Pregnancy after PGD for recessive dystrophic epidermolysis bullosa inversa: genetics and preimplantation genetics

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Introduction

Epidermolysis Bullosa (EB) is a group of inherited blistering disorders potentially life-threatening to newborns and infants. Stratified squamous epithelium is highly altered. Predominantly, it affects skin and mucous

Capsule Description of the molecular characterisation of a patient affected of dystrophic epidermolysis bullosa inversa followed by preimplantation genetic diagnosis in the family.

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membrane. The unifying diagnostic feature is the extreme fragility of the skin. There is a wide phenotypic variability from very mild to extremely severe manifestations [1]. Genetically, different types of EB have different inheritance patterns [2]. Furthermore, there exists genetic heterogeneity and 13 different genes have been associated with these blistering conditions [3]. Recessive Dystrophic EB Inversa (RDEB-I) (OMIM: #226600) is a subtype of RDEB with a very low prevalence (prevalence estimated of DEB is 0.4–0.6 per million live births [4]). It is caused by mutations in the *COL7A1* gene situated on chromosome 3p21.1, coding for the chain α -1 of Collagen VII. It is a 350 kDa polypeptide with a distinct structural domain organization and is the predominant component of the anchoring fibrils at the dermal-epidermal junction [5]. *COL7A1* is a complex gene, consisting of 118 exons and approximately 32 kb. RDEB mutations include nonsense, splice site, deletions or insertions, silent glycine substitutions and non-glycine missense mutations [6]. Very few cases of RDEB-I have been reported to date and about 29 genotypes have been recorded [7, 8]. RDEB-I is characterized by blistering from birth and early infancy. Later in infancy, blisters localisation change to body flexures, axis and mucous membranes [2]. Patients suffer healing with scarring and skin atrophy, oral, oesophageal, genital mucosal involvement, dental caries and variable nail dystrophy and presence or absence of milia.

From the reproductive point of view, if mutation/s is/are identified, prenatal diagnosis (PND) and genetic counseling is available for subsequent pregnancies. Nowadays, preimplantation genetic diagnosis (PGD) represents an alternative to PND. PGD was developed in the nineties [9] for couples at high risk of transmitting a genetic disease. Currently, PGD is available for a large number of genetic

disorders [10] and the number of cases increases year by year [11]. Briefly, PGD consists on a very early form of genetic diagnosis performed in preimplantation human embryos obtained after *in vitro* fertilization (IVF) treatments. Using micromanipulation technology, one or two cells are biopsied from embryos at the 6 to 8 cell stage. The single embryonic cells (blastomeres) are analysed individually by polymerase chain reaction (PCR). At this stage, the embryonic cells are totipotent and undifferentiated and the genetic status of the blastomere can be extrapolated to the complete embryo. The biopsied embryos are incubated for 24 to 48 h, awaiting the genetic diagnosis results. Finally, healthy embryos are transferred into the maternal uterus. In cases of PGD for single-gene disorders, the first premise is to have an accurate genetic diagnosis of the candidate couple. Dermatologically, more than 70 disorders are hereditary. In many cases, the genetic diagnosis is complex, time-consuming and costly.

In this paper we aim to present the design of the first *ad hoc*-PGD protocol applied clinically in a couple with a previous affected child of RDEB-I. The novelty of this case consists on the description of the complete process: accurate genetic diagnosis of the affected child, mutation detection in the parents, pre-clinical informativity study, single-cell validation studies, and pregnancy after clinical PGD. Unfortunately, at the time of writing this manuscript, a miscarriage was reported by obstetric problems at 15 weeks of gestation.

Materials and methods

Family history

An experienced geneticist provided comprehensive genetic counselling to the couple comprised by a 28 year old woman and a 33 year old man. The couple signed a specific informed consent. The genetic history (Fig. 1) revealed a Romanian family with a past of EB. The consultant couple had a five year old son with Down syndrome, hypothyroidism, cardiac malformations, dysphagia, Milium cyst, localised skin erosions, epidermal peeling on erythematous skin predominantly in flexural extremities and neck, anonychia, oral cavity completely eroded and conical dysmorphic teeth. Dermatologically, data were consistent with a type of DEB. Parents were completely asymptomatic. Paternal grandmother showed a very mild phenotype (toenail dystrophy), compatible with dominant DEB. The immuno-histological study of the child revealed normal staining of the collagen VII in the sample with dermo-epidermic integrity, and absence of staining in the dermal side of the blisters.

Fig. 1 Genealogical tree of the family requesting preimplantation genetic diagnosis for dominant dystrophic epidermolysis bullosa (DDEB). The arrow indicates the consultant patient. The asterisk indicates the family members involved in the informativity study. The grandmother and great uncle have got clinical but not genetic diagnosis

COL7A1 mutations survey

Mutations in the *COL7A1* gene are responsible for dominant and recessive forms of DEB. The screening study is a sequential process that begins by sequencing “high priority” exons 73, 74 and 75, as 75% of dominant glycine substitutions concentrate there. Genomic DNA from the index case patient was extracted from blood in EDTA using the High pure PCR template preparation kit (Roche, Switzerland). Two pairs of primers for the amplification of exons 73, 74 and 75 plus exon/intron boundaries were used (Table 1). Moreover, the 425A>G mutation was analysed by amplification of exon 3. A touchdown PCR was carried out in a total volume of 50 μ L containing 200 μ M dNTP, 2.5 mM $MgCl_2$, 1X PCR buffer, 1 unit *AmpliTaQ Gold* polymerase (Applied Biosystems, USA) and 0.4 μ M each of the forward and reverse primers. Furthermore, for the amplification of exon 3, betaine 1.3 M was also added. PCR conditions were as follows: a denaturation step at 95°C for 10 min followed by 15 cycles of denaturation at

Table 1 Primer sequences used for sequencing exons 3, 73, 74 and 75 of the *COL7A1* gene

Name	Sequences
<i>COL7A1</i> -E3_4-F	5' AGGGCCAGAAGAGATCCTG 3'
<i>COL7A1</i> -E3_4-R	5' CCCATCAGGACTCAGTCACC 3'
<i>COL7A1</i> -E73_75-F	5' AGAAGTGGCTCAGTGGGTTG 3'
<i>COL7A1</i> -E73_75-R	5' AAGCTAAGGGTGGCTTCCTG 3'

F forward, R reverse

95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 1 min. The same conditions were repeated for 10 more cycles, but the annealing temperature was decreased by 0.5°C every cycle until a touchdown of 55°C was reached. Then, 15 more cycles followed: denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension step was performed at 72°C for 10 min. PCR products were electrophoresed in 0.8% agarose and subsequently purified by the MultiScreen_{HTS} 96-well Filtration System (Millipore, USA). The fragment was double chain sequenced and capillary electrophoresed in an ABI 3730_{xl} (Applied Biosystems, USA). The results were analysed and compared with the reference sequence (NM_000094.2) using the SeqScape software (Applied Biosystems, USA).

Pre-clinical PGD study, lymphocyte validation and blastomere analysis

Blood samples in EDTA were requested from both members of the couple and DNA from the affected son was provided. DNA extraction was carried out following the protocol described in the High pure PCR template preparation kit (Roche, Switzerland). Mutation analysis was first performed on 10–30 ng of genomic DNA in a single PCR to confirm the mutations and to determine the informative short tandem repeat (STR) markers, then on 1 ng of diluted genomic DNA in a heminested multiplex PCR with the informative STR markers and finally on 50 single lymphocytes collected from both parents with the same PCR conditions that worked for 1 ng of genomic DNA. Oligonucleotides were tested in order to discard possible human DNA contamination.

Preclinical studies and lymphocyte validation were performed prior to the IVF cycle, following recommended international guidelines on PGD [12] and the norm UNE-EN ISO 9001:2008 [13, 14]. Four STR markers closely linked to the *COL7A1* gene were tested. The molecular analysis protocol was the same for lymphocytes (preclinical set-up) and blastomeres (clinical PGD cycle). Single cells were isolated and lysed as previously described [15]. PCR amplification was performed using the outer and inner oligonucleotide primers listed in Table 2. First round multiplex PCR containing the external primers was carried out in a total volume of 25 µL containing 200 µM dNTP, 1.5 mM MgCl₂, 1X PCR buffer, 1 unit *AmpliTaq Gold* polymerase (Applied Biosystems, U.S.A) and 0.4 µM each of the outer forward and reverse primers. One microlitre from the first round PCR was used as template for separate second round PCR reactions with fluorescently labelled inner forward and reverse primers in a total volume of 10 µL. PCR reactions were undertaken

using a 9700 PCR system (Applied Biosystems, U.S.A.). Twenty five thermal cycles were performed for the outer primers and 50 for the inner primers at 95°C for 1 min, 50°C for 45 s, and 72°C for 1 min. The cycling was preceded by a 5 min initial denaturation step at 95°C and ended with an additional elongation step of 7 min at 72°C. Amplified inner products were electrophoresed in an automated genetic analyser 3730_{xl} (Applied Biosystems, USA). Results were analysed with Genemapper software (Applied Biosystems, USA).

Five microlitres of exons 3 and 74 amplified inner products were purified with 2 µL of ExoSap-IT (Amersham biosciences). Minisequencing reactions were set up using the SnapShot multiplex kit (Applied Biosystems, U.S.A), following manufacturer's protocol. Resulting products were treated with Calph intestinal alkaline phosphatase (Roche) in order to remove unincorporated ddNTPs, incubating at 37°C for 1 h and heating to 75°C for 15 min to inactivate the enzyme.

IVF and embryo biopsy

The patient received a routine superovulation procedure based on the long protocol of down-regulation with gonadotrophin-releasing hormone agonist and ovarian stimulation with recombinant follicle stimulant hormone (FSH) for 12 days. Fourteen *cumulus*-oocyte-corona complexes were retrieved and 11 mature oocytes were obtained. Nine two-pronucleus zygotes were produced after intracytoplasmic sperm injection. Eight cleaving embryos were available for biopsy on day 3. Two blastomeres were removed per embryo and 16 single blastomeres were studied by PCR. IVF, embryo culture and biopsy procedures were performed according to previously described protocols [16].

Results

COL7A1 sequencing

The study of exons 73, 74 and 75 of the *COL7A1* gene on the affected son revealed the pathogenic mutation c.6205C>T. This mutation was double confirmed. It represents a cytosine to thymine replacement in nucleotide position 6205 of the cDNA. This mutation was described in a DEB inverse, a very rare recessive form occurring in early infancy with preserved immunoreactivity for type VII collagen [17]. This result is compatible with the clinical features of the affected son.

In addition, the c.425A>G mutation screening resulted positive. This mutation represents a splice-site mutation described in central European patients with DEB [18]. This

Table 2 Primer sequences for the STR markers used in the preimplantation genetic diagnosis of recessive dystrophic epidermolysis bullosa inversa

Name	Heterozygosity	Gene distance (Mb)	Primer sequences
D3S1581	0.88	0.35	Fout: 5' TGTCCCAGCCTAGAAGCCAT 3' R: 5' GCCATGATTGCACTATGTG 3' Fin: 5' 6FAM-GCCAGTCCTGACAGAACT 3'
D3S2420	0.77	0.53	Fout: 5' GATCGTACCACTGCACTCCAG 3' R: 5' CAGGAGCCTCTAAGTCAGC 3' Fin: 5' VIC-GCTACAAGTGCAGAACTCTG 3'
D3S643	0.49	0.04	Fout: 5'CCAGTCCCTGTATCTACTG 3' R: 5'CACTATGTGCTCCAGGCTG 3' Fin: 5' NED-GACAGAACTGCCAAACCATC 3'
D3S3629	0.78	0.95	Fout: 5' TCTACAACTGCCTGTCTG 3' R: 5' GAGATTCCGAGAGCAGCGAG 3' Fin: 5' PET-GCACTTTAGCTTAGTTATGCC 3'
<i>COL7A1</i> -E3 c.425	N/A	N/A	Fout: 5' CTGGATGCACTTGGCTCTG 3' R: 5' CCTGGACACTTCATTGGG 3' Fin: 5' 6FAM-CTCCATGTGGCTGACCATGTC 3' revSeq: 5' TGGTAGGGGTAGGGATCACC 3'
<i>COL7A1</i> -E74 c.6205	N/A	N/A	Fout: 5' TGGAAAGCCTGGTATTCCCG 3' R: 5' CAGCACAGCCTCCAGACAG 3' Fin: 5' 6FAM-CCGCTATTTCAGTTTCAGGG 3' Seq: 5' GAGAACGGGGAGAGAAAGGAGAA 3'

Fout forward outside primer; *Fin* forward inside primer; *R* reverse primer; *revSeq* reverse sequencing; *Seq* Sequencing; *N/A* not applicable, *Mb* megabases

GeneLoc: Exon-based integration of human genome maps. Bioinformatics 19(S1). URL: <http://genecards.weizmann.ac.il/geneloc>

confirmed the son's status of compound heterozygote. The inheritance of these mutations was confirmed: the mother carried the c.6205C>T and the father carried the c.425A>G. This mutation combination has been previously reported in RDEB-I [17].

Set up and informativity test

Four STR markers located at both sides of the *COL7A1* gene were tested, resulting in two informative (D3S1581 and D3S2420). The order respect to the *COL7A1* gene is shown in Fig. 2(a). Once the strategy of diagnosis to detect the c.425A>G and c.6205C>T mutations in the *COL7A1* gene was established, validation of the technique was carried out with 50 single isolated lymphocytes from the couple. Amplification failure and allelic drop out (ADO- the random non-amplification of one of the alleles) rates for each PCR amplicon were estimated. ADO rates were 20% (5/25) for c.425A>G mutation, 8% (2/25) for c.6205C>T mutation, 32% (16/50) for D3S1581 and 12% (6/50) for D3S2420. PCR efficiency for each amplicon was 88% (44/50) for the *COL7A1* gene exons 3 and 74, 100% for D3S1581 and 96% (47/50) for D3S2420.

Clinical PGD cycle

PGD results are shown in Fig. 2(b). Two blastomeres failed to amplify, as cells were lysed during the biopsy procedure

and DNA probably degraded. ADO rates were 14% (2/14) for c.425A>G mutation, 0% (0/14) for c.6205C>T mutation, 0% (0/14) for D3S1581 and 7% (1/14) for D3S2420. PCR efficiencies were 100% (14/14) for exons 3 and 74 mutations, 92.8% (13/14) for D3S1581 and 100% (14/14) for D3S2420.

An overall amplification rate of 98.21% was obtained and total ADO was 3.57%. The diagnostic rate was 100%. None of the blank controls signalled positive. Two embryos were diagnosed as healthy, four were carriers of the maternal mutation, one was carrier of the paternal mutation and one was affected. Electropherograms for some embryos are displayed on Fig. 2(c). Embryos number 6 and 8 were transferred and a singleton clinical pregnancy was achieved. PND was offered but the couple declined it.

Fig. 2 Preimplantation genetic diagnosis (PGD) of recessive dystrophic epidermolysis bullosa inversa (RDEB-I). **a** Position of the short tandem repeat markers (D3S2420 and D3S1581) with respect to the *COL7A1* gene on chromosome 3. **b** Pedigree showing haplotypes of the family members involved in the informativity study and embryo haplotypes generated in the PGD cycle. The arrow indicates the consultant patient. The thick black line represents the maternal RDEB-I-bearing haplotype and the discontinuous line represents the paternal RDEB-I-bearing haplotype. **c** Fragment analysis of the PGD cycle. Electropherograms from the couple and some embryos are displayed. Paternal affected alleles are in circles; maternal affected alleles are in squares. Tel: telomere; cen: centromere; Mb: megabases; G: guanine; A: adenine; C: cytosine; T: thymine; Mat: maternal; Pat: paternal; ET: embryo transfer

Discussion

Usually, couples with affected offspring or a family history of EB look for preconception assessment in order to avoid transmission of the disorder in subsequent pregnancies. In this reproductive context, PGD is a well-established procedure to avoid inheritance of serious hereditary diseases and it is a valid alternative to PND and termination of pregnancy. The accurate genetic diagnosis is a *sine qua non* requirement for PGD application. For inherited skin fragility disorders, the final genetic diagnosis requires a close collaboration between dermatologists and geneticists. In this sense, inherited EB is a prototype of phenotypic variability where the unifying diagnostic feature is skin fragility in several degrees.

In DEB cases, all forms are caused by mutations in the *COL7A1* gene. A genotype-phenotype correlation has been described [19] and the disease-causing mutations are usually specific for individual families, being recurrent mutations very unusual. In the present case, the positive collagen VII staining and family history suggested a dominant form inherited from the paternal side. However, genetic results from the hot spot exons sequencing revealed a previously described point mutation (c.6205C>T) associated to recessive forms of DEB. Moreover, this mutation was maternally inherited. These contradictory findings complicated the accurate diagnosis. A comprehensive revision of clinical features, immunostaining findings and literature, led to focus the attention in RDEB-I. RDEB-I was described in 1971 [20] and only a few cases have been reported to date [7, 8]. The main trait is positive staining for type VII collagen at the blister roof and anchoring fibrils are variably reduced and hypoplastic. From the clinical point of view, Kahofer *et al.* [17] described two children with DEB-I with very similar clinical features, in particular congenital blistering on the trunk and extremities shortly after birth and pronounced mucosal involvement. Dental aplasia, nail dystrophy, inguinal blistering, healing with atrophy, and milia were other traits. Genetically, these children were compound heterozygotes for two point mutations, c.6205C>T and c.425A>G. In particular, c.425A>G was described as a prevalent mutation causing DEB in central Europe [18]. This mutation was screened in the affected child because of his geographical origin (Romania). Finally, the affected son was a compound heterozygote exactly with the same genotype described previously (c.6205C>T/c.425A>G) [17].

The c.425A>G mutation at the position -2 of the donor splice site of exon 3 causes complex aberrant splicing pattern and three abnormal mRNA transcripts: in one form the exon 3 is skipped; in the second form 104 nucleotides at the 3' end of exon 3 are missed, as a consequence of the activation of a cryptic GT site inside this exon; in the third form, the entire intron 3 is

retained. The altered transcripts contain a premature termination codon before the end of exon 4, which results in shorter pro α 1(VII) chains, missing approximately 90% of the protein sequence [21, 22]. These truncated chains undergo premature degradation and are not incorporated into the collagen VII anchoring fibrils. However, the normal allele is sufficient to achieve a normal phenotype.

The large constellation of genetic mutations identified in DEB has allowed the establishment of a genotype-phenotype correlation between severe recessive forms. However, this correlation remains dark in particular subtypes as RDEB-I. Recently, van den Akker *et al.* [8] have proposed a recessive arginine and glycine substitution in the triple helix domain of ColVII as a predominant mutation. The study of Csikós *et al.* [18] suggests that the prevalent mutation c.425A>G is inherited in a recessive manner, and the phenotype is determined by the second mutation. In this sense, the second mutation (c.6205C>T) affects residue number 2069 of the protein that falls within subdomain COL12, considered crucial for the function of collagen VII [5]. The substitution of a large arginine (residue 2069) by a non-polar residue (cysteine), which preserves hydration of the polypeptide, with the concomitant effect of premature termination codon caused by c.425A>G, could cause the peculiar RDEB-I.

In the PGD context, the methodological aspects are noteworthy. Two different approaches have been proposed: indirect and direct PGD. Differences between both have been thoroughly discussed previously [23]. Basically, indirect PGD offers a wide-scope approach based on haplotype analysis in preimplantation embryos. Haplotyping is based on a battery of informative STR markers linked to the gene of interest. Recently, an indirect PGD based on haplotyping for the *LAMB3* gene has been proposed for Herlitz Junctional EB [24]. This wide-scope approach would be very useful in cases where DNA from the parents and at least one affected member, or sufficient unaffected members of a family are available in order to identify low and high-risk haplotypes. However, the major limitation is when STR markers result non-informative, family samples are not available or the patient carry a *de novo* mutation and it is impossible to establish haplotypes *a priori*. In agreement with the authors, direct PGD based on detecting the disease-causing mutation, represents the only alternative in these cases. Thus, *ad hoc* PGD protocols allow interrogating the disease-causing mutation on blastomeres, achieving the exact genotype of each embryo. Furthermore, it reduces the risk of misdiagnosis related to recombination of STRs markers, and permits the combination of methods in recessive disorders with different mutations [25]. In addition, in cases of rare inherited skin fragility disorders with a wide genetic heterogeneity, the setting-up and validation of a generic strategy is a

too laborious task for the very few cases expected. In our opinion a case by case strategy is the gold standard option for couples at risk of these extremely rare disorders.

Nowadays, in the molecular PGD laboratories, when the disease-causing mutations are diagnosed, the adaptation of PCR-based protocols to single cell conditions is a well-established, optimized, rapid and cost-effective approach. The preclinical work-up and validation on single cells results radically important. This informativity test allows confirming the mutations, to identify informative STR markers linked to the gene used as support for direct genotyping of the embryos, to validate PCR efficiency, to validate ADO rates for the primers used and to optimize the entire protocol on single cells [12]. Respecting ADO and amplification efficiencies for each STR marker, in some cases the estimated rates obtained in the preclinical setup differ from the rates obtained in the PGD cycle. In some cases, these rates are not in line with ESHRE's guidelines (20% of ADO for the c.425A>G mutation and 32% of ADO for D3S1581). Nevertheless, it has been previously published that ADO and amplification efficiency rates may vary depending on the cellular type analysed [12]. For example, ADO of the STR marker D3S1581 was 32% in the informativity study but 0% in the PGD cycle; or amplification efficiency for the c.425A>G mutation was 88% in the informativity study but 100% in the PGD cycle.

In summary, the origin of PGD is closely linked to the development of IVF technologies. Typically it is said that the close collaboration between human geneticist, reproductive medicine expertises and embryologist is a necessary condition. Nevertheless, it is not enough. In cases of serious inherited skin disorders, the accurate clinical management results radically important. In these rare conditions, where specific treatments are not available and symptomatic therapy is the only therapeutic option, avoiding inheritance is possible by means of PGD, where the disease-causing mutation/s is/are properly identified.

Conflicts of interest None declared.

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