Research Paper

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Mutational study of Extracellular Matrix Protein 1 in a case of Lipoid Proteinosis from South India.

Medical Science

KEYWORDS : ECM1, mutational study, lipoid proteinosis.

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ABSTRACT

Lipoid proteinosis (LP), also known as Hyalinosis cutis et mucosae or Urbach–Wiethe disease (OMIM 247100) is a rare, autosomal recessive disorder characterized by generalized thickening of skin, mucosae and certain viscera [1]. Histologically, there is widespread deposition of hyaline material and disruption/reduplication of basement membrane [2]. It is caused by loss-of-function mutations in the extracellular matrix protein 1 (ECM1). We conducted mutation analysis of the ECM1 gene in a 23 year old male with lipoid proteinosis. He presented with hoarse voice, asymptomatic skin lesions, scarring beaded eyelid papules and a thickened sublingual frenulum since his childhood. Skin biopsy showed widespread deposition of hyaline material in the dermis and thickened basement membrane. The coding region of ECM1 was amplified and sequenced and were shown to have mutations in introns 5, 6, 9 and exons 6, 7, 8. These mutations were concurrent with the mutations of previous studies.

Introduction

Lipoid proteinosis (LP) otherwise known as Urbach-Weith disease or Hyalinosis cutis et mucosae is a rare autosomal-recessive genodermatosis which affects skin and other systems. Around 250 cases were reported so far [3]. Chronologically, the cutaneous presentations of LP in skin are in three stages (i) vesicles and blisters, lead to scarring (ii) waxy papules and (iii) verrucous lesions at the site of trauma. Banerjee et al [4] had described a case of vesicular eruptions with hyertrichosis which is not seen in our case. Interestingly, Parlak AH et al [5] reported verruca vulgaris superimposed on papules of LP. The systemic manifestations are due to the deposition of hyaline like material in larynx (hoarseness of voice) and other organs. This hyaline like material is Periodic Acid Schiff (PAS) positive and diastase resistant. The clinical features are due to the loss of normal function of extra cellular matrix (ECM1) protein. This gene encodes a protein which is involved in endochondral bone formation, angiogenesis, and tumor biology. It also interacts with an array of extracellular and structural proteins, contributing to the skin integrity and homeostasis [6]. In this study, we tried to find out any new mutations in ECM1 which could modify the disease pattern in LP.

Materials and methods

Patient

A 23 year old male born of non-consanguineous parents presented with hoarseness of voice and multiple asymptomatic skin color papules over the eyelids, neck and shoulder area since childhood. He had fragile skin with history of ulcers, following minor injuries and healed with scarring. Patient had hoarseness of voice since infancy and subsequent development of blisters in the lower back during his childhood lead to atrophic scarring. Around 12 years he had waxy papules over the eyelids, neck and shoulder. Subsequently he developed verrucous skin lesions over both elbows. There was no history of similar skin lesions in the family members. He had no history of respiratory problems, seizures, photosensitivity, and headache except for restricted tongue movement. He could carry out his daily routine activities.

On examination, the patient had hoarseness of voice and waxy, skin colored papules over the eyelids (moniliform blepharosis), neck and shoulders (Fig.1, 2). Atrophic scarring was seen in lower back (Fig.3). Also he had hyerpigmented verrucous skin lesions in both elbows (Fig.4). He could not protrude his tongue out of the mouth because of the thickened frenulum (Fig.5). Psychiatric evaluation using Binet Kamat Test showed borderline intelligence with IQ of 72. The patient was subjected to video-laryngoscopy for the hoarseness of voice, which showed multiple, yellowish deposits in the arytenoids and inter-arytenoid area (Fig.6). Examination of eye and other systems was unremarkable.

Skin Biopsy

Skin biopsy of a papule from the left shoulder area revealed amorphous, eosinophilic protein like material in the upper dermis and around the eccrine glands in hematoxylin and eosin stain (Fig.7). The material was PAS (periodic acid-schiff) positive and diastase resistant (Fig.8)

Detection of mutations in ECM1 Gene in the patient.

PCR amplification and sequencing

About 10 ml of blood was collected from the affected patient in a vacutainer tube containing eight per cent EDTA K3 solution (Improvacuter®, Europe). Family members of the patient did not give consent to give blood for screening.Immediately after collection, samples were labelled and transported in an ice-packed container to the laboratory for further processing. DNA samples were isolated from blood samples using a modified high-salt method (Miller *et al.*, 1988). The procedure followed was as follows:

- Ten ml of blood and 25 ml of RBC lysis buffer were taken in a 50 ml centrifuge tube. This tube was inverted several times and incubated in ice with shaking for 10 minutes for complete lysis of red blood cells. Nuclear material was pelleted by centrifugation at 4,000 rpm at room temperature for 10 minutes and discarding the supernatant.
- Nuclear pellet was washed with 10 ml of RBC lysis buffer and centrifuged once again. This step was repeated three to four times till the clear nuclear pellet was obtained.
- Nuclear pellet was resuspended in 10 ml Tris buffer Saline (pH 7.4) and centrifuged at 3000 rpm for 10 minutes. The pellet was collected and the procedure was repeated for three times.
- Nine ml of TE buffer (pH 8.0) was added to the pellet and vortexing was performed. To this tube 50 µl of Proteinase-k and 500 µl of 0.5M EDTA were added and mixed thoroughly. To this solution 500 µl of SDS was added with gentle mixing and incubated overnight at 50 0C.
- After incubation, 4.3 ml of saturated sodium chloride was added and shaken vigorously. To this mix, equal volume of Chloroform: Isoamyl alcohol (24:1) was added, mixed and centrifuged at 4000 rpm for 15 minutes.
- Aqueous upper phase was transferred to fresh tube and steps involving chloroform:Isoamyl alcohol was repeated

twice.

- Finally, the DNA was precipitated by adding double the volume of 95 per cent ethanol to this supernatant and inverted several times until the DNA was precipitated.
- Precipitated DNA strands were transferred to a micro centrifuge tube containing one ml of ice-cold 70 per cent ethanol and centrifuged at 2,000 rpm for five minutes. Supernatant was discarded and the pellet was air-dried.
- Dried DNA pellet was resuspended in 400 µl of TE buffer and kept at 65 oC for 15 minutes for dissolution.

Primers were designed for amplification of all exons of the ECM1 gene (Table 1). For PCR amplification, 250 ng of genomic DNA was used as the template in an amplification buffer containing 5 pmol of each primer, 2.5 mmol $MgCl_2$, 0.5 mmol of each nucleoside triphosphate and 1.25 U of AmpliTaq Gold polymerase in a total volume of 50 µl. Mastermix was prepared for one additional sample to cover pipetting error. All the reactions were carried out in 0.2 ml thin walled PCR tubes. PCR tubes containing mixture were tapped gently and the tubes were placed in a thermal cycler (Mastercycler ep gradient S, eppendorf, Germany) and subjected to PCR amplification. The amplification conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, annealing temperature (see Table 1) for 45 s, 72°C for 45 s. PCR products were analyzed by 2.5% agarose gel electrophoresis and purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) for sequencing. The typing of amplified products was made by capillary electrophoresis using on 3130xl ABI Prism Automated Genetic Analyzer (Applied Bio-systems, Germany) at SciGenom Labs Pvt Ltd. CSEZ, Kerala, India. The reference sequence (NC_000001.11 (150508011..150513789) has been compared with the patient sequence which is sequenced from the region intron 5 to 3'UT (150510663 to 150513615) with a total base pair of 2952 using DNAStar Lasergene –Version 7.1.0 (DNASTAR, Inc., Madison, Wisconsin USA)

Results

Mutation analysis identified a major allele in this patient with LP.

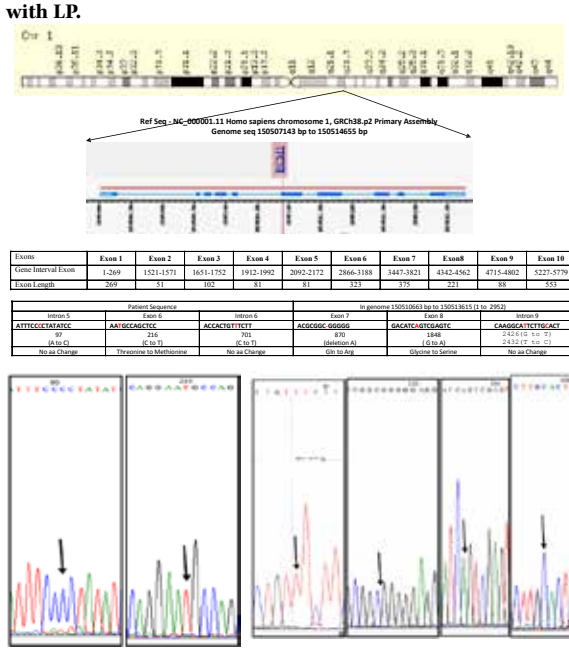
Amplified DNA from the patient disclosed the following mutation in different exons

- Direct sequencing of the PCR products amplified from the affected individual revealed a homozygous C to T **transition mutation** at nucleotide position 216 (216 C>T) (genome sequence 150510879) in the exon 6 of the *ECM1* gene. This mutation converts the amino acid Threonine (ACG) to Methionine (ATG).
- Amplified product of the same patient revealed a mutation of 870delA in exon 7 (genome sequence 150511533) of the *ECM1* gene. This **frame shift mutation** changes the amino acid glutamine (CAG) to arginine (CGG).
- Amplification of the Exon 8 revealed a homozygous G to A **transition mutation** at nucleotide position 1848 (c.1848 G>A) (genome sequence 150512511) of the *ECM1* gene. This mutation converts the amino acid Glycine (GGT) to Serine (AGT).
- In addition, mutations were noticed in intron 5 (97A/C) (genome sequence 150510760), intron 6 (701C/T) (genome sequence 150511364) and intron 9 (2426G/T, 2432 T/C) (genome sequence 150513089 and 150513096) without any change in amino acid sequence.

Discussion

LP is a rare autosomal recessive disorder with heterogeneous clinical manifestations from varying degrees of skin infiltrations and scars, hoarseness of voice, respiratory distress and extracutaneous features. Genetic studies of LP patients had revealed mutations in ECM1 gene, located on chromosome 1q21 [7]. Till date, 41 distinct germ line missense, nonsense, splice site, small and large deletions and insertions, have been reported [8]. Approximately 50% of the mutations cluster to exon 6 and 7 of the gene. Almost all the mutations of ECM1 published in LP are supposed to lead to low or absent mRNA or protein expression except very few missense mutations [9]. In our study the following observations were made (i) homozygous C to T transition mutation at nucleotide position 216 (216 C>T) (genome sequence 150510879) in the exon 6 of the *ECM1* gene. This mutation converted the amino acid Threonine (ACG) to Methionine (ATG), (ii) revealed a mutation of 870 delA in exon 7 (genome sequence 150511533) of the *ECM1* gene. This frame shift mutation leads to change of amino acid glutamine (CAG) to arginine (CGG). (iii) Amplification of the exon 8 revealed a homozygous G to A transition mutation at nucleotide position 1848 (c.1848 G>A) (genome sequence 150512511) of the *ECM1* gene. This mutation converted the amino acid Glycine (GGT) to Serine (AGT) and (iv) mutations were noticed in intron 5 (97A/C) (genome sequence 150510760), intron 6 (701C/T) (genome sequence 150511364) and intron 9 (2426G/T, 2432 T/C) (genome sequence 150513089 and 150513096) without any change in amino acid sequence. In concurrent with previous studies most of the mutations in this patient were restricted to exons 6 and 7 [10]. The types of mutations observed in exons of our patient were in agreement with the study by Hamada et al [11]. Also other unpublished Indian studies showed that mutations of ECM1 in LP patients occurred in exons 3 and 7 [12].

Mutation analysis identified a major allele in this patient with LP.



Exons	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon8	Exon 9	Exon 10
Gene Interval Exon	1-269	1521-1571	1651-1752	1912-1992	2092-2172	2866-3188	3447-3821	4342-4562	4715-4802	5227-5779
Exon Length	269	51	102	81	81	323	375	221	88	553

Patient Sequence			In genome 150510663 bp to 150513615 (1 to 2952)		
Intron 5	Exon 6	Intron 6	Exon 7	Exon 8	Intron 9
ATTTCCCTATATCC	AATGCCAGCTCC	ACCACTGTTCTT	ACGCGGC-GGGGG	GACATCAGTCGAGTC	CAAGGCATTCTTGCACT
97 (A to C)	216 (C to T)	701 (C to T)	870 (deletion A)	1848 (G to A)	2426 (G to T) 2432 (T to C)
No aa Change	Threonine to Methionine	No aa Change	Gln to Arg	Glycine to Serine	No aa Change

Table 1. Primers used for PCR amplification of the ECM1 gene

Exon number	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temp. (℃)	Product size (bp)
1	agctgggactgagtcatggc	taaaggctccactggcctag	62	416
2/3	tcctacactcttgatctcca	agaaacctggagggtcactg	58	247
4/5	cagtgaccctccaggtttct	cagagcccaccgtcttgtct	56	484
6	agccttgagaagcaggagga	agtgaacgggacctgaggtt	54	671
7	aacctcaggtcccgttcact	acatggatggatggactggc	52	548
8	cacatcaacagttgcctcct	ggcatcttctggcatcagat	60	499
9	agttgcctagtccttcccca	aggccaggtcagagtgaaga	60	408
10	aatccagctgtgcaaggcag	gtaatgagtgttcagatggg	62	469

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Figure 1: papules distributed along the eyelids (moniliform blepharosis)

Figure 2: waxy papules in the neck and shoulder

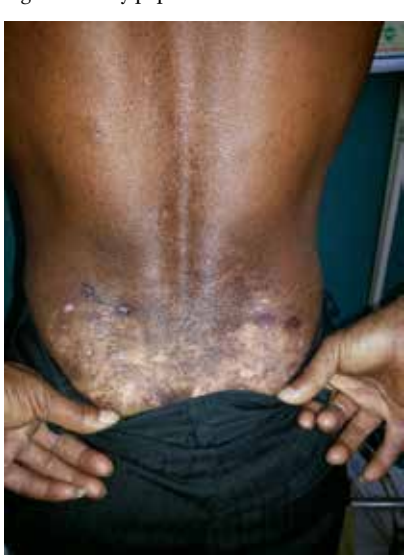

Figure 3: scarring in the lower back

Figure 4: photo showing verrucous lesions in both elbows

Figure 5: thickening and infiltration of the frenulum

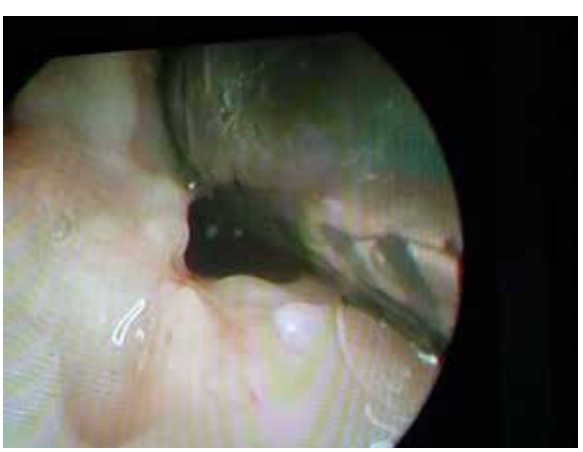

Figure 6: shows yellowish deposits in arytenoid and inter arytenoid region.

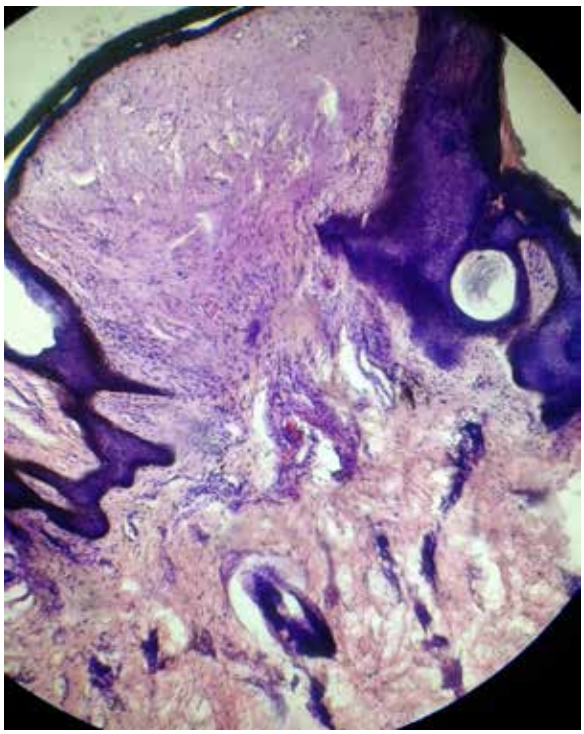

Figure 7: photo showing amorphous, eosinophilic material in papillary dermis and around eccrine glands.

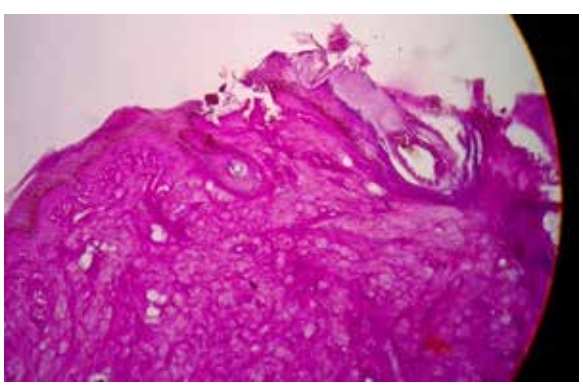

Figure 8: photo showing PAS (Periodic Acid Schiff) positive material in papillary dermis and around eccrine glands.

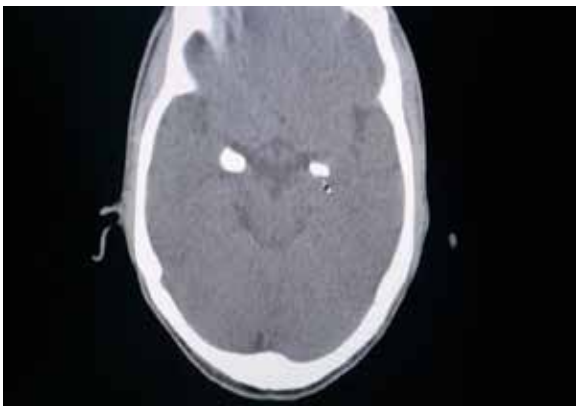

Figure 9: Brain CT showing calcification in both medial temporal lobe

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