Original Article

How genetic variation was analyzed in phenytoin-induced gingival enlargement using single-nucleotide polymorphism of candidate gene CYP2C9?

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Abstract

Background: Phenytoin-induced gingival overgrowth is an adverse drug reaction affecting few individuals, on phenytoin therapy for its antiepileptic effect. Analysis of genetic variation of CYP2C9*2 gene was done to identify the action of metabolic enzyme cytochrome P 450 on this drug. The main background of this publication is a quick review about one of the molecular techniques used to identify the single-nucleotide polymorphism (SNP) using polymerase chain reaction coupled with restriction fragment length polymorphism (PCR-RFLP).

Materials and Methods: Deoxyribonucleic acid (DNA) was extracted from 5 ml of venous blood withdrawn from the individual, who had gingival overgrowth following phenytoin therapy. DNA was isolated, using the phenol-chloroform method. Isolated DNA was used for SNP analysis of CYP2C9*2 presentation. The basic procedure used for SNP analysis in our case was PCR-RFLP.

Results: Genetic variation of CYP2C9*2 in our case was homomutant.

Conclusion: The etiology of phenytoin-induced gingival overgrowth is always an enigma, but it is now becoming clearer that a multifactorial role may be involved in the cause. One of the factors analyzed was polymorphism of CYP2C9*2 gene and it was found to be homomutant in our case. Adverse drug reaction can be minimized, by either reducing the drug dosage or drug substitution. However, larger scale genome-wide study has to be carried out to confirm one of the etiopathogenesis as mutation of the CYP2C9 gene, in phenytoin-induced gingival overgrowth.

Keywords: CYP2C9 gene, phenytoin-induced gingival overgrowth, single-nucleotide polymorphism

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INTRODUCTION

The human genome contains roughly 3.2 billion DNA base pairs. Within the genome, there are about 20,000 protein-coding genes, comprising only about 1.5% of the genome. These proteins variously function as enzymes, structural components and signaling molecules and are used to assemble and maintain the cell morphology and function.[1] The unraveling of our "genetic information" promises to unlock the pathogenesis of many human

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diseases. Gene is a nucleic acid sequence, which contains the information to make a polypeptide. A gene is a stable entity, but can suffer a change in the sequence. Such a change is called mutation. When the mutation occurs, the new gene is inherited in the stable manner.[2] Mutation can be at different levels. It can be either at the level of chromosome resulting in deletion, inversion and translocation or a single base pair change, which is called point mutation. A point mutation can result from the substitution, deletion or insertion of a single nucleotide base by a different base, resulting in the formation of different amino acid.[3] Mutations result either from errors in the DNA replication or from the damaging effects of mutagens, such as chemicals and radiation. Mutations are less frequent and occur at a frequency of <1% in a population, whereas polymorphism is a variation in the DNA sequence that is found in many individuals, at a specified frequency usually 1% or greater of a population.[4] Initially, polymorphism starts as mutations, but they become fixed in the population and achieve sufficient frequency after passing through few generations.

The most common polymorphism is single-nucleotide polymorphism (SNP), which constitutes about 90% of all human DNA polymorphism. The incidence of SNP is for every 1000 bp. A SNP is a single base pair mutation at a specific locus, usually consisting of two alleles (one from each parent). Suitable candidate genes are generally selected based on known biological, physiological or functional relevance to the disease in question. The candidate gene in our case is CYP2C9, which is located at 10q23.33 (long arm of chromosome 10 at position 23.33).[5] Genetic variation in this gene can be identified by analyzing the SNP. SNP can result in missense, nonsense, silent, frameshift and splice site mutation.

CYP2C9 gene which encodes for the protein cytochrome P450 is a metabolic enzyme. This enzyme helps in the metabolism of many drugs and xenobiotic substances. Cytochrome P450 (CYP) enzymes are a superfamily of mono-oxygenases that are found in all kingdoms of life. In mammals, these enzymes are found primarily in the membranes of the endoplasmic reticulum within liver cells, as well as many other cell types.[1] These enzymes use haem iron to oxidize molecules, often making them more water soluble for clearance. They achieve this by either adding or unmasking a polar group. CYP enzymes are responsible for oxidative metabolism of a wide number of compounds and are major enzymes involved in drug metabolism. Some 100 therapeutic drugs are metabolized by CYP2C9, including drugs with a narrow therapeutic index such as warfarin and phenytoin and other routinely prescribed drugs such as acenocoumarol, tolbutamide, losartan glipizide and some nonsteroidal anti-inflammatory drugs.[6] CYP2C9 defective enzymatic activity is the result of genetic variation present in that gene. Phenytoin takes up this pathway, hence we used CYP2C9 as candidate gene for our analysis.[7]

There are at least 33 variants of CYP2C9 (*1B through 34) being identified. Each variant differs by single base pair change which results in different amino acid formation. For example, CYP2C9*2 and CYP2C9*3 differ from the wild type CYP2C9*1 by a single base pair change (point mutation). CYP2C9*2 (rs1799853) is characterized by a 430 C > T exchange in exon 3 resulting in an Arg144Cys amino acid substitution, whereas CYP2C9*3 (rs1057910) shows an exchange of 1075 A > C in exon7 causing an Ile359 Leu substitution in the catalytic site of the enzyme.[8-10] A SNP is a single base pair mutation at a specific locus usually consisting of two alleles. Analysis by molecular techniques has tremendous advancement after the human genome project. There are various methods by which genetic variation can be identified using SNP. We are sharing our experience of identifying genetic variation of CYP2C9 by analyzing the SNP using polymerase chain reaction coupled with restriction fragment length polymorphism analysis (PCR coupled with RFLP).

MATERIALS AND METHODS

Various steps involved in our analysis to identify genetic variation

- Extraction of DNA by phenol-chloroform method
- PCR
  - Pre-PCR
  - PCR
  - Post-PCR.
- Digestion with restriction enzyme
- Analyzing the data
- Result obtained in our case.

Extraction of DNA by phenol-chloroform method

The process of isolating DNA from cells is the first step for genetic analysis. The technique used should be able to separate DNA from the unwanted substances of the cell. The isolated DNA, thus obtained should be stable and gentle enough to avoid denaturation (break up). DNA exists in cells in the form of chromatin structure, where it is associated with several proteins like histones. DNA has to be separated from this protein and cellular debris. Many different methods and technologies are available for the isolation of genomic DNA. In general, all methods involve disruption and lysis of the cell membrane, followed by removal of proteins and other

contaminants and finally recovery of DNA. We followed the phenol-chloroform method for the extraction of DNA from whole blood.[11,12]

Peripheral venous blood was obtained by standard venipuncture from a patient who had gingival enlargement following phenytoin therapy. It was collected in a polypropylene tube containing 100 µl of 10% ethylenediaminetetraacetic acid (EDTA) disodium salt. Whole blood will have cellular (red blood cells [RBCs] and white blood cells [WBCs]) and noncellular components (plasma). Collected blood was centrifuged at 2500 rpm for 10 min at 4°C to remove plasma. About 10 ml of RBC lysing solution was added after discarding the plasma, lysing solution caused the rupture of RBC. Centrifugation was done wherever required, nucleated WBC settled at the bottom. After separation of WBC, to rupture the WBC membrane, WBC lysing solution was added, which exposed the DNA content, impurities in the DNA were removed by the addition of anionic detergent which removed lipid in the cell membrane and proteinase K digested the protein including DNases and RNases and freed the DNA from the chromatin structure. After successful disruption of WBC membrane, chloroform was added which aided the removal of protein and excess proteinase K which will interfere with the action of restriction endonucleases used downstream. The addition of equilibrated phenol helped to maintain the DNA in the aqueous phase. Subsequent extraction with chloroform and octanol removed the traces of phenol from the nucleic acid. Finally, ice-cold ethanol was added, which precipitated the higher molecular weight DNA. Precipitated DNA [Figure 1] was adequately washed with 70% ethanol dried and dissolved in Tris EDTA buffer at 37°C overnight. After complete dissolution, the DNA samples are stored at 4°C for further use.

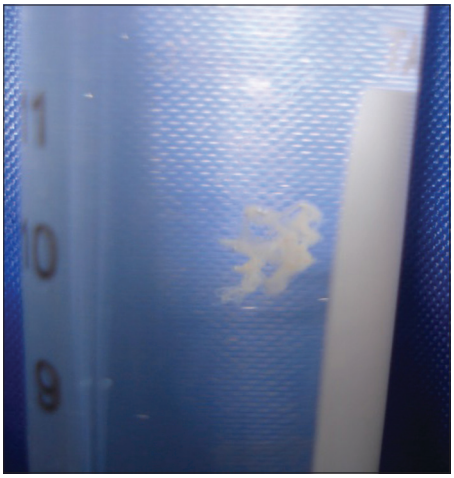

Figure 1: Extracted DNA from peripheral blood

Polymerase chain reaction

Three stages of PCR are pre-PCR, PCR and post-PCR.

Prepolymerase chain reaction stage

In the pre-PCR stage, the web-based design of PCR-RFLP procedure for CYP2C9*2 was done by accessing into the websites like Ensembl genome browser[12] and software tools like Primer designer,[13] sequence manipulation suits[14] and NEBcutter.[15] After surfing, the candidate gene was identified in the chromosome and that of the SNP location in EXON 3 (430C>T). The reference sequence identified was rs 1799853. After choosing the reference sequence, the selected sequence was copied to the primer designing software to determine the length of the forward and reverse primer and its sequence and neb cutter tool for choosing the restriction endonucleases enzyme. Based on the web search, primer designed for CYP2C9*2 with SNP position of 430 C > T was

Length of 2C9 F:5'TACAAATACAATGAAAATATCATG3'

Length of 2C9 R ;5'CTAACAACCAGACTCATAATG3'

The restriction enzyme chosen was Anabaena variabilis (AvaII). Palindromic sequence recognized by AvaII is

5'…G*GACC…3'

3'…CCAG*G…5'

The asterisk indicates the cutting sites in the sequence by the restriction enzyme AvaII. The total length of the PCR product is 690 bp. Restriction enzyme acts like molecular scissors. In case of wild type allele, the restriction enzyme will cut both alleles and the fragment size produced will be 521 bp and169 bp.

Polymerase chain reaction

PCR is an *in vitro* technique for the amplification of DNA sequence of interest. It involves the two oligonucleotide primers of varied length between 17 and 30 nucleotides which will be flanking the DNA sequence to be amplified. The length of the forward primer is 24 bp and reverse primer is 21 bp in our case. Master Mix containing template DNA, dNTPs, buffer, Mgcl2, Taq polymerase, water and forward and reverse primer. The primers hybridize to opposite strands of DNA after it has been denatured. Varied temperatures were maintained in the thermocycler for denaturation, hybridization and extension. Primers oriented such that DNA synthesis by polymerase proceeds through the region between the two primers. The extension

reaction creates two double-stranded target regions which, in turn, are subjected to repeated cycles of denaturation, hybridization and extension leading to the amplification of specific DNA sequences by an enormous factor. Amplification of about 10^6 fold is actually attained.

Postpolymerase chain reaction analysis

In the post PCR room, gel electrophoresis analysis of PCR amplified product was done. Gel electrophoresis analysis showed band at 690 bp. This PCR product was further subjected for restriction enzyme digestion as a downstream analysis.

Digestion with restriction enzyme

In a 0.5 ml microcentrifuge tube, digestion mix containing7.5 μl milli Q water, 2 μl buffer and 0.5 μl AvaII restriction enzyme was added. The digestion mix was centrifuged at 5000 RPM for 5 s. To this digestion mixture 10 μl of PCR product was added, was again centrifuged and incubated at 37°C overnight, restriction enzyme act like molecular scissors, that cleave the sugar-phosphate backbone of DNA at specific restriction sites. In the wild type allele, restriction site will be present, so PCR product which is 690 bp is cleaved into two fragments of 521 bp and 169 bp.

Analyzation of the data

In diploid organism, alleles are inherited through sexual reproduction as the resulting offspring inherit half of their chromosomes from the mother and half from the father. The cells in diploid organisms contain sets of homologous chromosomes, which are paired chromosomes that have the same gene at the same position. Although homologous chromosomes have the same genes, they may have different alleles for those genes. Different alleles can be identified by restriction fragment length, which codominate with polymorphism. Restriction fragment length was identified after digesting with restriction enzyme AvaII [Figure 2a]. In the analysis of SNP of CYP2C9*2, (430 C > T), it gives the information that 430 nucleotide of exon 3 of that gene, if cytosine is present in that position, it is the normal or wild type base pair. In case cytosine is substituted by thymine in that position, it is the polymorphic variant for that position. In wild type, both alleles contain cytosine (C/C) [Figure 2b], then the restriction site G*GACC will be recognized by the restriction enzyme AvaII and it will cut the fragment into two. In individuals having wild type allele, PCR product of 690 bp will be cut into 521 bp+169 bp. In individuals where both alleles have been substituted by thymine (T/T), the restriction enzyme will not be able to recognize the site and the fragment will remain intact. The resultant PCR product will be 690 bp [Figure 2c]. Then, the individual

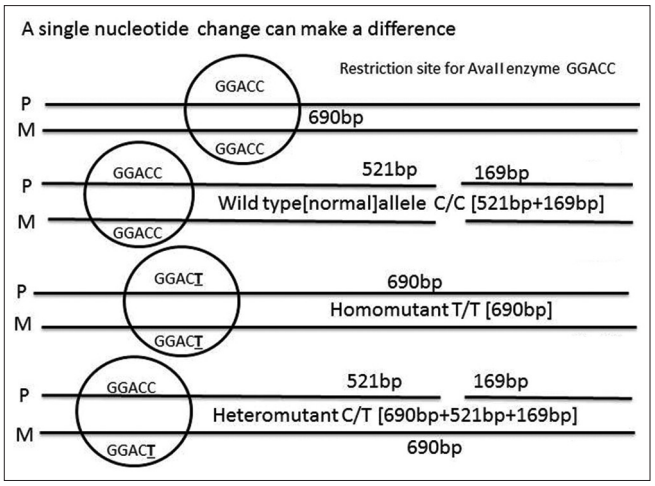

Figure 2: Schematic representation of PCR product when digested with Ava II

will be called homomutant. If one of the alleles has cytosine and other allele substituted by thymine (C/T), allele containing cytosine will be recognized by restriction enzyme and other allele will not be recognized. The resultant PCR product will have fragment of three different sizes, that is, 690 bp+521 bp+169 bp [Figure 2d]. The individual is heteromutant. The PCR product digested with restriction enzyme will give three different results. If two different size bands are produced, then the individual is having wild type allele. In case single band is present, the individual should be considered as homomutant. If three different bands are present, then the individual is heteromutant [Figure 3].

DISCUSSION

The etiology of phenytoin-induced gingival overgrowth is always an enigma, but it is now becoming clearer that a multifactorial role may be involved in the cause. One of the factors analyzed was polymorphism of CYP2C9*2 gene and it was found to be homomutant in our case. Adverse drug reaction can be minimized, by either reducing the drug dosage or drug substitution. However, larger scale genome-wide study has to be carried out to confirm one of the etiopathogenesis as mutation of the CYP2C9 gene, in gingival overgrowth. Three base pairs code for one amino acid, if there is going to be SNP of 430th nucleotide means 144th amino acid will be cysteine instead of arginine. Missense mutation of 144th amino acid has been identified in this case.

RESULTS

DNA was extracted from peripheral blood withdrawn from the individual, who had phenytoin-induced gingival enlargement. PCR amplification was done for the

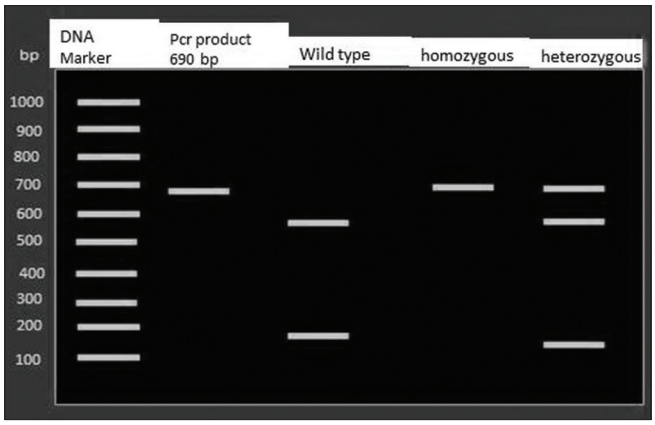

Figure 3: Gel electrophoresis band that will be produced in wild type and mutant variant

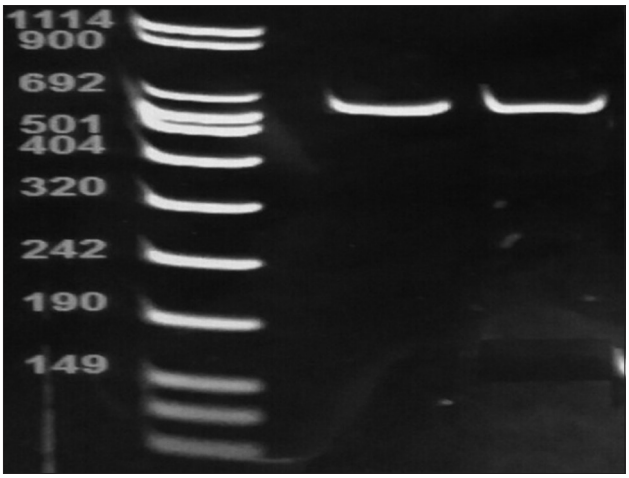

Figure 4: Homomutant band in our case

CYP2C9 gene. CYP2C9 is the metabolic enzyme for phenytoin. Restriction enzyme AvaII digestion of the amplified PCR product, when analyzed under the gel electrophoresis single band was produced [Figure 4]. The result obtained was homomutant (T/T), which infers that both alleles had the polymorphic variant and the individual will be poor metabolizer for phenytoin. Missense mutation of 144th amino acid, that is, cysteine secreted instead of arginine. The drug dosage was reduced after genetic analysis, following that gingival enlargement regressed.

CONCLUSION

Genomic study of drug-induced gingival enlargement will help us to understand the pathogenesis and as well metabolic status of particular individual for specified drug. Based on the genetic information, we can alter the drug dosage or drug substitution can be done. The genomic study helps in the personalized medicine. After the completion of the human genome project, basic tools exist to identify the biology behind human diseases. This publication mainly aims at how basic tools can be utilized to study the genetic variation using SNP.

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Conflicts of interest

There are no conflicts of interest.

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