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A novel deletion and two recurrent substitutions on type VII collagen gene in seven Iranian patients with epidermolysis bullosa

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ABSTRACT

Objective(s): Epidermolysis bullosa is one of the most important series of mechano-bullous heritable skin disorders which is categorized into four major types according to the layer that bullae forms within basement membrane zone. In dystrophic form of the disease, blisters are made in the sublamina densa zone, at the level of type VII collagen protein which produce anchoring fibrils. Type VII collagen gene is the only responsible gene for this form. The aim of this study was to survey causative mutations of type VII collagen gene among Iranian patients with epidermolysis bullosa.
Materials and Methods: For this purpose, exons 73-75 were investigated by polymerase chain reaction followed by direct sequencing.
Results: In current study, we found three different point mutations in type VII collagen alleles in 7 out of 50 patients. Four patients were homozygous for a new deletion which resulted in frame shift (p.Pro2089fs). Two patients were homozygous for a recurrent glycine substitution (p.G2031S) and one patient was detected with an allele carrying a substitution (p.R2069C).
Conclusion: The results emphasized heterogeneity in the type VII collagen gene and will provide a sign for early diagnosis and future study of the disease pathogenesis.

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Introduction

Epidermolysis bullosa (EB) is a series of mechano-bullous skin disorders in which skin and mucous membrane become very susceptible to fragility and blistering. Variable diagnostic methods such as skin biopsy, clinical examination, histological experiences have revealed four major subtypes (1). (a) In the simplex type of EB, the tissue is disrupted at the intraepidermal layer and mutations in genes KRT5 and KRT14 are involved in formation of this type of the disease. (b) Junctional form of EB is as a result of mutations in laminin 5, COL17A1, ITGB4 and ITGA6 genes and tissue separation occurs in lamina lucida (2). (c) Kindler syndrome, characterized by cleavage within the basal keratinocytes at the level of lamina lucida or below lamina densa and is caused by mutations in Kindlin-1 (3). (d) In dystrophic epidermolysis bullosa (DEB), abnormal anchoring fibrils structure, decreased number or lack of them make rupture under the basement membrane zone (sub-lamina densa) (2, 4). Both two forms of DEB, dominantly inherited and recessively inherited (DDEB; MIM# 131750 and RDEB; MIM# 226600) have been detected with extremely variable blistering trend. The only gene which has genetic linkage with both RDEB and DDEB is collagen, type VII, alpha 1 (COL7A1; MIM# 120120). This gene has been mapped to chromosomal region 3p21 and has 118 exon (2, 5). The protein type VII collagen is made of three identical subunits called α1 (VII) chains. Every α1 (VII) chains includes a 145 kilodalton (KD) zone (central triple helical collagenous region), flanked by a longer non-collagenous (NC-1) domain (exon 1-28) and a smaller carboxy-terminal non-collagenous (NC-2) domain (exon 112-118). The central triple helical collagenous part includes a repeatative Gly-X-Y sequence. Through out the producing anchoring fibrils (AFs), two collagene type VII molecules localize in an antiparallel direction which is fixed by disulfide bonds. Then these dimers assemble in a way that contain the NC-1 domain at both end of

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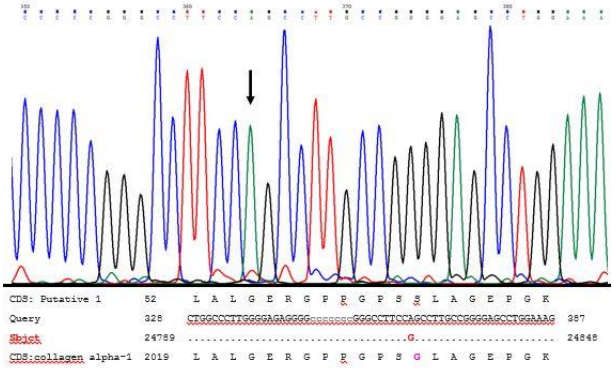

Figure 1. DNA sequence chromatogram of a homozygous affected individual with mutation c.6091G>A (p.G2031S) in exon 73. Arrow indicates the location of nucleotide substitution (upper part); BLASTn result of the chromatogram which reveals the changed base and aminoacid in color. Dot shows the indentities of bases (lower part)

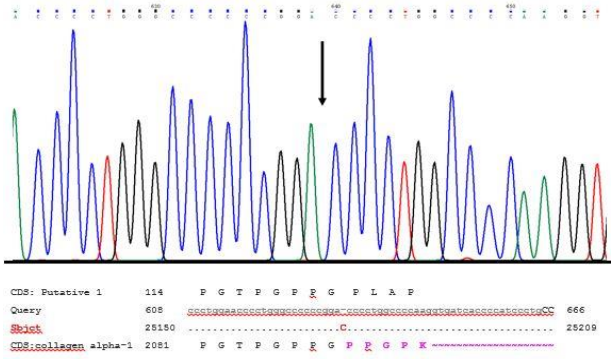

Figure 2. DNA sequence chromatogram of a homozygous affected individual with mutation c.6265delc (p.Pro2089fs) in exon 75. Arrow indicates the location of nucleotide deletion (upper part); BLASTn result of the chromatogram which reveals the deleted base and aminoacid changes in color. Dot shows the indentities of bases. (~) shows the noncoding sequences (lower part)

these structures and result in AFs' production (4). It has been reported that many mutations have trend to cluster in exon 73 (10.74%) and this indicates that this exon represents a section in which mutations normaly affect the function of AFs. Also mutations which impact exons 73-75, as part of a Gly-X-Y sequence, may lead to a more destructive glycine substitution than the other parts of the protein (5, 6). According to importance of exons 73-75 of COL7A1, we inclined to survey these exons of COL7A1 gene in order to get the picture of targeted exons' causative mutations (type and prevalence) among Iranian patients with EB. A novel variant and two recurrent mutations were discovered which resulted in revealing inheritance pattern among the cases under study.

Materials and Methods

Clinical diagnosis of participants was done by a skin specialist using phenotypic features like blistering (oral, dental, nail or mucosa involvement), dystrophic lesions, pseudosyndactyly of the hands and feet, milia, corneal scarring and so on. Detailed phenotype of probands have not been accessible. According to salting out method, genomic DNA was extracted from peripheral blood lymphocytes of 50 unrelated patients and 50 healthy control and used for amplification of COL7A1 gene sequences. Achieving this purpose, two oligonucleotide primers (forward and reverse) were designed around exons 73-75 and their flanking intronic regions (produced by Kawsar Biotech Company (KBC)). The primer sequences for generation of a 475 base pair (bp) fragment consisting exons 73-75 were as follows: Upstream primer sequence: 5' TCT GGT AGG TTC CTG CCT GT 3' and downstream primer: 5' AAT TCC AGG GTT ATG GCACA 3'. For polymerase chain reaction (PCR) amplification, 5 µl of 200 ng genomic DNA was used as template. A thermo cycler (Techne, UK) was employed for amplification. PCR cycles were initiated at 95 °C for 5 min for the first cycle. The PCR setting for the next 30 cycles was denaturing at 95 °C for 50 sec, annealing at 62 °C for 45 sec, and elongation at 72 °C for 1 min with a final elongation at 72 °C for 5 min. Amplification buffer (KBC) was contained of 100 mM $MgCl_2$ (0.5 µl) and 5 U of Taq polymerase (0.2 µl). Final volume of PCR was 50 micro liter. PCR product was applied for electrophoresis through a 1.0% agarose gel and also for gene sequencing in order to diagnose the causative mutations. The mutations were detected using sequence analyzer software (FinchTV version 1.4.0). Databases which are used in this study are: NCBI (http://www.ncbi.nlm.nih.gov), Ensembl Genome Browser (http://www.ensembl.org/index.html), human genome mutation database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php) (7) and human genome variation society (HGVS) guidlines in order to nomenclature of the new identified variant (http://www.hgvs.org/). The research protocol was approved by the institutional review board of Guilan University. All participants were informed about the purpose of our study and provided written consent to participate.

Results

Fifty unrelated patients who were suffering from EB were enrolled according to physician examination. Totally, 14% of causative mutations (3 different mutations in 7 out of 50 probands) were discovered at least in one allele. In 6 probands, mutations were found in both alleles of COL7A1 gene (homozygote) and in one patient a mutation was disclosed in an allele of the gene. Direct sequencing revealed a G to A transitional position 6091 within exon 73 of COL7A1. The mutation that causes a glycine codon (GGC) exchanged into a serin (AGC) is described as p.G2031S (Figure 1). This kind of mutation was detected in two unrelated patients. A newly detected variant in four unrelated patienets was a deletion of a C nucleotide at position 6265 within exon 75 of COL7A1. This deletion that consider as p.Pro2089fs, resulted in a frameshift within the collagenous domain of α1 (VII) chain (Figure 2).

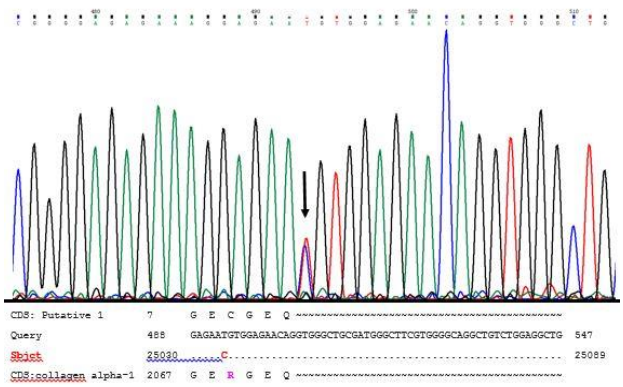

Figure 3. DNA sequence chromatogram of a heterozygous affected individual with mutation c.6205C>T (p.R2069C) in exon 74. Arrow indicates the location of nucleotide substitution (upper part); BLASTn result of the chromatogram which reveals the changed base and aminoacid in color. Dot shows the indentities of bases. (~) shows the noncoding sequences (lower part)

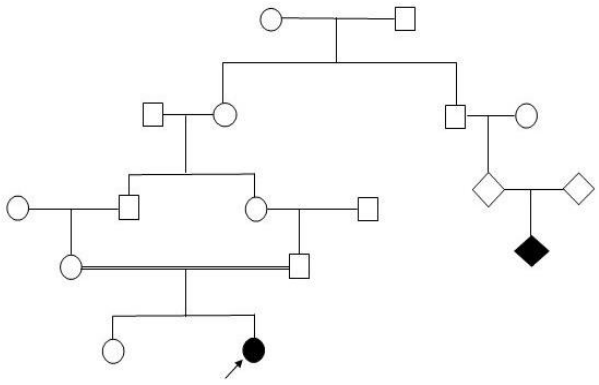

Figure 4. The pedigree of an individual with c.6091G>A (p.G2031S) mutation. The pedigree shows that proband 1 (identified by an arrow) was the affected child of clinically unaffected parents who were cousin. The proband have an affected distant relative

Genotyping of the probands' parents and 50 control did not detected the new variation. Only one patient had p.R2069C mutation that revealed as a C to T transitional position 6205 within exon 74 of COL7A1. This mutation had converted an argenine codon (CGT) into a cysteine codon (TGT) (Figure 3). parental consanguinity in 84% of studied families and no record of the disease among other members of 58% of studied families were detected. Three samples of probands' pedigree whose mutation were found are shown in Figures 4-6.

Discussion

Epidermolysis bullosa, a type of blistring disease, with substantial phenotypic and genotypic heterogeniety has been categorized into four subtypes. Dystrophic form of EB is triggered from mutations in COL7A1 with both autosomal dominant and autosomal recessive inheritance. In this study, we showed one novel variant and two discrete recurrent mutations within COL7A1 gene in 7 out of 50 probands from unrelated families. Three out of seven detected mutations was missense and all were located in collagenous domain of COL7A1 within exon 73-75.

In current study, the presence of parental consanguinity in 84% of studied families certified the direct relation between parental consanguinity and the probability of mutated alleles inheritated from a common ancester (8). However, considering the record of the disease among other members of 42% of studied families, our results and according to the pedigrees, most of the detected causative mutations (four out of seven) were just in the affected member of that family showing they were *de novo* mutations. There are some investigations suggesting that sequencing analysis of exons 73-75 of COL7A1 gene is able to detect approximately 75% of causative mutations in patients who are suspicious of DDEB (6, 9) (http://www.ncbi.nlm.nih.gov/books/NBK1304/) and also 95% of cases on the condition of exact diagnosis of DEB (10). In other words, 10.74% of known mutations in COL7A1 are gathered in exon 73 (5). In despite of most of other surveys in which all 118 exons of COL7A1 gene have been investigated (6, 9, 11-13), in our study, we examined the sequence of three exons (73-75) in 50 patients with EB that resulted in detection of 14% of causative mutations at least in one

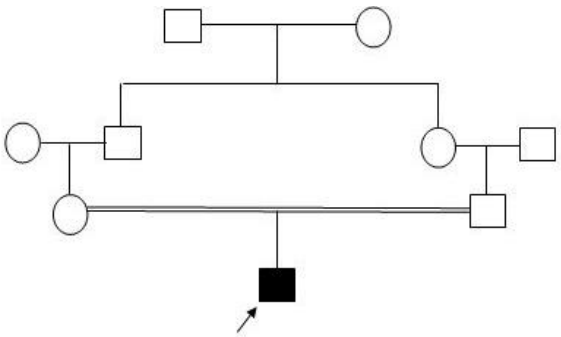

Figure 5. The pedigree of an individual with c.6205C>T (p.R2069C) mutation. The pedigree shows that proband 2 (identified by an arrow) was the affected child of clinically unaffected parents who were cousin

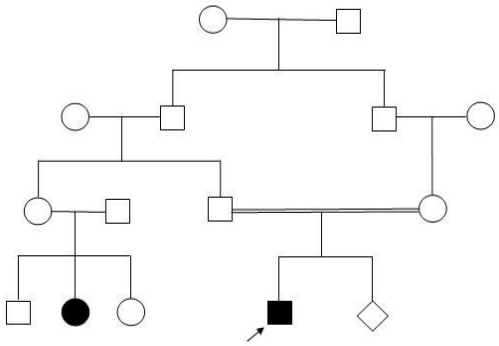

Figure 6. The pedigree of an individual with c.6265delc (p.Pro2089fs) mutation. The pedigree shows that proband 3 (identified by an arrow) was the affected child of clinically unaffected parents who were cousin. The proband have an affected cousin whose parents were not relative

allele which from high to low rate were belonged to exons 75, 73 and 74 respectively. Consequently, this study can be used as the first step in the screening of Iranians with EB, especially, the ones who are suspicious of having DEB.

Substitution in the triple helical domain of type VII collagen, especially in exons 73, 74, and 75 is the cause of catching DDEB in more than 75% of all cases (9). Mutations p.Gly2043Arg and p.Gly2034Arg are the most common mutations which include more than 50% of dominant mutations among biggest studied group of USA population (6). The tendency of Gly substitution to accumulating within exons 73-75 of COL7A1 disclose that the place of Gly in each third condition through triple repeat of type VII collagene is critical for triple helical structure stability (6). These substitutions could lead to anchoring fibrils instability and making them sensitive to mechanical stresses (5). In our study a recurrent glycine substitution (p.G2031S) was found in two patients in homozygous state. According to Nordal study (14), the probands that carry p.G2031S might have RDEB-HS phenotype. Whenever two missense mutations are identified in a proband genomic DNA, his/her parents are obligatory heterozygous carriers of the same mutation and the inheritance pattern is assumed autosomal recessive. This is important because the distinction between autosomal recessive and autosomal dominant is necessary for presenting accurate genetic counselling and prognosis the risk of childbirth with the same disorder in next generation (6).

The other missense mutation which was found in heterozygous state is p.R2069C that is a result of cytosine to tymidine transition in nucleotide 6205 in exon 74. So amino acid argenine is replaced by cystein in codon 2069 of collagen type VII protein in the triple helical collagenous domain. This mutation is reported by Kahofer *et al* in 2003, in compound heterozygous status whose carrier had RDEB-I phenotype (15).

The novel and most frequent variant among detected mutations was a deletion in exon 75 of COL7A1 gene which was found in four-unrelated families. This deletion resulted in a frame shift at the amino acids 2089-2093 of type VII collagene within triple helical collagenous domain and changing in the number and order of amino acids PPGPK in normal protein type VII collagene to PLAP in mutated type VII collagen. In this study the detected recessively inherited *de novo* variant suggests that parents of these families should be called gonadal mosaicism in order to precise genetic counseling, and focusing attention on this matter that the causative mutation might occure in other children of the family. It seems reasonable that we could find new deletion originated from Iranian people which lead to RDEB with regard to other reported mutations which are come from especial family from different countries such as R2814X- 7786delG- R578X originated from Great Britian, 6573+1G>C- E2857X- 5818delC originated from Japan, 425A>G- 8441-14del21-497insA originated from Italy, C-6527insC originated from Spain, and 2470insG originated from Mexico (16). As a result, our study imply the variety of mutational events which cause RDEB phenotype and emphasizes the molecular heterogenity of DEB.

Other studies that have dealt with the adjacent codons to codon 2089 have revealed some mutations exactly at backward and forward codons (11, 17, 18). This may be due to sensitivity of this location to mutations, especially, deletion mutation. Hence, it could develop a new screening method and underlines importance of further assessment on a larger population of Iranian patients with EB.

Conclusion

One of the things resulting from research like the current one on epidermolysis bullosa is the identification of specific and new mutations in COL7A1 gene. Also, extension of mutation data banks makes an accurate diagnosis and better DEB classification possible besides it might cause generation of other diagnostic methods based on clinical histological features and pedigree pattern. In addition, these kinds of studies are used for disease prediction, presenting better genetic counseling and prenatal diagnosis in families with high risk of the disease recurrence and for choosing appropriate candidates for gene therapy.

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