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Association of *Lysyl Oxidase* (LOX) Gene Polymorphisms with Keratoconus Disease in Samples of Iraqi Patients

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ABSTRACT

The current study aimed to investigate the possible associations between LOX polymorphisms, rs1800449, rs2288393, rs41407546 and rs2956540 and susceptibility to KC in sporadic Iraqi samples. keratoconus disease was counted as a multifactorial disease with obscure etiology that caused gradual loss of vision as a result of progressive change in corneal shape. *LOX* gene was responsible for collagen crosslinking in the human cornea that was necessary for clear vision and maintains the regular shape of cornea. Corneal collagen crosslinking (CXL) was a recent promising treatment which caused deceleration of disease progression**.** Association results were analyzed of single-nucleotide polymorphisms (SNPs) in the LOX gene by direct sequencing in two independent panels of 50 patients with keratoconus and 20 control groups. The exon 1 revealed the presence of rs41407546 only in KC patients and the absence of the rs1800449. The intron 1 rs2288393 was found only in CXL patients and the intron 4 rs2956540 was found in both KC, CXL patients and controls. The SNPs found only in KC patients are probably associated with keratoconus disease; as the exon SNP could cause conformational changes in 3D structure of the protein thus affect its function while the intron SNPs might increase susceptibility to the disease.

Keywords: Keratoconus; LOX; CXL; Polymorphisms; 3D structure.

1. INTRODUCTION

Keratoconus is a bilateral, asymmetric and progressive anterior protrusion and thinning of the cornea that cause corneal distortion which finally affect visual performance (1,2). Disease appearances ranging from inconsiderable irregular astigmatism, myopia to markedly failure to attain functional vision consequently making corneal transplantation is the proposed treatment thus keratoconus is known to be one of the most important indication for corneal transplantation in the world (3,4).

Keratoconus takes place in all ethnic groups with the age incidence rates being greatest in younger ages with significantly no difference by sex groups (5). KC is a multifactorial disease that is generated by the incorporation of environmental and genetic factors, however, the disease pathogenesis and etiology stay till know unclear but the genetic participation was proved by family studies that was demonstrated the presence of KC patients in family pedigree for two to three generations in addition to twin studies and genetic analysis that suggested several gene abnormalities which may be the essential cause that make the individuals susceptible to KC disease (6).

LOX gene that encodes the lysyl oxidase enzyme which catalyzes collagen crosslinking in cornea, therefore; one of the fundamental hallmark of KC disease is the lack of cross-links in stromal collagen this was combined with decreased in LOX expression in KC patients, thus collagen-crosslinking considered as the first line treatment for the disease by bonds formation between collagen fibers (7,8).

2. MATERIALS AND METHODS

The samples chosen to study had an age within the range of 11- 41 years old. Samples collection was carried for this study from LASIK Unit in Ibn al-Haytham Hospital (Baghdad) and from Al-Baseer eye specialist center (Baghdad / Palestine Street). All diagnosed keratoconus patients were informed about the study and gave written consent for their involvement in addition to filled a questionnaire form about their affection with keratoconus.

DNA extraction from blood samples were carried out using the genomic purification kit of Wizard® (Promega, USA) followed by evaluation of DNA concentration and purity for each sample using NAS99 nanodrop spectrophotometer. The DNA integrity was detected by agarose gel electrophoresis.

The *LOX* genes were chosen according to the study by Bykhovskaya *et.al.* (9) In which it was considered as a candidate gene in which there were SNPs associated with keratoconus. The specific sequences of primers used are listed in table (1).

Table 1: Primer sequences.

Gene	Target site for amplification	Primer sequences (F: Forward R: Reverse) 5`→3`	PCR product (bp)
LOX (10)	Exon 1-2	CCGTCACTGGTTCCAAGCTG ACGTCGAGAAGCCACATAGC	336
	Exon 2 alt	CCAGCTATGTGGCTTCTCGAC ACTTCCCAGCTCTTGTCCC	543
	Intron 4	TTCCCAAGATGCCAACTGCT GGTCAGTGTGGGTCCTTGTT	564

50µl of PCR reaction products that contains Go Taq® green master mix, 10 picomoles/µl forward and reverse primers, 100 ng/µl DNA and water.

The PCR reaction mixture was subjected to the following parameters initial denaturing for 5 min at 95 °C, 35 cycles of denaturing for 1 min at 95 °C, annealing for 1 min at 63 °C for exon 2-alt region and 61 °C for exon1-2 and intron 4 regions, extension for 1min at 72 °C, and a final extension for 10 min at 72 °C. To analysis the PCR products 1.5% agarose gel was used (1.5 gm in 100 ml of 1X TBE). After amplification of the targeted regions, 35µl of PCR product of several samples (control and patient) with primers were sent to Macrogen Company (South Korea) for direct sequencing. Alignment was established by tools that available online at the National Center Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) using BLASTn for nucleotide sequence and BLASTp for peptide sequence alignments by the translation of exons to their amino acids sequences (http://web.expasy.org/translate). The three dimensional structure was foretold using RaptorX tool which is the online available server for the prediction of protein structure (http://raptorx.uchicago.edu/).

3. RESULTS AND DISCUSSION

The exon 1-2 was designed to flank the exon 1-intron 1 junction Sequence results aligned using BLASTn with sequence ID: EF_094938.1 followed by SNPs analysis by the NCBI to discover the reference SNP if present with base pair sequence and allelic origin as shown in table (2) that revealed the presence of SNP detected in CXL patient.

Table 2: SNP analysis of exon 1-intron 1 region.

Sample type	SNP	BP sequence	Region	RefSNP	Sequence 5`→3`	Allelic origin
CXL patient	A>C	122077510	Exon 1	rs41407546	GC{A/C}GC	Heterozygote

The rs41407546 (A>C) located in base 3812 in the reference gene (EF_094938.1), while amino acid sequence within the polypeptide starts from the residue 140-168 in the reference gene (AAK58603.1) (NCBI). So a SNP in this exon was found to have an impact on the translated polypeptide due to the changing of the codon (CCG) which codes for proline to (CAG) which codes for glutamine as shown in figure (1).

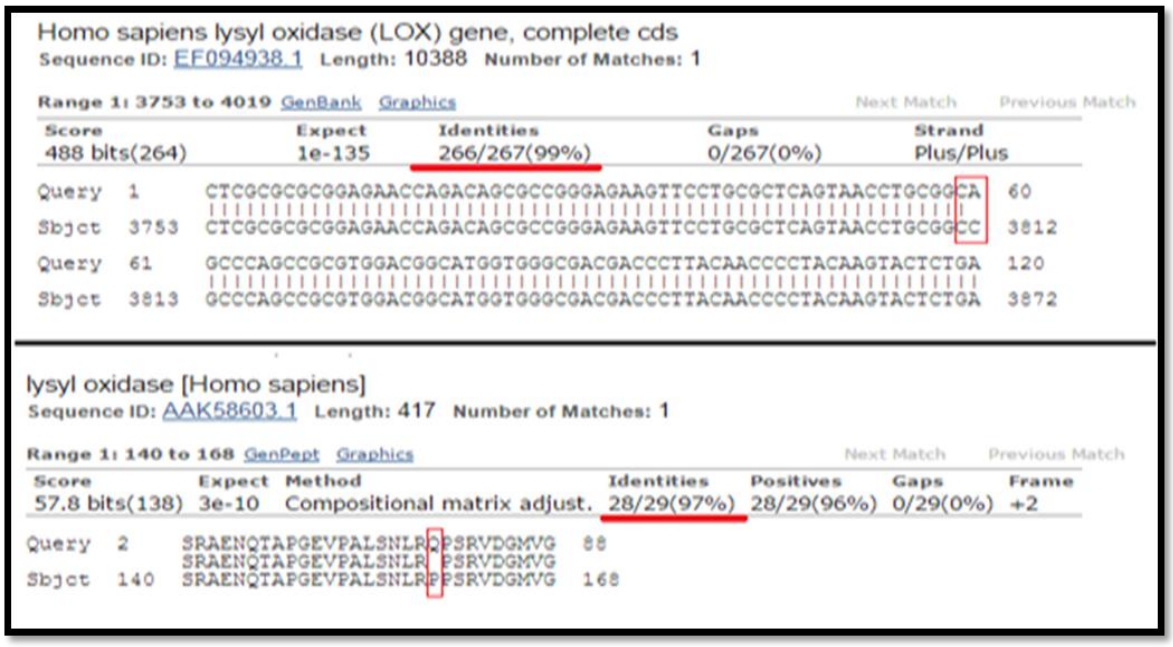

Figure 1: Alignment of exon 1 and its polypeptide product. A. exon1 BLASTn result shows 99% identity with the SNP in red box as the rs41407546. B. BLASTp result showing 97% identity with change in amino acid corresponds to the SNP.

The three dimensional structure for exon 1 to the amino acids sequence (140-168 aa) was portended using RaptorX tool that online available as presented in the figure (2) The predicted 3D structure in the natural state in the absence of is represent by loop region while in the presence of SNP there is loop and two separated α helix structures.

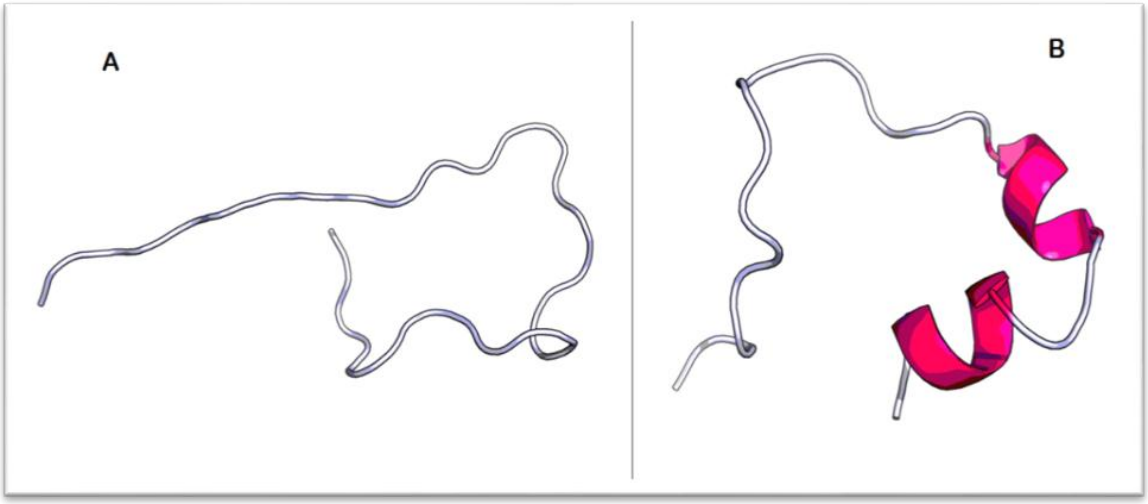

Figure 2: exon 1 predicted three dimensional structures.
A. Polypeptide sequence without SNP.
B. Polypeptide sequence with the SNP.

Exon 2 region this region comprises exon 2 in addition to the end of intron 1 and the beginning of intron 2 were amplified and sequenced the SNP analysis was demonstrated in table (4) which indicated that The SNP that was presented in three CXL Patients.

Table 3: SNP analysis of intron 1-exon 2-intron 3 region.

Sample type	SNP	BP sequence	Region	RefSNP	Sequence 5`→3`	Allelic origin
CXL patient	C>G	122077195	Intron 1	rs2288393	GC{C/G}CG	Heterozygote
CXL patient	C>G	122077195	Intron 1	rs2288393	GC{C/G}CG	Heterozygote
CXL patient	C>G	122077195	Intron 1	rs2288393	GC{C/G}CG	Heterozygote

Table 4: SNP analysis of intron 4 region.

Sample type	SNP	BP sequence	RefSNP	Sequence 5`→3`	Allelic origin
CK patient	C>G	122073485	rs2956540	CC{C/G}CA	Heterozygote
CK patient	C>G	122073485	rs2956540	CC{C/G}CA	Homozygote
CK patient	C>G	122073485	rs2956540	CC{C/G}CA	Heterozygote
CK patient	C>G	122073485	rs2956540	CC{C/G}CA	Heterozygote
CK patient	C>G	122073485	rs2956540	CC{C/G}CA	Heterozygote
CK patient	C>G	122073485	rs2956540	CC{C/G}CA	Heterozygote
CXL patient	C>G	122073485	rs2956540	CC{C/G}CA	Homozygote
CXL patient	C>G	122073485	rs2956540	CC{C/G}CA	Heterozygote
CXL patient	C>G	122073485	rs2956540	CC{C/G}CA	Homozygote
CXL patient	C>G	122073485	rs2956540	CC{C/G}CA	Heterozygote
Control	C>G	122073485	rs2956540	CC{C/G}CA	Heterozygote
Control	C>G	122073485	rs2956540	CC{C/G}CA	Homozygote

The Intron 4 region contained rs2956540 distributed among KC, CXL patients and control group. SNP analyses among samples were illustrated in table (5).

The rs41407546 in exon 1 was reported previously to be associated with keratoconic patients in Italian cohort (11). This is the first time to report the presence of this SNP in Iraqi patients and the rs1800449 (A>G) that also cause change in amino acid because of substitution of arginine to glutamine (10). This SNP showed marginal association with keratoconus disease in case/control studies with different populations due to the result of meta-analysis study that include three studies within Caucasian population and a study of Asian population that revealed the SNP was associated with keratoconus in Iranian population and there was no association with it in Caucasian and Chinas populations (12). So the absence of this SNP clarify there is no association with keratoconus disease in Iraqi population. Studies was revealed that the 417 amino acid protein consist of one alpha chain with the percentage 3.93% of helicase, 5.07% of beta sheets and the remaining are coiled configuration with loops which make the structure flexible moreover highly hydrophobic with better interaction with water (13). Loops are pieces in protein chains are predominately highly mobile even in stable proteins that connect secondary element in protein structure thus plays a vital role in protein folding and mediate significant biological processes, loops usually located on the surfaces of proteins consequently exposed to solvents in order to increase protein hydrophobicity (14).

As the amino acids sequence (140-168 aa) which located in the propeptide region because this region cover the sequence from (22-168 aa) which is N-glycosylated and separated at the 196 aa to release the mature active enzyme (15). But the N-glycosylation is not required for secretion but it is important for optimal enzyme activity (16). So that the SNP that located in the 159 aa will change the structure of LOX-propeptide by forming two alpha helix structures.

The C>G rs2288393 was previously evidenced marginal association with KC disease among case/control populations such as meta-analysis study in Caucasian and Chinese populations lacked adequate index to support the association of the SNP with the disease. Another study in Australian cohorts reported the presence of C>G rs2288393 in three KC patients in heterozygote form which lead to supposition that the presence of the minor alleles may influence the interaction and balance of the two isoforms in corneal tissue thus making the cornea weaken due to collagen crosslinking reduction and therefore making it easier to promote keratoconus (10,12). Consequently the presence of the SNP in CXL patients; While the activity and concentration of LOX enzyme in KC patients has been significantly reduced, but following CXL the enzyme has been remarkably up regulated nevertheless the precise mechanism of CXL at the molecular level has not until now been clarified (17).

The C>G rs2956540 demonstrate a strong association with KC disease in Iraqi cohorts, as it previously proved that the C allele was significantly associated with the disease by increasing susceptibility to keratoconus in Caucasian, Australian and Chinese populations (10, 12,18).

4. CONCLUSION

The present study is the first to examine the association of *LOX* gene with keratoconus patients in a sample of Iraqi population. The A>C rs41407546 that was found in exon 1 of *LOX* gene in keratoconus patient which cause change in the amino acid sequence thus lead to change in the 3D structure of protein that will effect on the optimal enzyme activity. This lead to suggest the presence of rs41407546 in a sample associated to keratoconus disease in. While the A>G rs1800449 is not associated with keratoconus patients. On the other hand the *LOX* intron 1 C>G rs2288393 is associated with keratoconus patients that undergo collagen crosslinking treatment. But the intron 4 *LOX* C>G rs2956540 indicate strong association to keratoconus disease in a sample of Iraqi population.

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