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Accurate Diagnosis of a Homozygous G1138A Mutation in the Fibroblast Growth Factor Receptor 3 Gene Responsible for Achondroplasia

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Satiroglu-Tufan, N.L., Tufan, A.C., Semerci, C.N. and Bagci, H. *Accurate Diagnosis of a Homozygous G1138A Mutation in the Fibroblast Growth Factor Receptor 3 Gene Responsible for Achondroplasia*. Tohoku J. Exp. Med., 2006, **208** (2), 103-107 —— Achondroplasia is the most common genetic form of dwarfism inherited as an autosomal dominant disorder. Individuals affected with achondroplasia have impaired ability to form bone from cartilage (endochondral bone formation). Homozygous achondroplasia is a neonatal lethal condition. The vast majority of patients with achondroplasia have a G-to-A transition at position 1138 of the fibroblast growth factor receptor 3 (FGFR3) cDNA sequence, resulting in the Gly-to-Arg substitution at position 380 of the FGFR3 protein. This mutation has been diagnosed by SfcI digestion of amplified genomic DNA. However, it has also been demonstrated that the SfcI digestion protocol does not consistently distinguish between DNA samples heterozygous and homozygous for the G1138A substitution. This study was designed to improve the molecular diagnosis based on the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques for the FGFR3 G1138A mutation. The newly designed forward primer contains one mismatch (G at position 1136) from the FGFR3 cDNA sequence (A at position 1136), thereby creating a PstI site (CT*G*CAG at positions 1134 to 1139) in the amplified DNA from alleles containing the G1138A mutation. The PCR-RFLP technique based on the PstI digestion of amplified genomic DNA with a novel forward primer shows 100% accuracy in diagnosis of the G1138A mutation in heterozygous and homozygous individuals. ———— homozygous achondroplasia; FGFR3; PCR; RFLP

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The genetic condition known as achondroplasia is characterized by an unusually large head (macrocephaly) with a prominent forehead (frontal bossing) and flat (depressed) nasal bridge; short upper arms and legs (rhizomelic dwarfism); unusually prominent abdomen and buttocks; and short hands with fingers that assume a "trident" or three-pronged position during extension. It occurs as a result of a fresh spontaneous mutation in the fibroblast growth factor receptor 3 (FGFR3) gene in about 90% of cases. However, it is inherited as an autosomal dominant disorder and the newborns of a couple where both parents are affected with the heterozygous form of the condition have a 25% chance of being affected with the homozygous achondroplasia, which is a neonatal lethal condition. It is important to diagnose homozygous achondroplasia at the earliest gestational age possible, which is achieved by molecular analysis.

The vast majority of patients with achondroplasia, i.e., more than 90%, have a G-to-A substitution at position 1138 of the FGFR3 cDNA sequence, resulting in the substitution of an arginine for a glycine residue at position 380 of the FGFR3 protein (Shiang et al. 1994). This mutation has been detected by SfcI digestion of amplified genomic DNA (Shiang et al. 1994; Ikegawa et al. 1995; Wang et al. 1996; Saito et al. 2000). Around 10% of the patients, on the other hand, have the G-to-C transition at the same nucleotide (Saito et al. 2000).

This study and others (Lanning and Brown 1997) have demonstrated that the SfcI digestion protocol does not consistently distinguish between DNA samples heterozygous and homozygous for the G1138A substitution. On the other hand, this study also demonstrated that the alternative procedure suggested by Lanning and Brown (1997) based on BsrGI digestion of amplified genomic DNA with modified primers does not reveal good quality polymerase chain reaction (PCR) products. Thus, this study was designed to improve the molecular diagnosis of homozygous achondroplasia based on the PCR-RFLP technique for the FGFR3 G1138A mutation.

Materials and Methods

Genomic DNA

DNA from 2 heterozygote individuals was isolated from blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the "blood and body fluid protocol" (Satiroglu-Tufan et al. 2005). DNA from 2 homozygote individuals (NA11317, and NA08859), on the other hand, was obtained from Corriell Cell Repositories (Camden, NJ, USA). This study was approved by the Pamukkale University Medical Ethics Committee.

Table 1. *Primer sequences used for PCR and conditions used for RFLP analyses*

PCR no.	Primer pairs (F, Forward; R, Reverse; 5′ to 3′)	Reference	Conditions for RFLP analyses	Mutation detected
1	F: AGGAGCTGGTGGAGGCTGA R: GGAGATCTTGTGCACGGTGG PCR product size: 164 bp	Shiang et al. (1994)	a) SfcI digestion at 37°C for 1 hr b) SfcI digestion at 25°C for 16 hr Resulting products: 109 bp, 55 bp	G1138A
2	F: AGGAGCTGGTGGAGGCTGA R: GGAGATCTTGTGCACGGTGG PCR product size: 164 bp	Shiang et al. (1994)	MspI digestion at 37°C for 1 hr Resulting products: 107 bp, 57 bp	G1138C
3	F: GTGTATGCAGGCATCCTCATGTAC R: GGAGATCTTGTGCACGGTGG PCR product size: 132 bp	Lanning and Brown (1997)	BsrGI digestion at 37°C for 4 hr Resulting products: 112 bp, 20 bp	G1138A
4	F: GTGTATGCAGGCATCCTCAGCTGC R: GGAGATCTTGTGCACGGTGG PCR product size: 132 bp	This study	PstI digestion at 37°C for 4 hr Resulting products: 107 bp, 25 bp	G1138A

DNA amplification and mutation detection conditions

Primers used in this study are listed in Table 1. The forward primer suggested by Lanning and Brown (2000) contained 2 base pair (bp) mismatches from the FGFR3 cDNA published sequence (Table 1, PCR no. 3, forward primer underlined sequence) creating a BsrGI site (TGTACA) in the amplified DNA from alleles containing the G-to-A mutation. On the other hand, the novel forward primer suggested in this study, containing 1 mismatch from the FGFR3 cDNA published sequence (Table 1, PCR no. 4, forward primer underlined sequence), creates a PstI site (CTGCAG) in the amplified DNA from alleles containing the G-to-A mutation. The strategy of the method developed in this study is summarized in Fig. 1.

All PCR amplifications were performed in a total volume of 50 μl containing extracted DNA, 20 pmol of each forward and reverse primer, and 25 μl of HotStarTaq Master Mix (containing 2.5 units of HotStarTaq DNA polymerase, 1 × PCR buffer with 1.5 mM $MgCl_2$, and 200 μM of each dNTP (Qiagen).

For PCR no. 1, and 2 (Table 1) the thermal cycling was as follows: initial activation of HotStarTaq DNA polymerase at 95°C for 15 min, followed by 30 cycles of 94°C for 0.5 min, 65°C for 0.5 min, 72°C for 0.5 min, and a final extension of 72°C for 7 min (Lanning and Brown 1997). For PCR no. 3, and 4 (Table 1) the thermal cycling was as follows: initial activation of HotStarTaq DNA polymerase at 95°C for 15 min, followed by 5 cycles of 94°C for 0.5 min, 60°C for 0.5 min, 72°C for 0.5 min, followed by 25 cycles of 94°C for 0.5 min, 65°C for 0.5 min, 72°C for 0.5 min, and a final extension of 72°C for 7 min (Lanning and Brown 1997).

All mutations were detected under the conditions summarized in Table 1 and analyzed on 1-4% Molecular Screening Agarose gel (Roche Diagnostics GmbH, Mannheim, Germany).

Results

First we analyzed the heterozygous patients to confirm their G1138A mutation (Fig. 2A). Fig. 2A, a representative gel image of 2 different experiments, each performed on a different thermal cycler for control, shows the products of 4 different PCR reactions listed in Table 1. The PCR no. 3 consistently revealed low quality PCR products when compared with other reaction products. RFLP analyses of products from PCR no. 1, 2, and 4 confirmed that both heterozygous patients had the G1138A mutation and that the PCR-RFLP technique based on PstI digestion of amplified genomic DNA with a novel modified forward primer does indeed work consistently (Fig. 2B).

We compared the success rate of SfcI vs PstI digestion protocol on homozygous patient samples, and confirmed that the SfcI digestion protocol does not consistently distinguish between DNA samples heterozygous and homozygous for

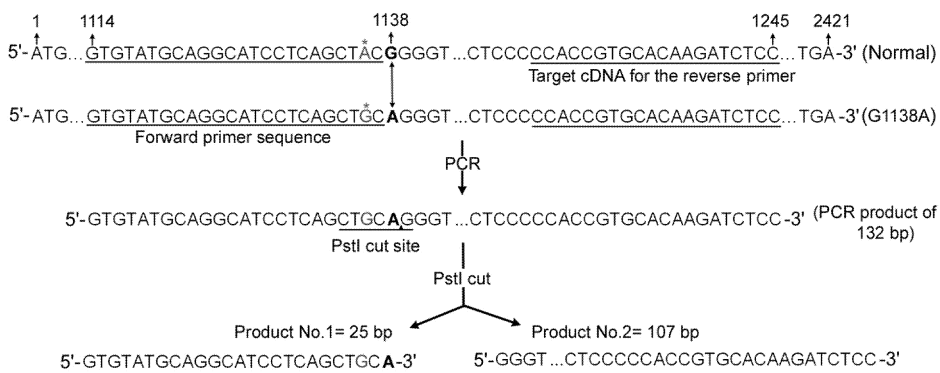

Fig. 1. Schematic diagram showing the strategy of the method developed. Numbers above the sequence indicate the nucleotide number within the FGFR3 cDNA sequence. First line indicates the FGFR3 cDNA sequence of a normal allele. Second line indicates the FGFR3 cDNA sequence of an allele affected by the G1138A mutation. Asterisk indicates the mismatch nucleotide introduced to the forward primer sequence in order to create a PstI cut site (CTGCAG) in the presence of the G1138A mutation. Third line demonstrates the 132 bp PCR product, and as fourth line shows the PstI digestion products of 107 bp and 25 bp of the amplicon.

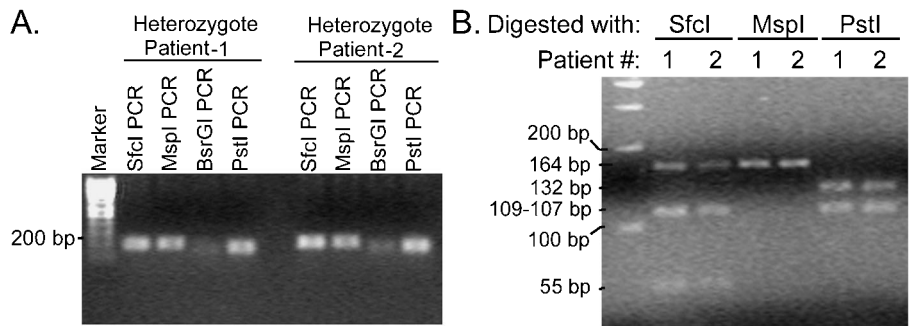

Fig. 2. Analyses of PCR products and conformation of the G1138A mutation in heterozygous patients. A) Analyses of the quality of PCR products obtained trough SfcI-PCR, MspI-PCR, BsrGI-PCR, and PstI-PCR given as PCR no. 1, 2, 3, and 4 in Table 1, respectively. B) Confirmation of the G1138A mutation of FGFR3 in both heterozygous patients by SfcI, MspI, and PstI digestions of amplified genomic DNA. Both SfcI and PstI digestions confirm the heterozygous G1138A mutation with one undigested allele (164 bp for SfcI digestion and 132 bp for PstI digestion) and one digested allele (109 bp and 55 bp for SfcI digestion and 107 bp and 25 bp for PstI digestion; however the 25 bp fragment can not be visualized on the gel shown here). The undigested amplicons after MspI digestion eliminates the possible G1138C mutation. See also Table 1 and relevant text for details.

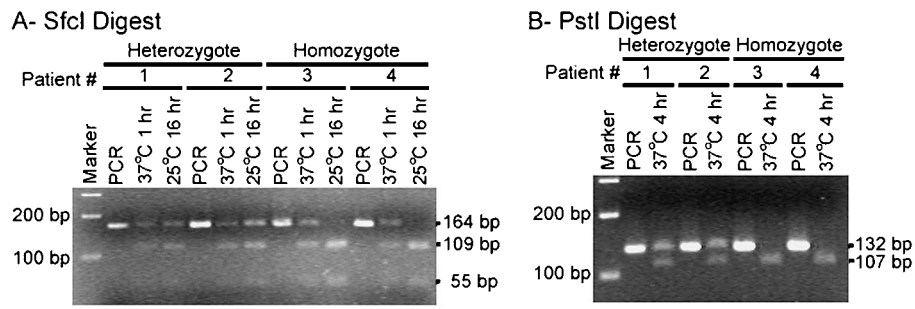

Fig. 3. Improvement of the mutation detection for homozygous G1138A patients. Comparison of the success rate of SfcI (A) vs PstI (B) digestion protocol of amplified genomic DNA on both heterozygous and homozygous patient samples with conditions for RFLP analyses given in Table 1 is demonstrated.

the G1138A substitution when the 37°C for 1 hr digestion protocol is used (Fig. 3A). The suggested PCR-RFLP technique with the endonuclease PstI, on the other hand, showed 100% accuracy in distinguishing between DNA samples heterozygous and homozygous for the G1138A substitution (Fig. 3B).

Discussion

It is important to distinguish between the heterozygote and homozygote forms of achondroplasia accurately during the course of prenatal diagnosis, since the homozygous achondroplasia is a neonatal lethal condition. This study suggests an improved PCR-RFLP procedure utilizing PstI digestion of amplified genomic DNA using a novel modified forward primer, which increases the accuracy of the technique to 100% and also shortens the diagnosis time significantly.

It has been demonstrated in this study that the classical endonuclease, SfcI, used for the diagnosis of achondroplasia is not reliable when it is necessary to distinguish between heterozygous and homozygous G1138A mutations of FGFR3. It is interesting to reveal that especially with the short digestion protocol, i.e., 1 hr at 37°C, SfcI digestion of amplified genomic DNA from homozygous patients consistently resulted in misdiag-

nosis of the G1138A mutation condition as heterozygous (Fig. 3A, patients 3 and 4). It has also been reported that different lots of SfcI enzyme differ in their enzymatic activity, probably contributing to the inconsistent results even with longer SfcI digestion protocol, i.e., 16 hr at 25°C (Lanning and Brown 1997).

The protocol suggested by Lanning and Brown (1997), on the other hand, may overcome the endonuclease activity problems by performing the PCR with a modified forward primer introducing a BsrGI site into the amplimer in the presence of the G1138A substitution and utilizing the BsrGI digestion for the mutation detection. However, this study demonstrated that the quality of the amplimer obtained by this modified PCR is consistently much lower than that obtained for the classical SfcI-PCR (Fig. 2A), which may simply be the result of the two mismatched bases at the 3´ end of the forward primer used to create the novel BsrGI site in the presence of G1138A mutation.

Taken into consideration that today's technology even allows isolation of fetal nucleated red blood cells present in the maternal circulation (Hennerbichler et al. 2003), it is possible to perform noninvasive prenatal diagnosis of single gene mutations such as achondroplasia at very early gestational ages. However, the very little yet very valuable DNA isolated for this type of diagnosis has to be analyzed by the best protocol available. Thus, this study suggests even one step further improved PCR-RFLP protocol with a novel forward primer, which includes only 1 base mismatch from the FGFR3 sequence and creates a PstI site in the presence of G1138A mutation of FGFR3. The quality of the amplimer obtained from so-called PstI-PCR was consistently as good as that of classical SfcI-PCR (Fig. 2A). This can be explained by the fact that the novel primer used in the present study had only one mismatched base but also higher G/C content. A "GC Clamp" at the 3´ end of the primer when compared to that of forward primer suggested by Lanning and Brown (1997) increased the yield of the PCR. The "GC Clamp" helps to ensure correct binding at the 3´ end due to the stronger hydrogen bonding of G/C residues. It also helps to improve the efficiency of the reaction by minimizing any "breathing" that might occur (Kwok et al. 1990).

In conclusion, the suggested protocol eliminates the drawbacks of the previous protocols and the need for more complex methods, such as DNA sequencing for the dependable and early prenatal diagnosis of homozygous achondroplasia G1138A mutation. It also reduces the cost of diagnosis.

Acknowledgments

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