Chapter 3

T920C mutation has no effect on the expression of butyrylcholinesterase messenger RNA
3.1. ABSTRACT

In an Indian community named Vysya, a SNP was identified in the \textit{BCHE} gene \((T920C)\) which alternates the leucine residue by proline at the 307 position in BChE protein and that leads to the disappearance of the protein from serum. Several SNPs are reported to have an up regulating or down regulating effect on its mRNA expression. The influence of the L307P mutation on the expression of \textit{BCHE} mRNA was evaluated by Taqman gene expression assay. The expression levels of mRNAs of silent variants and heterozygous were compared with the wild individuals from different ethnic group. The results showed that the expression of \textit{BCHE} gene is similar in all the Vysya individuals which specify that the absence of protein in serum is not because of the defective transcription mechanism. This conclusion has been corroborated by the DNA sequencing of the promoter regions that are involved in the transcription of \textit{BCHE} gene. No SNPs were present in these regions and thus confirming that these sites were impeccable in comparison with wild counterparts. Hence, we conclude that \textit{T920C} mutation in exon 2 of \textit{BCHE} gene has no association in its transcriptional regulatory mechanism and consequential mRNA expression.
3.2. INTRODUCTION

The precise physiological role of the hBChE enzyme is unclear; however, it is well documented to hydrolyse OP derivatives and several other similar esters of drugs on accidental ingestion. Certain individuals with lower levels of esterase activity and negligible BChE protein in serum are recorded who experienced a prolonged apnea following the administration of muscle relaxants like SuCh and mivacurium. Liddell et al., described such a patient whose serum was apparently devoid of BChE and its activity, therefore dubbed as the 'silent variant'. Such a silent variant was reported from our lab and it was identified in 4% of the Vysya population living in Tamil Nadu, India. All such silent BChE samples were homozygous for the point mutation at codon 307 (CTT→CCT; T920C) resulting in the substitution of leucine at 307 by proline. In these people, the esterase activity was less than 1% in the serum. In fact, the western blot analysis of plasma samples from these individuals showed the complete absence of BChE protein [Manoharan et al., 2006].

Till date, about 43 such silent variants are already identified in the BCHE gene. In all these silent variants, the cause for the absence of protein is not due to any mutations in the promoter region of the BCHE gene. But a defective promoter can also alter the translation of its protein. BCHE promoter appears to belong to the class of promoters which has no apparent TATA element, is not GC-rich, is not constitutively expressed but it is regulated during differentiation or developmental process. Additionally, initiation of transcription at only one or a few tightly clustered start sites was reported. Thus, the BCHE gene promoter has an initiator element within the transcription start site itself [Smale and Baltimore, 1989]. The transcription start site was found to be 157 bp upstream of Met^{28}, i.e. the translation start site. The 5' flanking regions of hBCHE encompass a single binding site for AP1 and multiple binding sites.
for Oct-1, PEAS, and topoisomerase II which are essential sites for the initiation of transcription. The AP1 binding site is situated 71 bp upstream of the transcription start site, thus the 200 bp fragment upstream of the translation site is defined as the promoter site in human BCHE gene [Jbilo et al., 1994]. Several SNPs in such regions of other genes are reported to have an up/down regulatory effect on their mRNA expression [Wang and Sadee, 2006]. However, no such modulatory role has been ascribed to BCHE gene. Hence in the present study, an attempt has been made to screen the promoter regions that are essential for transcription of BCHE for the possible presence of mutations that can interfere with mRNA synthesis and subsequent plasma levels of BChE. Also the influence of the T920C mutation on the gene expression of silent BCHE was evaluated.

3.3. MATERIALS AND METHODS

3.3.1. Biological Samples and DNA Extraction

Blood samples from 30 individuals were collected into tubes containing the anticoagulant trisodium citrate (3 mmol/l) from the Vysya community living in Coimbatore, Tamil Nadu, India. The 25 individuals consist of 10 homozygous mutant (C/C) and 15 heterozygous (T/C). 15 samples were collected from the non-Vysya individuals as wild (T/T) samples. All investigations were conducted according to Declaration of Helsinki principles. Informed consent was obtained from all subjects. Genomic DNA was extracted from 200 µl of blood using HiPurA blood genomic DNA miniprep purification spin kit (HiMedia, India).

3.3.2. PCR Reaction and DNA Sequencing

Two sets of primers were designed for the amplifying the entire promoter region of the BCHE gene. The sequences of the primers used are listed in table 3.6.1. Amplification was carried out in 20µl of reaction mixtures containing 20 ng of
genomic DNA, 2 µl Taq buffer (1X), MgCl₂ (2.5 µM), dNTP mixture (0.25 µM each), forward primer and reverse primer (1 µM each) and 1 U Taq polymerase. The DNA was amplified for initial denaturation at 94°C for 5 min followed by 35 cycles with denaturation at 94°C for 30 sec, annealing at 57°C and 58°C for 1 min for primer set 1 and set 2, respectively and extension at 72°C for 2 min, final extension was done for 10 min at 72°C. Amplified DNA samples were sequenced at Chromous Biotech Private Limited, India. DNA Cycle sequencing was performed using the ABI Prism 377 DNA sequencer with dye terminator chemistry.

3.3.3. RNA Extraction and Reverse Transcription

Total RNA was extracted from the blood samples with TRI REAGENT BD (Sigma, USA) according to the manufacturer’s protocol. The extracted RNA was treated with DNAase for 2 h at 37°C. Reverse transcription was conducted in 20 µl. RT reaction mix contains 1 µg of the extracted RNA, 1X reverse transcription buffer, 0.5 mM dNTP mix, 100 pmol random hexamer primer and 1U RevertAid premium enzyme mix (Fermentas Life sciences, Thermo Scientific Molecular Biology, USA). The mixture was incubated at 25°C for 10 min and subsequently at 50°C for 30 min. Reverse transcription was terminated by means of heating at 85°C for 5 min.

3.3.4. Gene Expression

Gene expression studies of BCHE were done by relative quantification according to the comparative ΔCt method in ABI 7500 real time system (Applied Biosystems, USA). The Taqman primer-probe set was used for the amplification of 172 bp length BCHE gene sequence (Hs00992319_m1). The probe sequence spans the third and fourth exons of BCHE gene. The TaqMan probes were labeled with a reporter fluorescent dye (FAM) at the 5’ end and a quencher fluorescent dye (NFQ-MGB) at the 3’ end, by which the reporter dye emission was quenched when the probe was intact.
GAPDH (Assay ID: Hs03929097_g1) and 18S rRNA (Assay ID: Hs99999901_s1) was used as the endogenous controls. Results of multiple reactions were analyzed together for compiling all the samples in a single experiment. PCR reactions were run in a total volume of 5 µl, containing 1 µl cDNA, 2.5 µl 1X Taqman universal master mix II without UNG and 0.25 µl 20 X Taqman primer-probe mix. Reactions were run with the following cycle parameters: 95ºC for 10 min; followed by 40 cycles at 95ºC for 15 sec and 60ºC for 30 sec.

3.4. RESULTS AND DISCUSSION

3.4.1. Promoter screening of BCHE gene

The whole promoter region of the BCHE gene was amplified and screened by DNA sequencing method for the presence of anonymous SNPs in the regions that are essential for the transcription of BChE mRNA. DNA sequencing showed that the AP1, PEAS and Oct-1 regions of the promoter were normal for the BChE silent variants (Fig.3.6.1). Previously reported that in cholesteryl ester transfer protein (CETP) gene, a SNP which substitutes G to A at the 269 nucleotide in the promoter region corresponding to the second nucleotide of the PEA3/ETS binding site located upstream of the TATA box causes the decreased transcriptional activity leading to hyperalphalipoproteinemia (HALP) [Nagano et al., 2001]. Lim et al., (2006) reported that in SLC6A4 gene that encodes the serotonin transporter protein, a repeat polymorphism in the promoter region (SERTLPR) has been suspected to affect expression and thus it has been implicated in depression and other mental disorders. But we observed that the promoter region for BCHE gene is normal even in the silent variants of BChE confirming that the disappearance of the protein in the biological system is not because of the defective transcription mechanism in BCHE gene.
3.4.2. Gene expression of BCHE

The human tissue that contained abundant BCHE mRNA was liver, followed by lung, brain, heart, skeletal muscle, pancreas and kidney [Jbilo et al., 1994]. A high level of BCHE mRNA in liver supports the finding that liver is the source of blood plasma BChE. Since liver and lung tissues are known as the principal detoxification sites of the human body, the abundance of BCHE mRNA in these tissues indicate a major role for BChE and it could be its role as the first line of defence against poisons that are ingested or inhaled. There was no detectable BCHE mRNA in placenta. The study by Jbilo et al., (1994) reported that neither red blood cells nor plasma contains mRNA, despite their high content of ChE enzyme activity. But in 2010, Assayag et al., (2010) showed prominent BCHE gene expression levels in nucleated blood cells by microarray tests. Esterase activity and mRNA ratios usually do not correlate directly with each other. Thus either lower or higher enzyme activity in any tissue does not necessarily predict the quantity of mRNA.

In the present study, total RNA was extracted and cDNA was synthesized from all the samples. The samples were grouped into three biological groups designated as wild (T/T), heterozygous (T/C) and mutant (C/C). The cDNA amplification graphs of BCHE, GAPDH and 18S rRNA from each biological group were shown in Fig. 3.6.2. From the figure it is clear that quantity of BCHE mRNA is same in all the three groups by observing the mean cycle threshold (ΔC₇) value of amplification (Table 3.6.2). Further, the mRNA expression analysis showed that the level of BCHE mRNA expression is same in all the biological groups (Fig. 3.6.3). The relative quantification value (RQ) of each gene in a group is mentioned in Table. 3.6.2. The RQ of wild group is considered to be 1 and in comparison with this group, the heterozygous and mutant groups show RQ values 0.96 and 0.93, respectively which shows similar mRNA
expression. Disagreeing with our observation, previous reports showed that several human SNPs affect the expression of their genes. For example, the mutation C3435T at exon 26 of the multi-drug resistant (MDR1) gene associated with the higher level of MDR1 mRNA in the duodenum in Japanese persons [Nakamura et al., 2002]. Also, in Dopamine receptor D2 (DRD2), C957T SNP altered the mRNA folding, led to a decrease in mRNA stability and thus the translation dramatically altered the dopamine-induced up-regulation of DRD2 expression [Duan et al., 2003]. Contrary to these reports our results showed that the T920C mutation in the exon2 of BCHE gene does not have transcriptional role on BCHE mRNA and thus the silence of the protein in the plasma is due to its inability to withstand against the plethora of protease due to the altered structure conformation and thus attributed to possible degradation.

3.5. CONCLUSION

In the present study, the promoter region of the BCHE gene that are essential for the transcription was sequenced for the presence of possible SNPs that can alter the transcription and subsequent protein synthesize. But to our surprise, these regions were normal when compared to the wild. Thus, we confirmed that the complete absence of BChE protein in plasma is not because of defective promoter of BCHE gene. Also, the effect of T920C mutation in BCHE gene on the expression of its mRNA levels were similar in the wild (T/T) as well as in the heterozygous (T/C) and the mutant (C/C) individuals of Vysya community. Thus we concluded that: i) the mutation (L307P; T920C) do not have any effect on transcription of BCHE mRNA, ii) the absence of the protein is not because of any defective promoter region and iii) conformational instability of L307P mutation is predicted which could be the reason for the absence of BChE in plasma. To the best of our knowledge, this study is the first attempt to analyze the influence of T920C mutation on BCHE mRNA expression.
### 3.6. TABLES AND FIGURES

**Table 3.6.1. Primers used for the amplification of promoter region**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-1</td>
<td>CAGCTGCTGCTGTAAGTTGAG</td>
<td>1.1kb</td>
</tr>
<tr>
<td>PR-1</td>
<td>CCACTAGTAGCTGTTGGAAAA</td>
<td></td>
</tr>
<tr>
<td>PF-2</td>
<td>CTGTTTTTCAACAGCTACTAG</td>
<td>1.2kb</td>
</tr>
<tr>
<td>PR-2</td>
<td>CCAGCCTGTAAATTGGACTGC</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.6.2. Normalized cycle threshold (ΔC)_T_ and RQ values of Vysya individuals**

<table>
<thead>
<tr>
<th>Biological groups</th>
<th>ΔC_T_ mean</th>
<th>RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild (T/T)</td>
<td>30.10</td>
<td>1</td>
</tr>
<tr>
<td>Heterozygous (T/C)</td>
<td>29.79</td>
<td>0.96</td>
</tr>
<tr>
<td>Mutant (C/C)</td>
<td>29.53</td>
<td>0.93</td>
</tr>
</tbody>
</table>
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Figure 3.6.1. Sequence alignment of the sequenced promoter region for wild and silent BCHE gene. The promoter region of the wild and silent BChE individuals was sequenced. Both sequences are aligned using BLAST. The PEA (yellow), Oct (blue) and AP1 (green) regions which are essential for the transcription of BCHE gene is highlighted and boxed. W and S denotes the wild and silent BCHE sequences respectively. Highlighted nucleotide A (cyan colour) is the transcription start site. Nucleotide changes shown as bold in figure are considered as synonymous mutations since it did not affect the mRNA expression.

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Figure 3.6.2. Amplification plots of BCHE, GAPDH and 18S rRNA in different biological groups. Amplification data of samples representing each biological group for the three genes are shown. The blue colour line denotes the 18S rRNA and green colour denotes GAPDH which are used as endogenous controls. The red colour line denotes the target gene BCHE. The cycle threshold (C<sub>T</sub>) of each sample is clearly perceived from the plot.
Figure 3.6.3. Expression of *BCHE* gene in different biological groups of Vysya individuals. The samples were grouped into different biological groups indicated in the figure. Wild (T/T) group consist of 15 non-Vysya individuals (i.e. controls), heterozygous (T/C) and mutant (C/C) groups consist of 15 and 10 Vysya individuals respectively. The expression levels are similar in all the three groups. The RQ values are given in Table 3.6.2