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ARTICLES

Research Article

The codon 17 polymorphism of the CTLA4 gene in type 1 diabetes mellitus in the Baghdad population
Najwa S. H. Ahmed

Mutation N308T of protein tyrosine phosphatase SHP-2 in two Senegalese patients with Noonan syndrome
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A study of correlation between CYP2C9 gene polymorphism and Warfarin maintenance dose in anticoagulant therapy among Han people in Yunnan of China
ZhiYu Chen, Jintao Li, Xuemei Dong, JianXing Liu, Hui Gao, Liping Zhao and BingYing Xu
The codon 17 polymorphism of the \textit{CTLA4} gene in type 1 diabetes mellitus in the Baghdad population

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The aim of this work was to study the polymorphism in \textit{CTLA4} gene in insulin-dependent diabetes mellitus (IDDM) type 1 patients in Baghdad population. To achieve this goal, blood samples were collected from 80 IDDM (40 males and 40 females) and 20 samples of healthy, DNA was isolated and the \textit{CTLA4} gene (A 152 bp fragment) were amplified by using specific primers for exon1 of this gene, and then found the sequence of this region. The DNA sequencing results of flank sense of \textit{CTLA4} gene from healthy patients was found to be compatible (100%) with wild type of \textit{Homo sapiens} from the Gene Bank, while 99% compatibility were found for the gene from 70 IDDM cases patients with wild type of gene. The difference may be attributed to one transition mutations, A/G at position 49 of the \textit{CTLA4} gene (from AGC to AAC). It is a missense mutation that leads to changes in amino acid from serine (S) to asparagine (N). Our results showed that the incidence of A/G mutation at nucleotide position 49 and diabetes was highly significant ($X^2 = 100$, $P < 0.01$). In total, 12% of patients with IDDM (10 cases) had two transition mutation +49 A/G and +47 C/T single nucleotide polymorphism from total cases, 98% compatibility were found for that gene from 10 IDDM cases patients with wild type of gene. The +47 C/T SNP was silent mutation which resulted in change of codon from GGT to GGC but no changes translated to amino acid (glycine to glycine). However, there was no significant correlation between diabetes and incidence of C/T at nucleotide 47 ($X^2 = 0.055$, $P > 0.05$). In conclusion, our case study suggests that the +49 A/G SNP of the \textit{CTLA4} gene is strongly associated with genetic susceptibility to type 1 diabetes mellitus in the Baghdad/Iraqi population.

\textbf{Key words:} \textit{CTLA4} gene, insulin-dependent diabetes mellitus, A/G polymorphism.

\section*{INTRODUCTION}

Cytotoxic T-lymphocyte antigen 4 (\textit{CTLA4}) also known as cluster of differentiation 152 (CD152) is a protein that plays an important role in the immune system regulations. \textit{CTLA4} is a member of the immunoglobulin superfamily, which is expressed on the surface of Helper T cells and transmits an inhibitory signal to T cells. The \textit{CTLA4} encodes the T cell receptor involved in the control of T cell proliferation and mediates T cell apoptosis (Yanagawa et al., 1997; Larsen et al., 1999). The receptor protein is a specific T lymphocyte surface antigen that is detected on cells only after antigen presentation. Thus, \textit{CTLA4} is directly involved in both immune and autoimmune responses and may be involved in the pathogenesis of multiple T cell-mediated autoimmune disorders. The human \textit{CTLA4} gene is located at chromosome 2q33 (Nistico et al., 1996; Donner et al., 1997a). This gene is a member of the immunoglobulin superfamily and encodes a protein which transmits an inhibitory signal to T cells. The protein contains a V domain of 116 amino acids, a
transmembrane domain, and a cytoplasmic tail. Alternate transcrip
tional splice variants, encoding different isoforms, have been characterized. The membrane-bound isoform functions as a homodimer which is inter-
connected by a disulfide bond, while the soluble isoform functions as a monomer (Kristiansen et al., 2000).

An A-to-G substitution at nucleotide 49 in exon 1 results in an amino acid substitution (Thr/Ala) in the leader peptide of the protein (Donner et al., 1997b). The Ala allele has been shown to predispose the individual carrying it to the development of various immune diseases including insulin-dependent diabetes mellitus, Graves disease, Hashimoto thyroiditis, celiac disease, systemic lupus erythematosus, thyroid-associated orbitopathy, and other autoimmune diseases (Anjos and Polychronakos, 2004).

Mutations and polymorphisms in this gene results in alteration of the CTLA4 activity and are believed to play an important role in the risk of developing autoimmunity (Anjos and Polychronakos, 2004). The CTLA4 (49+) GG homozygous genotype is associated with Type 1 diabetes in Egyptian children especially with younger age of onset and in younger patients and not associated with grades of diabetic control or diabetic complication (Hatem et al., 2008; Mosaad et al., 2012). The aim of this study was to assess the contribution of this CTLA4 poly-

Sequencing and sequence alignment

Sequencing of exon 1 of CTLA4 gene was performed by Macro gen company, USA. Homology search was conducted using Basic local alignment search tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and BioEdit program. The results were compared with data obtained from Gene Bank published eXPaSY program which is available at the NCBI online.

Statistical analysis

The statistical analysis is a very important final step in the research to analyses and evaluates the obtained results. Medical statistics of this study was conducted via computer based statistical program which was: X² for windows computer package. The statistical analysis tests used in this were as follows: P value < 0.01 is considered a significant correlation.

RESULTS AND DISCUSSION

CTLA4 gene was successfully amplified using specific PCR primers for exon 1. Figure 1 showed PCR amplification of exon 1 of the CTLA4 where a specific product at 152 bp was observed. Our result is in agreement with other studies (Hatem et al., 2008; Waterhouse et al., 1995). Sequencing of this gene was performed to detect variant +49A/G which related to development of diabetes. Sequences alignment using BLAST and BioEdit showed the 100% similarity or homology of healthy sample with wild type of the CTLA4 gene of H. sapiens from the Gene Bank (Figure 2). The CTLA4 gene from 70 diabetes patients shows 99% compatibility with the wild type sequences of CTLA4 gene from Gene Bank as shown in Figure 3A, there are one transition at position +49 A/G single nucleotide polymorphism that cause a serine to asparagine substitution in codon 17, there is a high significance between diabetes and incidence of + 49 A/G position in exon 1 of CTLA4 gene (X² = 100, P > 0.01), Table 1 shows the type of mutation and the effect of these mutations and Table 2 shows the translation of CTLA4 gene of all groups (healthy and patient) to a protein sequence, and two transition mutation at position +47 C/T and +49 A/G of CTLA4 gene from 10 diabetes patients was identified.

The sequence shows 98% compatibility with wild type CTLA4 gene as shown in Figure 3B. single nucleotide polymorphism at position +47 C/T that silent mutation, no change translate amino acid (Glycine to Glycine), there is lower significant correlation between type 1 diabetes and incidence of this SNP, (X² = 0.055, P > 0.05). Most molecular epidemiology studies have evaluated the role of the +49A/G single nucleotide polymorphism that causes a threonine to alanine substitution in codon 17 and associated with altered protein expression (Anjos et al., 2002) and T-cell activation (Maurer et al., 2002). Gribben et al. (1995) have suggested that this may be through antigen specific induction of the apoptotic pathway. The mentioned study investigated the A49G polymorphism in

MATERIALS AND METHODS

Samples and DNA extraction

Whole blood samples were obtained from 80 Baghdad patients affected by insulin-dependent diabetes mellitus (IDDM) (40 males and 40 females, age ranged from 4 to 25 years). Samples from 20 healthy individuals were used as a control group. In total, 4 ml whole blood was collected into an Ethylenediaminetetraacetic acid (EDTA) tube. The samples were stored at -20°C until further processing. DNA was extracted by DNA extraction kit (Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA) according to the manufacturer’s protocol.

Amplification of exon 1 of CTLA4 gene

A 152 bp fragment containing the +49 A/G polymorphism in exon 1 of CTLA4 was amplified using a forward primer (CTLA4: 5’-AAGGCTACGCTGAACCTGTT-3’) and a reverse primer (CTLA4: 5’-CTGCTGAACAAATGAACCC-3’) (Alpha DNA Company, Canada) (Marron et al., 1997). The polymerase chain reaction (PCR) amplification was performed in a total volume of 25 µl containing 5 µl DNA, 12.5 µl Go Taq green master mix 2X (Promega corporation, USA), 1 µl of each primer (50 pmol). The thermal cycling conditions were as follows: Denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 57°C for 1 min and 72°C for 1 min with final incubation at 72°C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem) (Genc et al., 2004; Hatem et al., 2008). The PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized by ultraviolet light (302 nm).
Figure 1. Agarose gel electrophoresis for detection of amplified CTLA4 gene. Bands were fractionated by electrophoresis on a 1.5% agarose gel (2 h., 5 V/cm, 1× Tris-acetic buffer) and visualized under UV light after staining with ethidium bromide. Lane: 12 (M: 100 bp ladder); Lane: N (negative control); Lane: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 (PCR product).

Homo sapiens chromosome 2 genomic contig, features in this part of subject sequence:

cytotoxic T-lymphocyte protein 4

Score = 150 bits (81), Expect = 2e-34, Identities = 81/81 (100%), Gaps = 0/81 (0%)

Query 16 CTTTGCAAGACAGGGATGAAGAGAAAGAAAAACAGGAGAGTGCAGGGCCAGGTCCTGG 75

Sbjct 264 CTTTGCAAGACAGGGATGAAGAGAAAGAAAAACAGGAGAGTGCAGGGCCAGGTCCTGG 205

Query 76 TAGCCAGGTTCAGCTGAGCCT 96

Sbjct 204 TAGCCAGGTTCAGCTGAGCCT 184

Figure 2. Sequencing of sense flanking the partial CTLA4 gene for healthy as compared with wild type CTLA4 obtained from Gene Bank.

exon 1 of CTLA4 gene in 40 Lebanese and 46 controls from the same ethnic background.

An increase in the frequency of the G allele was discovered in patients when compared to control subjects, this difference was statistically significant, despite the small sample size. Wafai et al. (2011) showed an association of CTLA4 with type 1 diabetes in Lebanese population. An association was detected between the CTLA4 gene polymorphism and younger-onset type 1 diabetes with autoimmune thyroid disease (AITD) (Gough, et al., 2005). The G variant was suggested to be genetically linked to AITD-associated type 1 diabetes of younger onset in this Japanese population (Mochizuk, et al., 2003). The defect in these patients presumably lies in a T-cell mediated autoimmune mechanism (Takara et al., 2000). Chistiaxov et al. (2001) reported that the CTLA4 gene is strongly associated with insulin-dependent diabetes mellitus (IDDM) in a fifty-six families each consisting of two siblings (one affected with IDDM diagnosed before the age of 18 years and one non-diabetic sibling).

It was reported that the CTLA4 49 (A/G) mutation conferred a risk of type 1 diabetes in the Chinese children but not in the West African children. On the other hand, the novel CTLA4 159 (C/G) mutation conferred a risk of type 1 diabetes in the West African children but not in the Chinese type 1 diabetic children (Hymaman et al., 2001). Donner et al. (1997b) showed that an alanine at codon 17 of CTLA4 is associated with genetic susceptibility to Graves disease as well as to IDDM. Lemos et al. (2009) states that the CTLA4 +49 A/G polymorphism is not associated with susceptibility to type 1 diabetes mellitus in the Portuguese population. This contrasts with positive associations that have been reported for the +49A/G polymorphism in case control studies in populations from Belgium, Germany, Poland, France, Japan, China, Italy, the Philippines, Lebanon, Estonia and Iran (Zalloua et al., 2004; Kavvoura and Ioannidis, 2005; Mojtahedi et al., 2005). However, lack of association for the + 49A/G polymorphism has also been reported in populations from the USA, Japan, Ghana, UK, France, Czech Republic, Morocco, Argentina, Brazil and Azerbaijan (Marron et al., 1997; Caputo et al., 2005; Hauache et al., 2005; Kavvoura
A: Sense of the partial CTLA4 gene, shown one transition mutation.

Score = 172 bits (93), Expect = 4e-41, Identities = 95/96 (99%), Gaps = 0/96 (0%)

Query 1 AAAAGTCTCACTACCTTTGCAAGAGACGGGATGAAGAGAAGAAAAACAGGAGAGTGCA 60

Sbjct 54942207 AAAAGTCTCACTACCTTTGCAAGAGACGGGATGAAGAGAAGAAAAACAGGAGAGTGCA 54942148

Query 61 AGGGCCAGGTCCTGGTAGACAGGTTCAGCTGAGCCT 96

Sbjct 54942147 AGGGCCAGGTCCTGGTAGACAGGTTCAGCTGAGCCT 54942112

B: Sense of the partial CTLA4 gene, shown two transition mutation.

Homo sapiens chromosome 2 genomic contig, features in this part of subject sequence: cytotoxic T-lymphocyte protein 4

Score = 165 bits (89), Expect = 7e-39, Identities = 93/95 (98%), Gaps = 0/95 (0%)

Query 1 AAAAGTCTCACTACCTTTGCAAGAGACGGGATGAAGAGAAGAAAAACAGGAGAGTGCA 60

Sbjct 54942206 AAAAGTCTCACTACCTTTGCAAGAGACGGGATGAAGAGAAGAAAAACAGGAGAGTGCA 54942147

Query 61 GGGCCAGGTCCTGGTAGACAGGTTCAGCTGAGCCT 95

Sbjct 54942146 GGGCCAGGTCCTGGTAGACAGGTTCAGCTGAGCCT 54942112

Figure 3. Sequencing of sense flanking the CTLA4 gene for diabetes as compared with wild type CTLA4 obtained from Gene Bank. (A: 70 diabetes patients have one mutation; B: 10 diabetes patients have two mutation)

Table 1. Types of mutations detected in partial CTLA4 gene of diabetes patients.

<table>
<thead>
<tr>
<th>No.</th>
<th>Location of gene bank</th>
<th>Nucleotide change</th>
<th>No. of sample</th>
<th>Amino acid change</th>
<th>Predicted effect</th>
<th>Type of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A/G 49+</td>
<td>AGC &gt; AAC</td>
<td>70</td>
<td>Serine (S)/ Asparagine (N)</td>
<td>Missense</td>
<td>Transition</td>
</tr>
<tr>
<td>2</td>
<td>C/T 47+</td>
<td>GGT &gt; GGC</td>
<td>10</td>
<td>Glycine (G) / Glycine (G)</td>
<td>Silent</td>
<td>Single nucleotide polymorphism</td>
</tr>
</tbody>
</table>

Table 2. Amino acid sequences of healthy and patient group.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequencing of amino acid</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K V S L T F A E D R E E K K Q E S A G P G P G S Q V Q L S L K</td>
<td>20 healthy</td>
</tr>
<tr>
<td>2</td>
<td>K V S L T F A E D R E E K K Q E S A G P G P G N V Q L S L K</td>
<td>70 Patient</td>
</tr>
<tr>
<td>3</td>
<td>K V S L T F A E D R E E K K Q E S A G P G P G N V Q L S L K</td>
<td>10 patient</td>
</tr>
</tbody>
</table>

K: lysine; V: Valine; S: Serine; L: Lysine; T: Threoine; F: Phenylalanine; A: Alanine; E: Glutamic acid; D: Asparagine; R: Arginine; Q: Glutamine; G: Glycine; P: Proline.

and Ioannidis, 2005; Ahmedov et al., 2006).

Conclusion

Our study showed that there was significant correlation between diabetes and incidence of A/G +49 position in exon 1 of CTLA4 gene, despite the limited size of our sample, our results together with population studies show an association of CTLA4 with type 1 diabetes mellitus, on the other hand, the novel of +47 C/T silent mutation was no significant correlation between type 1 diabetes in
REFERENCES


Baghdad population.
Full Length Research Paper

Mutation N308T of protein tyrosine phosphatase SHP-2 in two Senegalese patients with Noonan syndrome

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Noonan syndrome is a genetic autosomal dominant disorder characterized by facial dysmorphism, short stature, delayed puberty and congenital heart defects. The first gene implicated in this syndrome is PTPN11, encoding protein tyrosine phosphatase SHP-2. Several studies worldwide have identified missense mutations in this gene in patients with Noonan syndrome. Our objective focused on mutations screening of PTPN11 on a Senegalese population with Noonan syndrome. Six patients clinically diagnosed with Noonan syndrome were included in this study. DNA was extracted from whole blood by phenol chloroform. Mutation screening was performed by bidirectional sequencing of amplified polymerase chain reaction (PCR) products of PTPN11 exons frequently mutated in Noonan syndrome. This study identified in two patients, a c.923A˃C mutation in exon 8, predicting Asn308Thr (N308T) on SHP-2 protein. This is the first time that this mutation is described in Noonan syndrome in Africa, while codon 308 was reported as a hot spot mutation site in other populations. Frequently reported amino acid substitutions were Asn308Asp and Asn308Ser. All these mutations affected the protein tyrosine phosphatase domain (PTP) of SHP-2 protein exerting a gain of function which would likely explain observed phenotypes in patients.

Key words: Mutation, N308T, protein tyrosine phosphatise (PTP), SHP-2 protein, Noonan syndrome, Senegal.

INTRODUCTION

Noonan syndrome (NS, MIM 163950) is an autosomal dominant dsmorphic syndrome described first by Noonan (1968). The prevalence of NS is estimated to be 1 in 1000 to 2500 births. The disease is characterized by proportionate short stature, delayed puberty, congenital heart defects and multiple minor anomalies such as hypertelorism, malrotated ears, webbed neck, bleeding diathesis, cryptorchidism in males, mental retardation, and hearing difficulties (Marino et al., 1999; Roberts et al., 2013; van der Burgt et al., 2007). The most common congenital heart defect is pulmonary valve stenosis with displastic leaflets followed by hypertrophic obstructive cardiomyopathy (HC), atrial septal defects (Marino et al., 1999; Musante et al., 2003; Tartaglia et al., 2006).

Jamieson et al. (1994) mapped a gene for NS to the long arm of chromosome 12 (12q24). Tartaglia et al. (2001) reported that NS is caused by heterozygous missense mutations of the gene protein-tyrosinephospho-
PTPN11 mutations were detected in 45% of unrelated individuals with sporadic or familial NS (Tartaglia et al., 2002). PTPN11 gene consists of 15 exons and the expressed protein encodes cytoplasmic tyrosine phosphatase with two tandemly arranged Src homology 2 (SH2) domains (N-SH2 and C-SH2) at the N terminal region and a C terminal protein-tyrosine phosphatase domain (PTP) (Chan and Feng, 2007). The PTPN11 gene is widely expressed in various human tissues, especially in the heart, brain, and skeletal muscle (Ahmad et al., 1993). The protein plays a critical role in regulating the response of eukaryotic cells to extracellular signals through the RAS/MAPK pathway (Roberts et al., 2013).

All PTPN11 missense mutations associated with NS were clustered in the interacting portions of the N-SH2 domain and the PTP domains are involved in switching the protein from its inactive to the active conformation. Functional studies by energetic-based structural analysis of two N-SH2 mutants revealed that those mutations favoured the active conformation of PTPN11 protein, resulting in a gain-of-function effect (Tartaglia et al., 2001; Uhlen et al., 2006). Also, other studies have reported enhanced phosphatase activity of NS mutants located in the SH2 and PTP domains (Niihori et al., 2005; Tartaglia et al., 2003).

Understanding of the molecular genetic causes of NS, enable the study of the pathophysiological mechanisms underlying the varied medical and developmental features of NS. PTPN11 belongs to the RAS-MAPK pathway which is an important signal transduction pathway. Mutations that cause NS deregulated this pathway leading to the clinical features observed. Furthermore, all the other genes implicated in NS including SOS1, RAF1, and KRAS encode proteins integral to this pathway (Roberts et al., 2013).

Several studies have reported mutation analysis of PTPN11 gene and genotype-phenotype correlation in NS in different geographical regions (Bertola et al., 2004; Pierpont et al., 2009; Sznajer et al., 2007; Tartaglia et al., 2006; Yoshida et al., 2004). Mutation screening in NS patients from United States showed that all mutations are exonic changes with the majority clustering in exon 3 and 8. The most common mutation was a c.922A>G in exon 8, leading to the Asn308Asp substitution within the PTP domain (Tartaglia et al., 2002). The occurrence of an adjacent c.923A>G mutation predicting an Asn308Ser change and c.923A>C (Asn308Thr) indicated that codon 308 is a hot spot site for NS with a frequency of 36% in PTPN11 mutated patients (Tartaglia et al., 2002; Tartaglia et al., 2006; Tartaglia et al., 2003). Other studies have reported the occurrence of different mutations types of PTPN11 in European and Asian populations. In Germany, the most common mutation is c.188A>G (Tyr63Cys) followed by c.922A>G (Musante et al., 2003), while in Japan the most common mutation is c.236A>G (Gln79Arg) (Yoshida et al., 2004). In Africa, few studies have focused on mutation screening in NS. Only one report from Morroco identified the c.922A>G mutation in two affected siblings with normal parents (Elalaoui et al., 2010) and c.182A>G (Ratbi et al., 2008). In sub-Saharan Africa, no report from PTPN11 mutation is available to date. The objective in this study was to screen for PTPN11 gene mutations in 6 Senegalese patients with NS and summarized observed clinical features.

**RESULTS AND DISCUSSION**

Clinical features of studied NS patients are summarized in Table 1. In this study, 4 females and 2 males were recruited with ages ranging from 1 to 31 years. None of the patients had known family history of NS. Growth was delayed in all cases. Dysmorphic features were present with variable severity in all cases. Figure 1 illustrates some of these features (hypertelorism, low-set ears, webbed neck, chest deformity, and ptosis) in a male and a female patient. The most frequent cardiac abnormalities were hypertrophic cardiomyopathy (HCM) and pulmonary valve stenosis (PS). One of the male cases had cryptorchidism.

The phenotypes observed in NS are heterogeneous and vary in different ages. The diagnosis of Noonan syndrome is primarily clinical and is guided by the most common dysmorphic signs such as hypertelorism, low-
Table 1. Clinical features in 6 Senegalese patients with NS.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NS1</th>
<th>NS2</th>
<th>NS3</th>
<th>NS4</th>
<th>NS5</th>
<th>NS6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>17</td>
<td>31</td>
<td>4</td>
<td>15</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Growth</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
<td>delayed</td>
</tr>
<tr>
<td>Craniofacial dysmorpho</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertelorism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Webbed neck ptosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiovascular defects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deformity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genital defects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptorchidism</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS: Noonan syndrome; HC: hypertrophic cardiomyopathy; PS: pulmonary valve stenosis.

Table 2. PTPN11 codon 308 mutations in Senegalese patients with NS compared to other populations around the world.

<table>
<thead>
<tr>
<th>Exon 8</th>
<th>rs number</th>
<th>AA change</th>
<th>Senegal</th>
<th>USA</th>
<th>USA</th>
<th>Brazil</th>
<th>Japan</th>
</tr>
</thead>
<tbody>
<tr>
<td>c. 922A&gt;G</td>
<td>rs28933386</td>
<td>N308D</td>
<td>Ndiaye et al</td>
<td>40/204</td>
<td>4/33</td>
<td>-</td>
<td>2/18</td>
</tr>
<tr>
<td>c. 923A&gt;G</td>
<td>rs121918455</td>
<td>N308S</td>
<td>Tartaglia et al. (2006)</td>
<td>13/204</td>
<td>2/33</td>
<td>1/21</td>
<td>-</td>
</tr>
<tr>
<td>c. 923A&gt;C</td>
<td>ND</td>
<td>N308T</td>
<td>2/6</td>
<td>2/204</td>
<td>1/33</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

ND: Not determined.

set ears, chest deformities and short stature, associated cardiac abnormalities (Tartaglia et al., 2002; van der Burgt, 2007; van der Burgt et al., 1994). These morphological abnormalities were observed in most of our patients and were the key elements of diagnosis.

In addition to these morphological abnormalities, alterations in several genes have been implicated in NS. This is the case of the PTPN11 gene which is mutated in 40 to 50% of patients with NS (Tartaglia et al., 2001). Mutation screening of PTPN11 gene in studied exons have identified a heterozygote substitution in exon 8, c.923A>C, in two unrelated patients NS5 and NS6 (Figure 2). This missense mutation led to N308T substitution on the PTP domain of SHP-2 protein. This mutation has not been detected in 15 healthy Senegalese controls.

The mutation rate found in our study (2 of the 6 studied patients) is lower than reported in other populations (Hung et al., 2007; Tartaglia et al., 2002). This difference could be explained, first by the small number of patients enrolled, due to the absence of a clinical consultation in dysmorphology at health facilities in Senegal. The six patients recruited are indeed followed up in the Cardiology Unit of Aristide Le Dantec Hospital for their associated heart abnormalities. Secondly, this study only have sequenced the exons 3, 4, 7 and 8 which are most frequently mutated in NS but mutations may be observed in other exons not studied (Musante et al., 2003; Yoshida et al., 2004). Thirdly, PTPN11 is not the only gene associated with NS. Mutations have also been reported in genes such as KRAS, RAF, SOS1 all involved in the same signaling RAS/MAP pathway (Roberts et al., 2013).

The c.923A>C mutation observed in our study is heterozygous and confirms the autosomal dominant transmission of NS. This mutation leads to the substitution of N308T in SHP-2 protein. This mutation has not been detected in 15 healthy Senegalese controls.

The mutation rate found in our study (2 of the 6 studied patients) is lower than reported in other populations (Hung et al., 2007; Tartaglia et al., 2002). This difference could be explained, first by the small number of patients enrolled, due to the absence of a clinical consultation in dysmorphology at health facilities in Senegal. The six patients recruited are indeed followed up in the Cardiology Unit of Aristide Le Dantec Hospital for their associated heart abnormalities. Secondly, this study only have sequenced the exons 3, 4, 7 and 8 which are most frequently mutated in NS but mutations may be observed in other exons not studied (Musante et al., 2003; Yoshida et al., 2004). Thirdly, PTPN11 is not the only gene associated with NS. Mutations have also been reported in genes such as KRAS, RAF, SOS1 all involved in the same signaling RAS/MAP pathway (Roberts et al., 2013).

The c.923A>C mutation observed in our study is heterozygous and confirms the autosomal dominant transmission of NS. This mutation leads to the substitution of N308T in SHP-2 protein. It is the first time that this mutation is reported in African patients with NS. Two previous studies have reported this mutation in USA in 3 individuals without any details about their Caucasian or African-American ethnic origin (Pierpont et al., 2009; Tartaglia et al., 2006) (Table 2). It was hypothesized that N308T as de novo mutation may
Two NS patients: (A and B) a 4 year old boy (NS3) without mutations in studied exons of PTPN11 gene, typical signs of dysmorph are ptosis, hypertelorism, webbed neck, chest deformity; (C and D) a 4 year old girl (NS6) with mutation c.923A˃C in exon 8 of PTPN11 gene, typical signs of dysmorph are low set ears, hypertelorism, webbed neck.

Figure 2. Sequence plots alignment around position 923 of exon 8 of PTPN11 gene in studied patients. NS1 to NS4 are homozygous for the wild type allele at position 923 (genotype A/A); NS5 and NS6 have a heterozygous mutation c.923A˃C (genotype A/C).

Conclusions

This study focused on finding the PTPN11 gene mutations involved in Senegalese patients with Noonan syndrome. Two patients in six had a heterozygous mutation, c.923A˃C in exon 8 of PTPN11 gene, resulting in amino acid change Asn308Thr in SHP-2 protein. This is the first time that this mutation is described in Noonan syndrome in Africa although position 308 is considered as a "hot spot" site. The results presented are preliminary results of a pilot study of the PTPN11 gene in Senegalese patients with Noonan syndrome and currently followed in the health services in Dakar.

ACKNOWLEDGEMENTS

The authors thank the patients and their families for their interest in this study. They also thank clinicians of the Cardiology Unit of Aristide le Dantec Hospital and Fann Hospital for patient recruitment. Thanks to the Parasitology Laboratory of University Cheikh Anta Diop and the
Molecular Biology Laboratory of Aristide Le Dantec Hospital for providing technical facilities. Funding for this work came from the Third World Academy of Science (RGA/ N° 07-053RG/BIO/AF/AC).

ABBREVIATIONS

NS, Noonan syndrome; PTPN11, protein tyrosine phosphatase non receptor 11; PTP, protein tyrosine phosphatase domain; HC, hypertrophic cardiomyopathy; PS, pulmonary valve stenosis.

REFERENCES


Full Length Research Paper

A study of correlation between CYP2C9 gene polymorphism and Warfarin maintenance dose in anticoagulant therapy among Han people in Yunnan of China

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In this study, we investigated the correlation between CYP2C9 gene polymorphism and maintenance dose of Warfarin in 300 patients who were the Han population derived from the Affiliated Yan An Hospital of Kunming Medical University in Yunnan Province of China, subjected to the operation of cardiac valve replacement and Warfarin oral administration post operation from 2008 to 2009 by detecting the genotypes and Allele Frequency at the three candidate loci (CYP2C9*2, CYP2C9*3 and CYP2C9*c_65) of CYP2C9 gene from the blood samples. Polymerase chain reaction -restriction fragment length polymorphism (PCR-RFLP) assay and DNA sequencing were used to ascertain the genotypes and their corresponding distribution rate. The maintenance dose of Warfarin administered in anticoagulant therapy among the CYP2C9*3 genotypes found in our experiment showed: A/A wild type > A/C heterozygote>C/C homozygote, suggesting that patients with C mutation need the lowest maintenance dose of Warfarin among the three genotypes found in this study. Our results will shed a new light on the personalized medicine of Warfarin and provide basic and genetic experimental data and foundation for future studies with regard to multiple genes’ effect on Warfarin dosage in anticoagulant therapy.

Key words: Warfarin, maintenance dosage, CYP2C9, gene polymorphism, single nucleotide polymorphism (SNP), restriction fragment length polymorphism (RFLP), correlation.

INTRODUCTION

As a kind of common oral anticoagulant, Warfarin is extensively applied for the anticoagulant therapy in various diseases, including valvular heart disease or pathological conditions, valve replacement, fibrillation atrial, electrical conversion, coronary heart disease, pulmonary embolism, deep vein thrombosis and stoke e.t.c. Along with the elevation of incidence rate in chronic fibrillation atrial, cardiovascular and cerebrovascular diseases related to thrombo embolism occur more than ever before. Additionally, the popularity of artificial cardiac valve replacement results in more and more patients receiving long-term oral administration of Warfarin for anticoagulant therapy.

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#ZhiYu Chen and Jintao Li contributed equally to this work.
therapy. However, in the clinical practice of anticoagulant therapy, it is difficult to control the effective maintenance dose of Warfarin, due to the large variation with regard to intra-individual difference in maintenance dose of anticoagulant agent, its therapeutic effect and side effect (Aithal et al., 1999).

Inadaptable dose of Warfarin administration, especially over dose may lead to some serious complications including hemorrhage or thrombosis, even a threat to life. In the early stage of Warfarin administration, the incidence rate of hemorrhage is about 12%, and death rate resulted from hemorrhage in patients is 2% (Levine et al., 2001). It is reported that in America, there are about 2 million patients who were suffered from the side effect of drugs, half of which were lethal (Lazarou et al., 1998). Therefore, to make a change for traditional medication mode into personalized medicine will shed a new light on the settlement of this difficulty by adjusting the Warfarin dose as optimal one suitable for patients and, at the same time, reducing the side effect of it.

To date, among the studies of gene polymorphism of CYP2C9, the most studied and extensive ones were involving in the correlation between mutation of either CYP2C9*2 or CYP2C9*3 and clinical Warfarin maintenance dosage (Higashi et al., 2002). Furthermore, there exists large variation in Allele Frequency of CYP2C9*2 and CYP2C9*3 among different races. However, until now, there are few reports about the roles of gene loci of CYP2C9 in Warfarin administration, and the guidance of Warfarin personalized medication needs intensive study.

In this study, we investigated the correlation between the gene polymorphism of three important gene loci (CYP2C9*2, CYP2C9*3 and CYP2C9*c_65) in CYP2C9 gene and Warfarin maintenance dosage administered in anticoagulant therapy, so as to pave a new way for the personalized medicine of Warfarin according to different genotypes of different patients.

MATERIALS AND METHODS

Object of study

Sample harvesting and Admission standard of patients

A total of 300 patients of Han population who were subjected to cardiac valve replacement from 2008 to 2009 in Affiliated Yan An Hospital of Kunming Medical University in Yunnan Province of China were registered and recruited according to the strict standard (see below) in this study. After informed consent was signed, a total of 3ml peripheral venous blood was extracted from each patient. The blood samples were anticoagulated by addition of ethylenediaminetetraacetic acid (EDTA) and preserved at -48°C.

All 300 patients, more than 18 year-old, were orally administered with Warfarin for anticoagulant therapy under strict monitor persisted for one month post of operation, whose intentional normalized ratio (INR) range from 1.5 to 3.0. In the retrospective whole therapies of the patients were performed at this Hospital. The Warfarin tablets administered to all these patients were produced from the same pharmaceuticals company (Orion Corporation). The strict monitor index consisted of normal hepatic function and obeying dietetic contraindication according to the medical order. Clinical therapeutic and laboratory data were recorded in detail.

Exclusion standard of samples

Under following circumstances, the patients were excluded outside of this study: The patients who had liver diseases at present and before, or his (her) serum transaminase was >120 μmol/L. In the prospective cases, Warfarin was orally administered three months prior to this test. Patients who were administered or administering the drugs influenced the metabolism of Warfarin Herman et al. (2006) Basal INR scale>1.4 - patients who were not appropriate for Warfarin administration due to other reasons.

Definition of target International normalized ratio (INR) scale and stable dosage of Warfarin

International normalized ratio (INR)

Since Professor Armand Quick (1935) set up routine prothrombin time (PT) blood coagulation assay in 1935, until now, it is still an important screen test to measure factors and associated inhibitors in exogenous blood coagulation system. However, the outcome of PT assay was influenced by various factors. Therefore, it must undergo standardization and quality control so as to elevate its precision, accuracy and reliability. In recent years, international normalized ratio (INR) detection was adopted extensively to measure the clotting time, which avoids the differential outcomes in different detections due to different reagents used in this assay.

Target International normalized ratio (INR) in this study

In this study, the scales of INR measured from blood samples of patients were screened by the standard ranging from 1.5 to 3.0, downward for 0.2 was considered as the normal. The patients whose INR were outside of this range were excluded from this study, because under this situation, the Warfarin dose could not attain stability, which is unfavorable to our study and may produce a misleading and even false outcome.

Stable dosages of Warfarin

Stable dosage of Warfarin was referred to: under the same dosage of Warfarin, sequential INR detection of patients ranged from 1.5 to 3.0. The interval of two INR tests was at least above 7 days.

Primer synthesis

The primers used in the PCR assay were synthesized by Sai Bai Sheng Gene Technology Co. Ltd. Primers of these 3 candidate gene loci were as follows:

CYP2C9*2: locates at 3rd exon

Single Nucleotide Polymorphism (SNP) name: rs1799853

Upriver primer 5' - ATGAAACAGACACTACAGAGTG - 3'

Downstream primer 5' - CACGTAAGTCACTGATGAGTAG - 3' CYP2C9*3: locates at 7th exon SNP name: rs1057910

Upriver primer 5' - CTGAATGGCTACAAACTATCTGCCA - 3'

Downstream primer 5' - AGGCTGTGGGGAGAGGCTCC - 3'

CYP2C9*c_65: locates at 3rd exon SNP name: rs9332127

Upriver primer 5' - TTTTGTGTGATGAGGAATTTG - 3'

Downstream primer 5' - CAATTCCAGCTTGATCCATG - 3'
**Table S1. Contents and quantities of PCR reaction system.**

<table>
<thead>
<tr>
<th>Contents of PCR reaction system</th>
<th>Gene locus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP2C9*2 (quantity: μl)</td>
</tr>
<tr>
<td>Distilled water (PH8.2)</td>
<td>14</td>
</tr>
<tr>
<td>10×PCR Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>DNA template (250ng/μl)</td>
<td>1.0</td>
</tr>
<tr>
<td>dNTP</td>
<td>2.0</td>
</tr>
<tr>
<td>Upriver primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Downstream primer</td>
<td>0.5</td>
</tr>
<tr>
<td>TaqDNA polymerase (5U/μl)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Table S2. Contents and quantities of the digestion system of CYP2C9*2, CYP2C9*3 and CYP2C9*c_65 gene locus.**

<table>
<thead>
<tr>
<th>Contents in Digestion system</th>
<th>Gene locus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP2C9*2 (Quantity: μl)</td>
</tr>
<tr>
<td>Distilled water (PH8.2)</td>
<td>7.7</td>
</tr>
<tr>
<td>10 x Buffer (Ava II)</td>
<td>0.8</td>
</tr>
<tr>
<td>Restrictive endonuclease (10u/ μl)</td>
<td>0.5</td>
</tr>
<tr>
<td>Products of PCR amplification</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Selection of restrict endonuclease**

CYP2C9*2: restrict enzyme of Ava II recognition site: GGTC*C
CYP2C9*3: restrict enzyme of Mva1 recognition site: CCA*GG
CYP2C9*c_65: restrict enzyme of Hpa I recognition site: GTT*AAC

**DNA extraction**

Saturated phenol/chloroform method was employed according to traditional procedure described previously by Joanna et al. (2012) to extract DNA from the blood samples of 300 patients who were recruited for this study.

DNA purification and concentration assaying were performed as described in Alessandra et al. (2011) report.

**Primer dilution**

The pairs of primers used in PCR amplification were diluted and adjusted at the concentration of 20 pmol/μl referred to following formula:

\[ \text{Volume of distilled water added (μl)} = \frac{2 \times \text{number of nmol of synthesized primer} \times 10^3}{40} \]

Following dilution, the primer solution was subpackaged and stored at -48°C, awaits further usage.

**PCR amplification**

PCR reaction systems are shown in Table S1. Pure water, 10×PCR reaction buffer, DNA template, dNTP, primers and Taq DNA polymerase was added into 0.2 ml sterile microcentrifuge tube respectively and orderly, covered by paraffin oil. The tubes were placed into the PCR amplifier (GeneAmp PCR SYSTEM 9700, AB applied Biosystems) under conditions set up as following:

PCR reaction conditions of CYP2C9*2, CYP2C9*3 and CYP2C9*c_65 were shown below respectively:

- CYP2C9*2: 97°C 5 min → 80°C 5 min → 94°C1 min → 72°C 1 min → 72°C 10 min 30 cycles
- CYP2C9*3: 97°C 5 min → 80°C 5 min → 94°C 30 s → 64.5°C 30 s → 72°C 30 s → 72°C 10 min 30 cycles
- CYP2C9*c_65: 97°C 5 min → 80°C 5 min → 94°C 30 s → 55°C 30 s → 72°C 30 s → 72°C 10 min 30 cycles

After PCR reaction was finished, the products obtained from PCR were taken out and stored at -20°C.

**Digestion by nucleate endonuclease**

Restrictive endonuclease Ava II, Mva1 and Hpa I were used for digestion in target fragment of CYP2C9*2, CYP2C9*3 and CYP2C9*c_65 gene loci respectively. The contents and quantities of the digestion system of CYP2C9*2, CYP2C9*3 and CYP2C9*c_65 gene loci were shown in Table S2. The digestion reaction was performed in a thermostatic waterbath at 37°C for 14 h.

**Electrophoresis test for products from PCR amplification and digestion**

In this test, 8% vertical native polyacrylamide gel electrophoresis...
Figure 1. Samples 1 to 5 showed the digestive products following PCR, their genotypes were all C/C wild type Sample 6 showed PCR product of CYP2C9*2 locus, whose segment size was 309 bp. Number 1 was heterozygotemutant A/C (127, 105, 75 and 22 bp), Number 2 was homozygote mutant C/C (105, 75 and 22 bp), Number 3 to 5 was wild type A/A (127 and 75 bp), Number 6 was the product of PCR amplification of CYP2C9*2 locus. with segment size of 202 bp.

was employed. Silver Nitrate staining was used to observe the outcomes of the electrophoresis. By using 50 and 100 bp DNA ladder as standard molecular weight markers, the lengths of DNA fragments of target genes were ascertained and their genotypes were determined. Silver Nitrate staining method was used for coloration. Camera (Canon, IXY DIGITAL) was used to take pictures of the staining outcome. Dry gelatin was made so as to preserve the outcome.

DNA sequencing

DNA sequencing of three loci of CYP2C9 gene following PCR amplification

The products of PCR amplification of CYP2C9*2 (rs1799853), CYP2C9*3 (rs1057910) and CYP2C9*c_65 (rs9332127) were subjected to DNA sequencing. A total of 5 DNA samples from each locus were used for DNA sequencing in order to ascertain whether the PCR amplification products was the expectant target fragments. 3130-Gene Analysis Apparatus (ABI Company, America) was employed to perform automatic DNA sequencing.

DNA sequencing of three loci of CYP2C9 gene following PCR amplification and digestion

The digestive products underwent DNA sequencing at CYP2C9*3 locus consisted of 5 cases of A/A wild type (127 bp, 75 bp), 1 case of C/C homozygote mutation type (105 bp, 75 bp, 22 bp) and 2 cases of A/C heterozygote mutant (127 bp, 105 bp, 75 bp, 22 bp).

The DNA sequencing for the digestive products of CYP2C9*c_65 locus following PCR amplification included 5 cases of G/G wild type (317 and 54 bp) and 2 cases of G/C heterozygote mutant (371, 317 and 54 bp).

Statistical analysis

Experimental data were expressed as mean±SD and analyzed by SPSS15.0 and PLINK software. Hardy-Weinberg law of genetic equilibrium was employed to detect the goodness of fit-test of genetic balance. The genotypes and allelotype frequency of three gene loci of CYP2C9 were calculated. χ²-test was used to analyze the data of genotypes and allelotype-frequency. A level of p<0.05 was considered as statistical significance.

RESULTS

Products of PCR amplification and genotypes of three loci of CYP2C9 gene

The segment size of CYP2C9*2 locus derived from the PCR and digestion in 300 DNA samples was all 309 bp (Figure 1) With the digestion by restrictive endonuclease AvaII, when restriction enzyme cutting site was at base C, fragment of 309 bp-product following PCR was digested into three fragments, whose segment size was 195, 91 and 23 bp respectively. When restriction enzyme cutting site was at base T, the fragment of product following PCR could be digested into two fragments, whose segment size was 286 and 23 bp respectively. Only one allele- C (195, 91 and 23 bp) and one kind of genotype- C/C wild type (Figure 1) was checked out in the 300 samples. Among the 300 DNA samples, there was no mutational site was detected at CYP2C9*2 gene locus. As the digestive product of 23 bp fragment at CYP2C9*2 locus was the smallest one, when polyacrylamide gel electrophoresis (PAGE) finished, this fragment have run out of the gelatin, image of this fragment could not be observed within the gelatin. Only the 195 and 91 bp DNA fragments could be observed.

The segment size of CYP2C9*3 locus obtained from the PCR and digestion in 300 DNA samples was all 202 bp.
Samples 1 to 5 showed the digestive products following PCR, their genotypes were all C/C wild type. Sample 6 showed PCR product of CYP2C9*2 locus, whose segment size was 309 bp.

Number 1 to 6 showed PCR amplified fragments of CYP2C9*c_65 locus, with segment size of 371 bp.

bp (Figure S1). With restrictive nuclease va1 digestion, when the restriction enzyme cutting site was at base A, the 202 bp DNA fragments were digested into two fragments, whose segment size was 127 and 75 bp respectively. When the restriction enzyme cutting site was at base C, the DNA fragments (202 bp) were digested into three fragments, with segment size of 105, 75 and 22 bp respectively. Among 300 samples, two kinds of alleles were detectable, which were A and C. There were three kinds of genotypes found in these 300 samples, exhibiting A/A wild type (127 bp, 75 bp), C/C homozygosis mutant (105, 75 and 22 bp) and A/C heterozygote mutant (127, 105, 75 and 22 bp) in genotypes (Figure S1). Among these 300 DNA samples, there were 276 cases of A/A wild type for CYP2C9*3 locus, which accounted for 92%. There were 22 cases of A/C heterozygote mutant, accounting for 7.3%. There were 2 cases of C/C homozygote mutant, accounting for 0.7%. The frequency of allele A was 95.67%, and that of allele C was 4.03%.

The DNA fragment of PCR and digestive product at CYP2C9*3 locus was 22 bp. As it was too small, when the gel electrophoresis finished, this fragment has run out of the gelatin, this fragment could not be seen within the gelatin. Only DNA fragments sized 127, 105 and 75 bp could be observed.

The segment size of CYP2C9*c_65 locus derived from the PCR and digestion in 300 DNA samples was all 371 bp (Figure S3). By using restrictive nuclease Hpa I, when enzyme cutting site located at base G, 371 bp sized DNA fragment obtained from PCR was digested into two fragments, whose segment size was 317 and 54 bp respectively. When the enzyme cutting site located at base C, the DNA fragment of 371 bp could not digested by Hpa I, leaving a single fragment of 371 bp. Among the 300 DNA samples, there were two kinds of alleles detectable, which were G and C. There were two kinds of genotypes found in these 3000 DNA samples, exhibiting G/G wild type and G/C heterozygote mutant (Figure S2). Among these 300 DNA samples, there were 281 cases showing G/G wild type, accounted for 93.7%. There were 19 cases showing heterozygote mutant, accounting for 6.3%. The frequency of allele G was 96.83%, and that of allele C was 3.17% (Figure S3).
Figure S3. Showed genotypes of CYP2C9*c_65 locus. Number 1 to 4 were G/G of wild type (317 and 54 bp), Number 5-6 were heterozygote mutant (371, 317 and 54 bp).

Table 1. Hardy-Weinberg goodness of fit test of genotypes of CYP2C9*3 locus.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>A1/A1</th>
<th>A1/A2</th>
<th>A2/A2</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual frequency</td>
<td>276</td>
<td>22</td>
<td>2</td>
<td>4.002</td>
<td>0.177 (&gt;0.05)</td>
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<tr>
<td>HW theoretical frequency</td>
<td>274.56</td>
<td>24.87</td>
<td>0.56</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 2. Hardy-Weinberg goodness of fit test of genotypes of CYP2C9*c_65 locus.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>A1/A1</th>
<th>A1/A2</th>
<th>A2/A2</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual frequency</td>
<td>281</td>
<td>19</td>
<td>0</td>
<td>0.321</td>
<td>0.451 (&gt;0.05)</td>
</tr>
<tr>
<td>HW theoretical frequency</td>
<td>281.30</td>
<td>18.40</td>
<td>0.30</td>
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</table>

Table 3. Sex distribution of CYP2C9*3 locus.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Genotype frequency</th>
<th>CYP2C9*3 locus</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CYP2C9*3 locus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP2C9*3 locus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Allele frequency</td>
<td></td>
</tr>
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<td>cases</td>
<td>A1/A1</td>
<td>A1/A2</td>
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<tr>
<td>Male</td>
<td>106</td>
<td>94</td>
<td>10</td>
</tr>
<tr>
<td>Female</td>
<td>194</td>
<td>182</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>χ²</td>
<td>0.581</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.434 (&gt;0.05)</td>
<td>0.714 (&gt;0.05)</td>
</tr>
</tbody>
</table>

DNA sequencing

Genetic analysis apparatus (3130 Genetic Analyzer, AB applied Biosystems) was used for sequencing the DNA fragments from PCR and restrictive nuclease digestion was conducted to confirm them as target DNA fragments. Results showed that sequencing outcome was in accordance with the standard DNA sequences of target DNA checked out from GeneBank.

Statistical analysis

Hardy-Weinberg balance test (HWSIM) statistical analysis revealed that the observed number coincided well with the expected value in CYP2C9*3 and CYP2C9*c_65 locus (P>0.05), which was in accordance with the Hardy–Weinberg balance law, suggesting that the DNA samples possessed group representativeness (Tables 1 and 2). Sex distribution of CYP2C9*3 and CYP2C9*c_65
DISCUSSION

In the present study, only the population of patients subjected to the operation of cardiac valve replacement was investigated. Because, in this population, patients routinely administered Warfarin, which provides an appropriate opportunity to examine the correlation of genotypes of special loci in CYP2C9 gene and Warfarin maintenance dosage. As for other diseases apart from cardiovascular disorders, such as diabetes, hypertension, were not involved in this study due to their independent with our study.

Influence of CYP2C9 gene mutation on Warfarin dosage in anticoagulant therapy and possible mechanism

The molecular mechanism of CYP2C9 gene polymorphism leading to metabolic defect of Warfarin lies on the base mutation, which results in alteration of sequences of DNA base, and then, amino acid replacement occurs, ultimately changes the catalytic activity of proteins, expressing as weak metabolic pattern and enzyme deficiency pattern. So far, researches involving in the correlation between CYP2C9 mutation and maintenance dosage of Warfarin in anticoagulant therapy demonstrated that CYP2C9 gene mutation could decrease the Warfarin metabolism. Therefore, patients with CYP2C9 gene mutation needed relatively lower dosage of Warfarin.

Higashi et al. (2002) firstly reported that the correlation between genotype of CYP2C9 gene and anticoagulation or hemorrhage. Subsequently, Sconce et al. (2005) found mutation of CYP2C9*2 or CYP2C9*3 reduced the Warfarin dosage needed in patients subjected to anticoagulant therapy, and CYP2C9*3 mutation led to a more large extent of Warfarin dosage reduction (30% reduced). There exists much variation of allele frequency in CYP2C9*2 and CYP2C9*3 loci among different races (Margaglione, 2000; Taube, 2000; Loebstein, 2001; Sanderson, 2005; Yu, 2004; Mizutani, 2003; Hong, 2005; Bae, 2005) as well. In White People, obvious uniformity is commonly seen in the distribution of allele frequency of CYP2C9*2 gene in that the allele frequency ranges from 8 to 19% (Nakai, 2005; Scordo, 2001; Garcia-Martin, 2001). Higashi et al. (2002) reported CYP2C9 gene has high genetic polymorphism, especially in the mutation of CYP2C9*2 and CYP2C9*3, because the activity of enzymes encoded by them decreased 30 and 80% respectively when compared with that of wild type CYP2C9*1, which is the main cause of CYP2C9 mutation, leading to the lower dosage of Warfarin administered in patients with CYP2C9 mutation. Although the correlation relationship between CYP2C9*2 and/or CYP2C9*3 and Warfarin maintenance dosage in the Han people in China was few reported. Additionally, the crucial role of gene polymorphism of CYP2C9*2 and/or CYP2C9*3 in the intra-individual variation of Warfarin dose attracts more and more attention. Therefore, in this study, our finding that the influence of CYP2C9 gene polymorphism on the substrate drug metabolism has gene dosage effect sheds a new light on the personalized medication of Warfarin by applying Warfarin according to different genotypes of patients. Importantly, results from CYP2C9*3 locus study showed that the maintenance dosage of Warfarin administered in anticoagulant therapy among the CYP2C9*3 genotypes exhibited: A/A wild type >A/C heterozygote>C/C homozygote, suggesting that patients carried with C/C mutation needed the lowest maintenance dose of Warfarin among the three genotypes found in this study. Our investigation is the first time to elucidate the conclusive correlation between the mutant in CYP2C9*3 gene locus and Warfarin maintenance dosage in Han people for clinical practice, providing a novel, useful and effective guidance for Warfarin personalized medication according to different genotypes in different patients.

Correlation between CYP2C9*c_65 locus mutation and Warfarin maintenance dosage

In the present study, CYP2C9*c_65 locus-genotypes and their corresponding distributed rates suggesting the mutation at CYP2C9*c_65 locus may not correlated with maintenance dosage of Warfarin. However, Chern et al.
(2006) found in Chinese Taiwanese population that the mutation in CYP2C9*c_65 locus could reduce the Warfarin dosage administered, and in patients carried c_65 heterozygote or homozygote only needed one half of conventional dosage of Warfarin. To further ascertain the conclusive correlation between mutation of CYP2C9*c_65 locus and the Warfarin maintenance, we investigate whether or not this correlation exist in the Han people of China. The results of ours and other authors above mentioned were much different, it is indicative that CYP2C9*c_65 mutation has obvious variation among different populations and races. At least until now, there are no definite evidence revealing CYP2C9*c_65 mutation has conclusive correlation with Warfarin maintenance administered in anticoagulant therapy. This is another first report proposed by us from this study in Han people of China. We also found that there was no significant difference in genotype-distribution and allele frequency in CYP2C9*3 and CYP2C9 c_65 gene loci between male and female patients, suggesting CYP2C9 gene polymorphism had no definitive correlation with gender variation.

Taken together, the maintenance dose of Warfarin administered in anticoagulant therapy among the CYP2C9*3 genotypes found in our experiment showed: A/A wild type> A/C heterozygote>C/C homozygote, suggesting that patients with C mutation need the lowest maintenance dose of Warfarin among the three genotypes found in this study. This will provide a conclusive and effective guidance for the Warfarin maintenance dose application according to different genotypes of patients. In future, intensive studies should focus on the correlation of more gene loci with Warfarin maintenance dose administered in the anticoagulant therapy and how to perform personalized medication of Warfarin in clinical practice.

ABBREVIATIONS

INR, International normalized ratio; PT, prothrombin time; SNP, single nucleotide polymorphism.

REFERENCES


UPCOMING CONFERENCES

Human Genome Meeting (HGM) :: HGM 2014

The Biology Of Genomes May 6 - 10, 2014
Conferences and Advert

April 2014
Human Genome Meeting (HGM) :: HGM 2014

May 2014
The Biology Of Genomes May 6 - 10, 2014

September 2014
2nd International Conference on Genomics & Pharmacogenomics September 08-10, 2014 Raleigh, North Carolina, USA
Journal of Medical Genetics and Genomics

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