promoter, compared to other CpG sites of the gene.[23,24] Promoter is in unmethylated form in the normal allele of *FMR1*, and as the repeated sequence numbers pass the specific threshold, hypermethylation takes place. Accordingly, we supposed unmethylated CGG repeats in nonaffected cases. As a result of unmethylation statue, there are no bisulfite-converting impediments. On the other hand, the methylation status of the gene in affected ones changed the conversion rate in an inverse relation with methylation percentages. Even in 100% methylation form of repeats, one of the cytosine in CCG unmethylated and GC percentages scaled down to 67%. Thus, the effect of bisulfite treatment is more in imperfect methylation of full mutant alleles.

As proposed by performing this treatment, it is simpler to amplify the GC-rich track, which is the decision-making mutation of the fragile X. As it is clear in Figure 3, the candidate track in a normal individual is amplified with GC-rich PCR and also using normal Taq DNA polymerase after bisulfite-treated genomic.

Since all the available techniques in amplifying GC-rich sequence of genomic DNA are problematic and conventional PCR is unable to amplify high repeated GC-rich sequence, this study demonstrates the effectiveness of bisulfite therapy on template DNA.

Although bisulfite treating leads to amplification of GC-rich track, there is a drawback that should not be overlooked. Bisulfite-treating DNA could cause fragmentation which may generate difficulties in analyzing repeated sequence numbers. However, fragmentation of DNA was scaled down using Zymo-Research kit, with a 12–16 h stage of incubation.

Conclusions

Although there are several developed techniques for amplifying repetitive track in fragile X disorder, there are still difficulties with amplification of sequence. Bisulfite treatment of the track to decrease GC content was an initiation to overcome problems of amplifying the candidate sequences, yet it requires further studies to be acquired as one of the common diagnostic methods.

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Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Budworth H, McMurray CT. A brief history of triplet repeat diseases. In: Trinucleotide Repeat Protocols. Methods Mol Biol Springer; 2013. p. 3-17.
2. Orr HT, Zoghbi HY. Trinucleotide repeat disorders. Annu Rev Neurosci 2007;30:575-621.
3. La Spada AR, Taylor JP. Repeat expansion disease: Progress and puzzles in disease pathogenesis. Nat Rev Genet 2010;11:247-58.

4. Cummings CJ, Zoghbi HY. Fourteen and counting: Unraveling trinucleotide repeat diseases. Hum Mol Genet 2000;9:909-16.
5. Albrecht A, Mundlos S. The other trinucleotide repeat: Polyalanine expansion disorders. Curr Opin Genet Dev 2005;15:285-93.
6. Kanwal M, Alyas S, Afzal M, Mansoor A, Abbasi R, Tassone F, *et al*. Molecular diagnosis of Fragile X syndrome in subjects with intellectual disability of unknown origin: Implications of its prevalence in regional Pakistan. PLoS One 2015;10:e0122213.
7. Garber KB, Visootsak J, Warren ST. Fragile X syndrome. Eur J Hum Genet 2008;16:666-72.
8. Gabis LV, Gruber N, Berkenstadt M, Shefer S, Attia OL, Mula D, *et al*. Fragile X premutation carrier epidemiology and symptomatology in Israel-Results from a tertiary child developmental center. Cerebellum 2016;15:595-8.
9. Grigsby J. The fragile X mental retardation 1 gene (FMR1): Historical perspective, phenotypes, mechanism, pathology, and epidemiology. Clin Neuropsychol 2016;30:815-33.
10. Strien J, Sanft J, Mall G. Enhancement of PCR amplification of moderate GC-containing and highly GC-rich DNA sequences. Mol Biotechnol 2013;54:1048-54.
11. Meguid NA, Ismail MF, El-Mahdy RS, Barakat MA, El-Awady MK. Simple molecular diagnostic method for fragile X syndrome in Egyptian patients: Pilot study. Acta Biochim Pol 2014;61:259-63.
12. Bhagya CH, Wijesundera Sulochana WS, Hemamali NP. Polymerase chain reaction optimization for amplification of Guanine-Cytosine rich templates using buccal cell DNA. Indian J Hum Genet 2013;19:78-83.
13. Chakrabarti R, Schutt CE. The enhancement of PCR amplification by low molecular weight amides. Nucleic Acids Res 2001;29:2377-81.
14. Rajan-Babu IS, Chong SS. Triplet-repeat primed PCR and capillary electrophoresis for characterizing the fragile X mental retardation 1 CGG repeat hyperexpansions. In: Clinical Applications of Capillary Electrophoresis. Methods in Molecular Biology Springer; 2019. p. 199-210.
15. Chen L, Hadd A, Sah S, Houghton JF, Filipovic-Sadic S, Zhang W, *et al*. High resolution methylation PCR for fragile X analysis: Evidence for novel FMR1 methylation patterns undetected in southern blot analyses. Genet Med 2011;13:528.
16. Grasso M, Boon EM, Filipovic-Sadic S, van Bunderen PA, Gennaro E, Cao R, *et al*. A novel methylation PCR that offers standardized determination of FMR1 methylation and CGG repeat length without southern blot analysis. J Mol Diagn 2014;16:23-31.
17. Sasaki M, Anast J, Bassett W, Kawakami T, Sakuragi N, Dahiya R. Bisulfite conversion-specific and methylation-specific PCR: A sensitive technique for accurate evaluation of CpG methylation. Biochem Biophys Res Commun 2003;309:305-9.
18. Li LC, Dahiya R. MethPrimer: Designing primers for methylation PCRs. Bioinformatics 2002;18:1427-31.
19. Smith ZD, Gu H, Bock C, Gnirke A, Meissner A. High-throughput bisulfite sequencing in mammalian genomes. Methods 2009;48:226-32.
20. Warnecke PM, Stirzaker C, Song J, Grunau C, Melki JR, Clark SJ. Identification and resolution of artifacts in bisulfite sequencing. Methods 2002;27:101-7.
21. Woo HD, Kim J. Global DNA hypomethylation in peripheral blood leukocytes as a biomarker for cancer risk: A meta-analysis. PLoS One 2012;7:e34615.
22. Antequera F, Bird A. Number of CpG islands and genes in human and mouse. Proc Natl Acad Sci U S A 1993;90:11995-9.
23. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415-28.
24. Shen L, Guo Y, Chen X, Ahmed S, Issa JP. Optimizing annealing temperature overcomes bias in bisulfite PCR methylation analysis. Biotechniques 2007;42:48, 50, 52 passim.