Identification of Novel Mutations in LCT-Gene of Pakistani Patients with Lactose Non-persistence

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ABSTRACT

Lactose non-persistence (LNP) develops due to the downregulation of the lactase-phlorizin hydrdolase (LPH) enzyme. LPH is encoded by the LCT-gene located on chromosome 2q21. In this study, 50 patients with LI were investigated for the identification of mutations in the LCT-gene. In comparison of 30 subjects with lactose persistence (LP) considered as healthy group. A total of 13 genomic mutations were identified, 1 in the promoter region and 12 in the intronic/exonic region of LCT-gene. Among these 12 mutations, 6 of them are novel in origin. The novel mutations were found in intron 4 c.(918+116)A>C, intron 12 c.(4877+20)G>A; intron 15 c.(5346+35)T>C, UTR of exon 17 (c.5865C>T), exon 6 c.1147A>G and c.1095A>G. Four mutations resulted in an altered amino acid sequence. The frequency of each mutation observed in both groups was: mutation I of exon 1 (0.64/1), mutation II of exon 2 (0.34/0.67), mutation III, IV and V of exon 6 (1/0.12/0.0, 0.64/1) mutation VI of exon 13 (0.64/1), mutation VII, VIII, IX of exon 17 (0.64/1, 1/1, 0.12/0), mutation X of intron 4 (0/1), mutation XI of intron 12 (1/0), mutation XII of intron 15 (1/0) and mutation XIII of promoter region (0.36/0.67). In this study, molecular identification and screening in LNP subjects provide the mutational variants of the LCT-gene in the region of Pakistan. This is a major step in clinical management and accurate genetic counseling of the pre-symptomatic diagnosis of LNP. Among the six novel mutations found, mutation XI was found in all of the LNP subjects and was absent in the lactose persistent group. This study, for the first time, focuses on molecular analysis of LCT-gene from Pakistani patients with LNP.

INTRODUCTION

Lactose non-persistence (LNP) (OMIM#223100) is a genetically determined, developmental phenomenon, which occurs after weaning in different populations (EFSA, 2010). LNP is a phenotypic enzyme deficiency caused by decreased lactase enzyme production. It can cause different kinds of intestinal and systemic symptoms: headache, vomiting, nausea, flatulence, abdominal pain, gut distension, diarrhea, constipation, loss of concentration, muscle pain, allergies, heart arrhythmia, mouth ulcers, sore throat and increased Micturition (Asmawi et al., 2006; Babu et al., 2010; EFSA, 2010; Freund et al., 1989; Hovde et al., 2009; Montgomery et al., 1991; Potter et al., 1985; Sahi et al., 1983; Welsh, 1970). Most of the studies related to LNP were reported from Finland. According to one of the reports, Finnish children had an onset age from 0-20 years (Rasinpera et al., 2004; Vesa et al., 2000). Whereas in the present study, the reported age of onset for this enzymatic deficiency varies from 2-17 years (Cook, 1967; Lehtimaki et al., 2006), the highest prevalence in Pakistani patients was found in the range of 2-5 years. In the past, LNP was diagnosed with help of tests like glucose-oxidase test, blood glucose test, lactose tolerance test, glucose-galactose tolerance test, urinary galactose test, stool pH and breath hydrogen test. All of these tests did not provide reliable results (EFSA, 2010; Asmawi et al., 2006; Hovde et al., 2009; Sahi et al., 1983; Bayless et al., 1966; Boll et al., 1991; Cuatrecasas et al., 1965; Dahlqvist, 1961; Ferguson and Maxwell, 1967; Gray et al., 1969; Krawczyk et al., 2008; Lee et al., 1998; Lukito et al., 2015; Raz et al., 2013; Sahi et al., 1973; Sahi, 2001; Tandon et al., 1981; Tomar, 2014; Witte et al., 1990). However, with the advent of PCR and genetic marker analysis techniques, the research work of Scandinavian countries, the United States and Europe were concluded by analyzing various
aspects of LCT-gene along with its cis-acting factors. All these researches used various tests in comparison of genetic markers in order to give a final conclusion about the diagnosis, prevention, treatment and mechanism in development of LNP (Burger et al., 2007; Campbell et al., 2005; Ennatah et al., 2008; Jarvela et al., 2003; Krawczyk et al., 2008; Miquel et al., 2011; Rasinpera et al., 2004; Raz et al., 2013; Sahi et al., 1983; Sahi, 2001; Semenza et al., 1989; Swallow, 2003; Szilagy et al., 2007; Tandon et al., 1981; Tarabra et al., 2010; Troelsen, 2005; Vesa et al., 2000).

MATERIALS AND METHODS

Formal approval was obtained from Institutional Review Board (IRB), at the Institute of Biochemistry and Biotechnology (IBBT), University of Veterinary and Animal Sciences (UVAS) Lahore, Pakistan Vide Letter No DAS/3067 dated 11.12.12. The NCBI Reference Sequence used was NC_000002.12 (www.ncbi.nlm.nih.gov/gene/3938). Written informed consent was taken from the LNP patients specifically belonging to Province Punjab of Pakistan, blood samples of 50 patients were collected. All these cases were diagnosed for LNP based intolerance symptoms after drinking milk. Blood DNA samples of 30 healthy subjects from Punjab were also collected as a control group. The DNA extraction was performed on whole blood according to the standard phenol-chloroform extraction method (Sambrook and Russel, 2012). The equipment used for this process is “Thermo Scientific Gene JET Whole Blood Genomic DNA Purification Mini Kit” #K0781, #K0782 Pub. No.A00012667 manufactured by Thermo Fisher Scientific Inc. The software for designing the primers, of the LCT-gene and promoter region were: ‘Primer 3’, ‘ApE’, Clustalw and others reported by Ranciaro et al. (2014) as shown in Table I. The rest of the work was compiled in the research lab of the Max-Planck Institute for Biophysical Chemistry, Gottingen, Germany. A PCR approach similar to that adopted by Ranciaro with an annealing temperature of 60°C for intron spliced exons and 61.5°C for the promoter region (Ranciaro et al., 2014) was implemented, which amplifies and performs melting analysis. After PCR amplification, PCR products showing a clear band of required lengths, purification was done using GENECLEAN Spin Kit for rapid and user-friendly purification of DNA in three basic steps. The PCR products were eluted with 15µl elution buffer and diluted 1:10 with water. The sequencing was done using an automated ABI PRISM 3100 Genetic Analyzer (SeqLab, Germany). Sequencing data results were analyzed by using ApE a plasmid editor software.

Table I.- Primers used for LCT-gene amplification.

| No. | Primer | Sequence (5'-3') Forward | Sequence (5'-3') Reverse | Size (Bp) |
|-----|--------|--------------------------|--------------------------|----------|
| 1   | EXON 1 | TATTGCATTAGTGAACCGTGTTACTG | TAGCTACCCTCCCTCCTATCGCC | 953      |
| 2   | EXON2  | CAACACACACACACACACACACAC | GAAGCTGACACTTGAGCAACAAGCA | 495      |
| 3   | EXON3  | AAGAGAGAGGTTAGAGGTGGGGG | CCCCATTGATGAGTTGCGCTT | 700      |
| 4   | EXON4  | TAAAGTCTGGTATGCCCATGGT | TCCCTGACTGAGTTGACC | 1050     |
| 5   | EXON5  | CTCAGTGTTTCTGGAATCTTTTC | TAGCTGGGACCATAGGCTAGT | 970      |
| 6   | EXON6  | ACTTTGTGACAGCTCGCAG | TAGTGGTTGGGACTAAGAGAG | 925      |
| 7   | EXON7  | CTTGTAATGGAGGGCTGGCA | GAGTAACCCACCCTTCCA | 840      |
| 8   | EXON8  | ACTTTGTGACAGCTCGCAG | TGGAGTGGGACCTAAGAGAG | 920      |
| 9   | EXON9  | CTTGTAATGGAGGGCTGGCA | CCATGGGCTGGTCTGAAATCT | 700      |
| 10  | EXON10 | AAGAGGGCTGTAGGTCCTTTTG | TGATAAAATGCTGCTCCCCAGC | 425      |
| 11  | EXON11 | CCCATGGAAAGCACTAGCTGA | CAGAGAGCTCCCCACCTGAG | 700      |
| 12  | EXON12 | CTGCCATTTAACCAGAGAG | CCAAGCTCACAATACTAGGC | 848      |
| 13  | EXON13 | GTGAGACTGAGGCTGCAACAG | CCGAGAACACAAAGAGAGCTCAGT | 700      |
| 14  | EXON14 | GACCTAGGGAAGCCAGGTTT | TTCTCTCCCTTCTTGTTGCT | 700      |
| 15  | EXON15 | GCCCTTCTCGTCTCTAAGTCC | TTCTCTTCTACTGTTGGCCCA | 422      |
| 16  | EXON16 | ACTGCACTGCAAAGAAAGGGG | AACAGACTGAGAGAGGCC | 700      |
| 17  | EXON17 | TCAATGCTGCTGGCTGATTGG | AGACCCACTAAAGGGTGTTG | 410      |
| 18  | LCT-P1* | TTGAGACCAAGGCTGGG | TGCCATATGATTTCATGCCATT | 783      |
| 19  | LCT-P2* | GGGCTGAGGCACTCCCTACAG | TTGACCTCCCTGCGGAGAGC | 953      |
| 20  | LCT-P3* | AGAGAAAAATTTGCGGCAAAATAC | ACCCCACGATGAAAAACTT | 980      |

*From Ranciaro et al. (2014).
Fig. 1. Molecular model of LCT-gene (NCBI) 2012.

Fig. 2. Predicting molecular modeling of LCT-gene with alterations.

Table II.- Mutations in LCT-gene associated with lactose non-persistence/lactose persistence.

| Mutation No. | Mutation position | Variant | Exonic/ intronic/ promoter | Novel/ reported | Frequency- LNP/LP | % in LNP (% heterozygosity) | % in LP (% heterozygosity) | Change in amino acid | P-value |
|--------------|-------------------|---------|-----------------------------|-----------------|------------------|----------------------------|----------------------------|----------------------|---------|
| I            | c.593C>T g.593C>T | C>T     | Exon 1                      | R               | 0.64/1           | 64 (20%)                   | 100 (66%)                  | -                    | 0       |
| II           | c.666G>A g.4005G>A | G>A     | Exon 2                      | R               | 0.34/0.67        | 34 (100%)                  | 66 (100%)                  | Val 219 Ile          | 0.005   |
| III          | c.1095A>G g.19206A>G | A>G     | Exon 6                      | N               | 1/1              | 100 (0%)                   | 100 (0%)                   | Ile 362 Val         | -       |
| V            | c.1430C>A g.19541C>A | C>A     | Exon 6                      | R               | 0.64/1           | 64 (0%)                    | 100 (33%)                  | -                    | 0       |
| IV           | c.1147A>G g.19258A>G | A>G     | Exon 6                      | N               | 0.12/0           | 12 (100%)                  | NIL                       | Glu 379Arg          | 0.049   |
| VI           | c.4927A>G g.30981A>G | A>G     | Exon 13                     | R               | 0.64/1           | 64 (20%)                   | 100 (33%)                  | Asn 1639 Ser         | 0.001   |
| VII          | c.5579T>C g.48360T>C | T>C     | Exon 17                     | R               | 0.64/1           | 64 (10%)                   | 100 (66%)                  | -                    | 0.001   |
| VIII         | c.5845G>C g.48896G>C | G>C     | Exon 17                     | R               | 1/1              | 100 (44%)                  | 100 (66%)                  | -                    | -       |
| IX           | c.5865C>T g.48916C>T | C>T     | Exon 17                     | N               | 0.12/0           | 12 (0%)                    | NIL                       | -                    | 0.049   |
| XII          | c.(5346+35)T>C g.49004.T>C | T>C     | Intron 15                   | N               | 1/1              | 100 (0%)                   | 100 (0%)                  | -                    | -       |
| X            | c.(918+116)A>C g.15842A/C | A>C     | Intron 4                    | N               | 0/1              | NIL                       | 100 (0%)                  | -                    | -       |
| XI           | c.(4877+20)G>A g.39039G>A | G>A     | Intron 12                   | N               | 1/0              | 100 (0%)                   | 0                         | -                    | 0.05    |
| XIII         | c.-956 C>T g.-956C/T  | C>T     | Promoter                    | R               | 0.36/0.67        | 36 (100%)                  | 66 (100%)                  | -                    | 0.008   |
Fig. 3. Exonic and intronic alterations in LCT-gene
RESULTS

A total of thirteen genomic mutations were identified in the LCT-gene, including three in intronic, one in the promoter region and nine in the exonic region. The six novel mutations included mutations III, IV (in exon 6), IX (in UTR of exon 17), X, XI and XII (in intron of 4, 12 and 15). Rest of the seven mutations: I, II, V, VI, VII and VIII were already reported in different populations (Burger et al., 2007; Matthews et al., 2005; Enattah et al., 2008; Jarvela et al., 2003; Krawczyk et al., 2008; Morales et al., 2011; Rasinpera et al., 2004; Raz et al., 2013; Sahi et al., 1983; Sahi, 2001; Sebastio et al., 1989; Swallow, 2003; Szilagy et al., 2007; Tandon et al., 1981; Tarabra et al., 2010; Troelsen, 2005; Vesa et al., 2000). A cis-acting factor present in the promoter region was studied at position -956 (mutation XII). The results showed it at a higher frequency in lactose persistent species group with 100% heterozygosity in both groups. As the mutations III, X, XII were also present at a frequency of “1” in the control group, their effect on the development of LNP was nullified. Mutations IV and IX were found at a frequency level of “0.12” in the same patients. The intronic region mutation: XI was found at a frequency of “1” in LNP and “0” in LP. The final change of amino acids was observed in four genomic mutations, but only mutation IV of exon 6, (nucleotide position 379) was novel in origin and was reported in LNP patients, mutation III was presented in both groups at same frequency levels. In intronic region, the mutation XI in intron 12 was found 100% in LNP patients, while among novel exonic mutations (IV and IX) only mutation IV was presented by 100% heterozygosity. All mutations are shown in Table II and Figures 3 and 5. In terms of frequency levels, only mutation IV, IX and XI were only reported in lactose intolerant group. Rest were found at different frequencies in both groups i.e. LNP and LP. All exonic mutations were presented with heterozygosity of 20% to 100% in both groups, which was not the same in intronic region as shown in Table II.

DISCUSSION

LCT enzyme is formed as a protein, which is made up of 1927 amino acids and is further processed to a final main protein of 1059 amino acids (Matthews et al., 2005; Troelsen, 2005). Mutation of LCT-gene has been implicated in LNP, as reported in different earlier studies (Boll et al., 1991; Jarvela et al., 2003; Harvey et al., 1998; Hollox et al., 2001; Wang et al., 1995, 1998). Primers for exon 1, 2, 6, 10, 13 and 17 had a size of 953, 495, 925, 425, 376 and 410 bases, respectively, while the primers for promoter region had a size of 783, 953 and 980 bases, as shown in Table I (Jarvela et al., 2003; Johnson et al., 1993; Tishkoff et al., 2007) (NCBI dated 2.4.16 & 5.5.17). The already reported mutations occurred at codon 194, 219, 371, 473, 819, 1096, 1443, 1499, 1536, 1639 and 1856 in different exons of LCT gene (Boll et al., 1991; Harvey et al., 1998).

Later with the advancement in ongoing research, different studies reported the involvement of cis-acting factors in the nearby regions of LCT-gene and in the development of LP/ LNP in some populations (Boll et al., 1991; Montgomery et al., 1991). In one of the previous studies, the promoter region was also analyzed for its cis-acting factor (Ranciaro et al., 2014). Our study confirmed the same codonic positions 194, 219, 362, 379, 473, 1639, 1856 of exon 1, 2, 6, 13 and 17 named as mutations I, II, V, VI, VII, VIII, and one promoter region named as mutation XIII at position 956 (Figs. 1, 2, 3, 4 and 5) (Harvey et al., 1998; Hollox et al., 2001; Wang et al., 1998).

LNP is the most common phenotype in human and its incidence varies as a function of ethnicity in different populations (Troelsen, 2005). It was found at a higher percentage in Southern Europe as compared to North-Western Europe (Sahi, 2001; Vesa et al., 2000). Likewise, North Americans were more LP as compared to African Americans with West African ancestry (Asmawi et al., 2006). In the Israeli and Afghani population, the incidence of lactose intolerant was found higher i.e. 82-97% and 83%, respectively (Rahimi et al., 1976). In a similar manner, Southern Indians were more LNP as compared to Northern Indians which are in the closest neighbourhood and are collectively considered as the descendants of Aryans (Asmawi et al., 2006; Tandon et al., 1981; Tomar, 2014).
In the current study, among the 12 mutations in LCT-gene, 3 were found in the intronic region and 2 in UTR. The rest of the 7 mutations caused the change of nucleotide in 3 codonic structures. Among the 7 mutations, three produced the same amino acids whereas the other four produced different amino acids.

Mutation I at position c.593 ACC>ACT resulted in the production of Threonine even after a change of one nucleotide. Mutation V at position c.1430 GGC>GGA produced Glycine with both codons. Mutation VII at position c.5779 (GCT>GCC) coded for Alanine simultaneously. Mutation I was also considered to be involved in decreased production of lactase in correlation of transcriptional regulation (Boll et al., 1991; Jarvela et al., 2003; Wang et al., 1995, 1998; Betts et al., 2003). In the present study, it was found at 0.64 frequency level in LNP subjects and 20% of heterozygosity. Mutation V was already reported (Betts et al., 2003; Boll et al., 1991) at encouraging levels of frequency in both LP and LNP groups. Mutation VII was found at a frequency of 0.644 in LNP and 1 in LP, which nullifies its relevance with mutational correlation.

The four important mutations, which resulted in the change of amino acids along with the final protein structure include mutations II, III, IV and VI. Mutation II GTT>ATT at position c.666 caused the change of Valine to Iso-Leucine. This particular mutation is mostly observed in LP individuals. This mutation was also reported to act as a cis-acting factor on LCT-activity. It was also found involved in a population study for reporting 14 haplotypes (A to N) (Boll et al., 1991; Harvey et al., 1998; Hollox et al., 2001; Jarvela et al., 2003; Wang et al., 1995, 1998). Mutation II was observed at a frequency level of 0.67 in LP and at 0.34 in LNP patients with 100% heterozygosity in both groups. Thus, in order to have conclusive data for mutational analysis, we suggest including this exon on a larger scale. Mutation III at position c.1059 (ATC>GCT) which caused the production of valine from iso-leucine. Both mutations II and III are mirror images of each other. As Valine and Iso-Leucine both are non-polar/hydrophilic
in nature. Mutation III, however, is novel in origin, but due to its occurrence at the same frequency level in both LNP and LP patients and homozygosity, its significance is nullified. Mutation IV was found at position c.1147 (CAG > CGG), which is a missense mutation of novel origin. It resulted in the change of Glutamine to Arginine at a frequency level of 0.12 in LNP patients and zero in LP. Mutation VII had been reported at the same level as mutation II (Boll et al., 1991; Harvey et al., 1998; Holloxo et al., 2001; Wang et al., 1998). Mutation VI at position c.1430(GCC>GCA) resulted in a change of Asparginine to Serine. In current data analysis, mutation VI was observed at a frequency level of 0.64 in the LNP group, however, it could not be correlated to the development of LNP due to co-presence in the control group.

In the untranslated region, mutation No. VIII and IX were observed at position c.5845 G>C and c.5865 C>T, respectively. Mutation VIII was also reported (Boll et al., 1991). Wang (1998) also aligned the data for the presence of heterozygosity along with the lactase sucrase activity, supporting cis-activity. The first variant was present in 100% of LNP and LP groups, having a frequency of “1” in both. Therefore, this mutation cannot be relied upon as one of the causes of the intolerant state (Boll et al., 1991; Holloxo et al., 2001; Jarvela et al., 2003). The last mutation of the untranslated region was novel in origin and was found at a frequency of 0.12 in LNP group, whereas none of the LP group showed its presence. Mutation IV and IX (at exon 6 and 17) must be investigated at a larger population scale in order to deduce a conclusion. These two novel mutations were found at codon No. 379 and 1856 were observed in the same province and attributed to environmental factors and genetic variations amongst different racial/ethnic groups. Alternatively, it may be related to a lack of clinical studies on mutations in the LCT-gene along with the promoter region, other than those at codons 593, 666, 1430, 4927, 5579 and 5845 (Boll et al., 1991; Harvey et al., 1998; Jarvela et al., 2003).

The identification of novel mutations in Exon 6, 17 and intron 12, is unique to Pakistani subjects from Punjab province and attributed to environmental factors and genetic variations amongst different racial/ethnic groups. Alternatively, it may be related to a lack of clinical studies on mutations in the LCT-gene along with the promoter region, other than those at codons 593, 666, 1430, 4927, 5579 and 5845 (Boll et al., 1991; Harvey et al., 1998; Jarvela et al., 2003).

The probable reason may be, that Dravidians were original inhabitants of our neighboring country India, Aryans established their cultures in the northern part and forced Dravidians to move to the southern part. The current Punjabis of Pakistan and North India are considered descendants of Aryans who had pastoralist backgrounds with the habit of regular milk consumption (Babu et al., 2010; Baseer and Rab, 1975; Simoons et al., 1978). Two studies were carried out using the conventional methods in Pakistan, both of them were contradictory to each other (Ahmad and Abhas, 1983; Baeer and Rab, 1975). Pasteurization and milking began in Punjab 5000 years ago, but due to cultivation trends of cereals, pulses and conversion of milk to other dairy products e.g. ghee, yogurt and butter, the selective mechanism may be correlated to LP in subjects belonging to Punjab (Pigott et al., 1977). The possibility of the introduction of the LP gene to the Indian subcontinent by the migration of Aryan nomads exists but is not supported by data from Afghanistan and Iran, showing a relatively low incidence of LP (Rahimi et al., 1976).
groups. The high frequency level in LP clearly indicates its vital role in the development of LP after weaning. Further investigation must be conducted with more emphasis on the role of Promoter and enhancer for lactase production. In recent studies, the genetic analysis of two cis-acting factors: C/T-13910, G/A-22018 at MCM-6 gene is presented as reliable genetic testing tools, they must also consider the LCT Promoter region. They can give more reliable results for the genetic screening of LNP/LP individuals, whereas the single nucleotide polymorphisms (SNPs) reported in exon 1 and 2 at positions 593 and 666, respectively may also regulate LCT expression and the enhancer effect of LCT-Promoter (Wang et al., 1995, 1998). Troelszen (2014) hypothesized that post-weaning decline/LNP is a result of a decrease in recruitment of transcriptional activators or increase recruitment of repressors in promoter of the LCT-gene (Ennata et al., 2007; Pigott et al., 1977).

In LNP individuals, avoidance of milk and milk-containing products could result in low Ca²⁺ intake resulting in osteoporosis and fractures (Amiri et al., 2015; Beto, 2015; Escher et al., 1992; Flatz et al., 1973; Lewinsky et al., 2005; Gueguen et al., 2006; Sahi et al., 1978). The results of the current study give a firm foundation to the conclusion of high reliability on genetic markers or genetic testing for the diagnosis of LNP. That every patient with LNP should have information about the dietetic sources of lactose and instructions to regulate it on their own, in order to become symptom-free.

CONCLUSION

Introns, Exons and the Promoter region responsible for mutations are analyzed. The general public in Subcontinent is unfamiliar with illnesses such as Lactose Non-persistence. Thus a ratio of 40% of tested patients having LNP, indicates that there could be a significant relationship in LNP and Mutations of LCT-gene in Subcontinent, in contrast to other continents. A larger study sample should be tested for future works.

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Disclosure statement

No competing Financial intersets exists

Statement of conflict of interest

The authors have declared no conflict of interest.

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