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Research article

Biological effect of *LOXL1* coding variants associated with pseudoexfoliation syndrome

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ABSTRACT

Pseudoexfoliation (PEX) syndrome is a systemic disease involving the extracellular matrix. It increases the risk of glaucoma, an irreversible cause of blindness, and susceptibility to heart disease, stroke and hearing loss. Single nucleotide polymorphisms (SNPs) in the *LOXL1* (*Lysyl oxidase-like 1*) gene are the major known genetic risk factor for PEX syndrome. Two coding SNPs, rs1048861 (G > T; Arg141Leu) and rs3825942 (G > A; Gly153Asp), in the *LOXL1* gene are strongly associated with the disease risk in multiple populations worldwide. In the present study, we investigated functional effects of these SNPs on the LOXL1 protein. We show through molecular modelling that positions 141 and 153 are likely surface residues and hence possible recognition sites for protein–protein interactions; the Arg141Leu and Gly153Asp substitutions cause charge changes that would lead to local differences in protein electrostatic potential and in turn the potential to modify protein–protein interactions. In RFL-6 rat fetal lung fibroblast cells ectopically expressing the LOXL1 protein variants related to PEX (Arg141_Gly153, Arg141_Asp153 or Leu141_Gly153), immunoprecipitation of the secreted variants showed differences in their processing by endogenous proteins, possibly Bone morphogenetic protein-1 (BMP-1) that cleaves and leads to enzymatic activation of LOXL1. Immunofluorescence labelling of the ectopically expressed protein variants in RFL-6 cells showed no significant difference in their extracellular accumulation tendency. In conclusion, this is the first report of a biological effect of the coding SNPs in the *LOXL1* gene associated with PEX syndrome, on the LOXL1 protein. The findings indicate that the disease associated coding variants themselves may be involved in the manifestation of PEX syndrome.
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1. Introduction

Pseudoexfoliation (PEX) syndrome is a systemic disease of the extracellular matrix. It primarily presents as an ocular condition characterised by the deposition of pathological greyish-white extracellular proteinaceous material (PEX material) on the anterior surface of the lens. Histologically, the extracellular pathological material is found in most of the tissues of the anterior and posterior ocular segments (Schlotzer-Schrehardt et al., 2012; Schlotzer-Schrehardt and Naumann, 2006). Deposition of PEX material in the trabecular meshwork can obstruct the aqueous outflow and lead to elevation in intraocular pressure that in turn increases the risk of glaucoma. Consistently, PEX syndrome is the most common known risk factor for glaucoma worldwide. It can also predispose the eye to a range of potentially sight threatening conditions and increases the rate of complications from cataract surgery. Systemic

Abbreviations: PEX, Pseudoexfoliation syndrome; LOXL1, Lysyl oxidase-like 1; SNP, single nucleotide polymorphism; BMP-1, bone morphogenetic protein-1; RT-PCR, reverse transcription-polymerase reaction; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; GFP, green fluorescent protein; RMSD, root mean square difference; kDa, kilo Dalton; MT, mock transfected.

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  1 MALARGSRQL GALVWGACLC VLVHGQQAQP GQGSDPARWR QLIQWENNGQ VYSLLNSGSE
 61 YVPAGPQRSE SSSRVLLAGA PQAQQRRSHG SPRRRQAPSL PLPGRVGSDT VRGQARHPFG
121 FGQVPDNWRE VAVGDSTGMA RARTSVSQQR HGGSASSVSA SAFASTYRQQ PSYPQQFPYP
181 QAPFVSQYEN YDPASRTYDQ GFVYYRPAGG GVGAGAAAVA SAGVIYPYQP RARYEEYGGG
241 EELPEYPPQG FYPAPERPYV PPPPPPPDGL DRRYSHSLYS EGTPGFEQAY PDPGPEAAQA
301 HGGDPRLGWY PPYANPPPEA YGPPRALEPP YLPVRSSDTP PPGGERNGAQ QGRLSVGSVY
361 RPNQNGRGLP DLVPDPNYVQ ASTYVQRAHL YSLRCAAEEK CLASTAYAPE ATDYDVRVLL
421 RFPQRVKNQG TADFLPNRPR HTWEWHSCHQ HYHSMDEFSH YDLLDAATGK KVAEGHKASF
481 CLEDSTCDFG NLKRYACTSH TQGLSPGCYD TYNADIDCQW IDITDVQPGN YILKVHVNPK
541 YIVLESDFTN NVVRCNIHYT GRYVSATNCK IVQS
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Fig. 1. The human LOXL1 protein sequence. The 574 amino acid long pre-pro-protein sequence (GenBank accession NP_005567) is shown. The signal peptide (green), pro-region (purple), PEX syndrome associated variant amino acids (red), and BMP-1 cleavage sites (blue, orange, pink) are indicated. BMP-1 cleavage sites identified in the bovine protein are in blue (Borel et al., 2001), putative sites conserved between the human and bovine protein are in orange and putative site not-conserved between the human and bovine protein is in pink. The aspartic acid residue preceding the Flag-tag inserted in the protein in this study is underlined.

accumulation of the pathological material in blood vessels, skin, heart, lung, liver and cerebral meninges is thought to increase the risk of cardiovascular and cerebrovascular diseases (Akarsu and Unal, 2005; Andrikopoulos et al., 2009; Kan et al., 2015; Mitchell et al., 1997; Streeten et al., 1990, 1992). Additionally, several studies indicate an association with hearing loss (Cahill et al., 2002; Samarai et al., 2012). The presence of extracellular matrix proteins and remodelling enzymes, complement proteins, cell adhesion molecules and stress response proteins in PEX material, and deregulated expression of genes encoding some of these proteins in the affected ocular anterior segment tissues suggest that increased production/reduced turnover of extracellular matrix, inflammation and oxidative stress contribute to the pathogenesis of this disease (Ovodenko et al., 2007; Schlotzer-Schrehardt and Naumann, 2006; Zenkel et al., 2005).

Due to clustering of the disease in families, concordance in monozygotic twins, and ethnicity-based differences in prevalence, genetic factors had been thought to contribute to this disease (Allingham et al., 2001; Damji et al., 1999; Gottfredsdottir et al., 1999). Consistently, common single nucleotide polymorphisms (SNPs) in the *LOXL1* (*Lysyl oxidase-like 1*) gene have been found to strongly associate with the disease in various populations around the world (Abu-Amero et al., 2010; Chen et al., 2009b; Fan et al., 2008; Hayashi et al., 2008; Hewitt et al., 2008; Jaimes et al., 2012; Malukiewicz et al., 2011; Mayinu and Chen, 2011; Ramprasad et al., 2008; Thorleifsson et al., 2007). Although nominal associations of SNPs in the *CLU* (*Clusterin*) and *CNTNAP2* (*contactin associated protein-like 2*) genes have been reported in some Caucasian populations and of SNPs in *CLU* also in an Indian population (Burdon et al., 2008; Krumbiegel et al., 2009, 2011; Padhy et al., 2014), polymorphism in the *LOXL1* gene is by far the major known genetic risk factor associated with PEX syndrome.

Two common coding variants, rs1048861 (G > T; Arg141Leu) and rs3825942 (G > A; Gly153Asp), in the *LOXL1* gene are significantly associated with PEX syndrome in genetically diverse populations (Abu-Amero et al., 2010; Fan et al., 2008; Hewitt et al., 2008; Pasutto et al., 2008; Ramprasad et al., 2008; Thorleifsson et al., 2007). In most populations, the G_G haplotype of these variants confers the highest disease risk (Caucasian, Indian, Arabian and some Chinese). However, the risk allele in the Japanese and Han Chinese populations is the T_G haplotype, and in Black South Africans the G_A haplotype (Chen et al., 2009b; Hayashi et al., 2008; Lee et al., 2009; Rautenbach et al., 2011; Tanito et al., 2008; Williams et al., 2010). The T_A haplotype of the two associated SNPs has not been identified in any population to date hence is likely non-existent. Additionally, association of an intronic SNP, rs2165241 (T > C), in *LOXL1* with the disease has been reported (Jaimes et al., 2012; Thorleifsson et al., 2007). In the Scandinavian populations, the T allele of this SNP tags the G_G risk haplotype of the associated coding variants (Thorleifsson et al., 2007), whereas in the Mexican population this T is the risk allele (Jaimes et al., 2012). Furthermore, association of SNPs in the promoter region of *LOXL1* has been reported in Caucasian Americans (Fan et al., 2011), however, the risk allele at one of these SNPs (rs16958477) is reversed in an Indian population (Dubey et al., 2014).

LOXL1 is a member of the lysyl oxidase family of enzymes that are involved in cross-linking elastin and/or collagen fibrils in the extracellular matrix (Smith-Mungo and Kagan, 1998). It is ubiquitously expressed including in all the tissues of the anterior ocular segment (Hewitt et al., 2008). Consistent with its role and genetic association of the *LOXL1* gene with PEX syndrome, the LOXL1 protein is present in PEX material (Schlotzer-Schrehardt et al., 2008; Sharma et al., 2009a). However, the biological significance of genetic association of this gene with the disease is poorly understood. Some studies have suggested that regardless of the disease, carriers of the G allele at the coding SNP rs1048861 (Arg141Leu) have lower levels of *LOXL1* expression in tissues than carriers of the T allele (Schlotzer-Schrehardt et al., 2008, 2012; Thorleifsson et al., 2007); whether this difference in expression levels contributes to disease susceptibility is as yet unknown.

The LOXL1 protein is encoded as a pre-pro-protein and after cleavage of the N-terminal signal peptide is secreted as a pro-protein (Borel et al., 2001; Decitre et al., 1998). The secreted pro-peptide has an N-terminal pro-region and a C-terminal catalytic domain (Thomassin et al., 2005). The protein binds to tropoelastin monomers through the pro-region that is followed by cleavage of the catalytic domain by BMP-1 (Bone morphogenetic protein-1) giving rise to the active enzyme. The enzymatically active LOXL1 cross-links tropoelastin monomers through oxidative deamination of lysine residues (Borel et al., 2001). The PEX-associated variant amino acid residues reside downstream of the pro-region and proximal to potential BMP-1 cleavage sites (Fig. 1). To gain further insight into the biological significance of the genetic association of *LOXL1* with PEX syndrome, we aimed to investigate the biological effects of the disease-associated coding variants on the LOXL1 protein. Here we present data supporting the effect of the disease-related variants on cleavage of the LOXL1 protein.

2. Material and methods

2.1. Molecular modelling

Molecular modelling studies were undertaken on the LOXL1 protein, accession number Q08397. The reference sequence without the signal peptide and with Arg141 and Gly153 residues was modelled. Homology modelling was performed using a

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