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First successful preimplantation genetic diagnosis of epidermolysis bullosa with pyloric atresia: Case study of a novel c.4505-4508insACTC mutation

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Introduction

Epidermolysis bullosa (EB) is an inherited blistering disorder characterized by the fragility of the skin and mucous membranes [1–4]. The disease can be traumatic and lethal especially for newborns and infants however non-lethal variants were reported [5]. Epidermolysis bullosa is categorized in three major groups: EB simplex(EBS), junctional EB (JEB) and dysthrophic EB (DEB) [3, 4, 6] . EB with pyloric atresia (EBPA) is classified as a form of JEB. EBPA; which is inherited in an autosomal recessive manner is characterized by fragile skin and congenital atresia of the gastrointestinal tract (especially pylorus) [7–9].

α6β4 integrin levels are shown to be reduced or absent in EBPA patients. α6β4 of hemidesmosomes attaches the keratinocyte to the basal membrane and is encoded by ITG A6 and ITGB4 genes [8, 10–13] Sequencing analysis shows that ITGB4 accounts for%80, ITGA6 accounts for%5 of EBPA patients [14]. Another binding protein of hemidesmosomes, Plectin also interacts with α6β4. Plectin is encoded by PLEC1 gene and as sequencing analysis shows; accounts for the%15 of EBPA patients [15, 16].

Recently, more than a hundred disease causing mutations for EBPA have been described. [1, 10, 11, 17–23]. In this case study we report an in vitro fertilization (IVF)- preimplantation genetic diagnosis (PGD) cycle with a novel mutation of ITGB4 (c.4505-4508insACTC) to a family with a history of 3 deceased children diagnosed as EBPA. A singleton pregnancy was confirmed at 7-week gestation by ultrasonography. After an uneventful pregnancy period, a healthy baby was born.

Capsule This is the first succesful PGD for Epidermolysis Bullosa with pyloric atresia with the subsequent delivery of a healthy baby, as the literature revealed not one single result as such.

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Methods

Family history

The consulted couple was comprised of a 30 years old woman and a 28 years old man with a history of 3 deceased babies diagnosed as EBPA. The latter was operated for pyloric stenosis and died as a result of pulmonary failure and sepsis postoperatively. The woman had also experienced a ruptured ectopic pregnancy which resulted in an emergency operation. The laboratory findings of the woman and the semen parameters of her husband were normal.

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ITGB4 mutation survey and pre-clinical PGD study

Genomic DNA from the parents were extracted from blood in EDTA using the GeneJET Genomic DNA purification kit (Fermentas, Life Sciences). Outer and inner pairs of primers (forward and reverse) for nested-polymerase chain reaction (PCR) amplification of sequences encompassing the insertion mutation (c.4505-4508insACTC) were designed using the Primer3 software based on the ITGB4 gene sequence (NCBI) (Table 1). Mutation analysis were 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. Four STR markers located at both sides of the ITGB4 gene were tested. Marker primers suitable for multiplex PCR were carefully designed and one of the inner primers was flourescently 5′-labelled. The primer sequences were described in Table 1.

IVF, blastomere biopsy and cell lysis

The patient was treated with an antagonist protocol in which Gonal F300IU/day SC was started on the second day of the cycle. At day 10, Ovidrelle 250mcg SC was given. Twenty eight oocytes were collected and 18 mature oocytes were obtained. Ten embryos were achieved after intra-cytoplasmic sperm injection. Ten cleaving embryos were available for biopsy on the morning of post-fertilization day 3. Following mechanical assisted hatching, blastomeres containing a nucleus were gently aspirated through the opening. The single blastomeres were collected and put into the small eppendorf tubes containing 5 μl lysis buffer (including H2O, 10X Buffer, 10% Tween 20, 10% TritonX, Proteinase K). Cells were lysed via a lysis protocol: 45°C for 15 min, 96°C for 15 min and 72°C for 10 min.

PCR analysis

First round multiplex PCR containing the external primers was carried out in a total volume of 30 μl using HelixAmp Taq DNA polymerase (including 10x Reaction Buffer, TuneUp solution, dNTP Mix) (Nano-helix Cat no: T5000N). The first and second round PCRs were performed in a thermalcycler (Applied Biosystems, GeneAmp PCR System 9700) using the following PCR protocol: 1 cycle at 95°C for 2 min; 35 cycles at 95°C for 30 s, 57°C for 50 s, and 72°C for 1 min; 1 cycle at 72°C for 7 min.

For the second round PCR reactions, 2,5 μl first round PCR product was used as a template DNA. The total volume of each second round reaction was 30 μl containing the inner primer pairs for the mutation and STRs. Mutation primer amplicons were purified and directly sequenced using automated DNA sequencer (Model 3100, Applied Biosystems, Foster City, CA). Fragment analysis were performed for the analysis of STR markers.

Results

Set up and informativity test

Preliminary genetic analyses on genomic DNA from the couple were carried out in order to verify the genetic status of each family member and to determine the STR alleles linked to ITGB4 mutation. As expected, the mutation in the *ITGB4* gene was confirmed in both parents. It represents an insertion of 4 bases (ACTC) into the nucleotide position 4505 of the cDNA. Three STR markers (D17S1839, D17S785, D17S1817) were found to be informative among the four markers tested. Fragment analysis of the STR markers confirmed that there were no allelic drop out (ADO-the random non amplification of one of the alleles).

Table 1 Nested primer sequences for mutation and STR markers

Primers	Orientation	Primer sequences (Outer)	Primer sequences (Inner)
ITGB4	F	CAGAGCACCTGGTGAATGG	CAGAGCACCTGGTGAATGG
	R	ATGCGTGTGCACGTGTGT	GGAAGGGTTAGTGGGAGAGG
D17S1839	F	CGCCTCAGTCTCCCAAAGT	GCCCAGCGGACTTTAGATTT
	R	GGAGGTTAAGGCTGCAGTGA	GCCCCTGCCTTATAAAAAGA
D17S785	F	CACATAGAGTTTGGTCACAGTGG	TCCCTGGAGAGTGAAAATGG
	R	GCGTCTGTTTCCCTGTGTTT	CGTAAGGCCAACCTGAAAAC
D17S1817	F	TGGTTCTTAGGACTGGGAAGG	AGGCTTTCTTTAGGGGGTGA
	R	AAGTGGAGGTTGGCAGTGAG	GCTCTAGCCTGGGTGACAGA
D17S801	F	TGGAAAGGCAGGATAGATGC	TGCCTCTCTGGGCCTGAT
	R	CTTGAGCCCAGGAGTTTGAG	CAAGCCAGAGAGCAAGATCC

F forward; *R* reverse

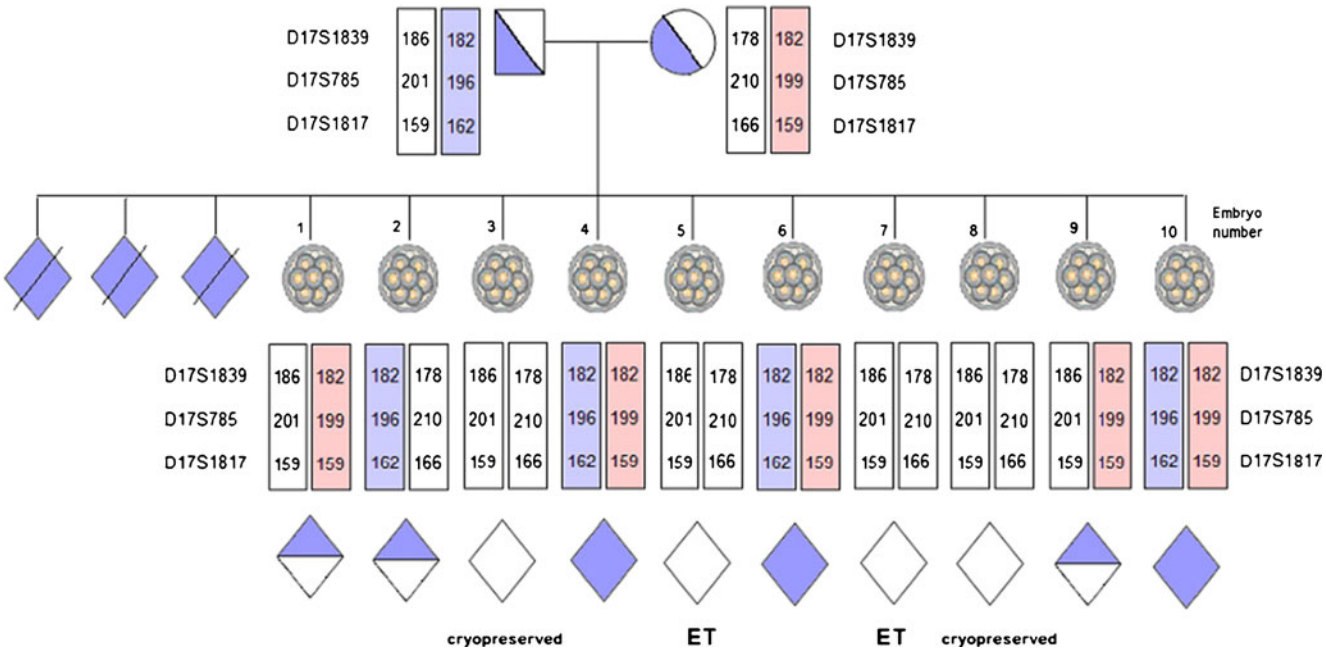

Fig. 1 Pedigree displaying haplotypes of the family members and embryos generated in the PGD cycle

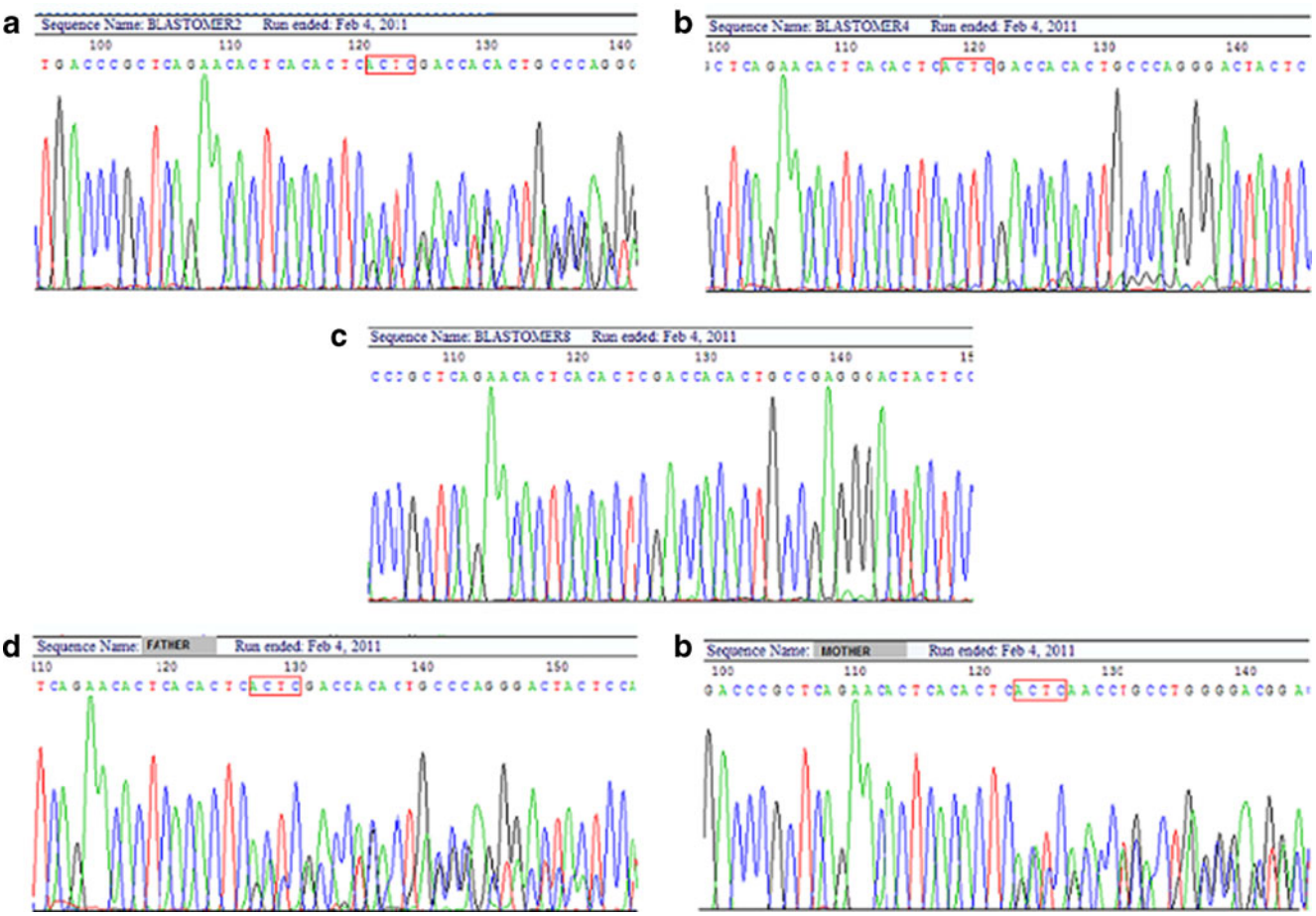

Fig. 2 Sequence analysis of the PGD cycle including the parents and some embryos. ACTC insertion is displayed in rectangle. **a** Embryo no.2, heterozygous; **b** Embryo no.4, affected; **c** Embryo no.8, normal; **d** Father, heterozygous; **e** Mother, heterozygous

Clinical PGD cycle

From the genetic point of view, results of the simultaneous PGD for EB-PA were summarized in Fig. 1 and the sequencing results of the parents and some embryos were displayed in Fig. 2. All 10 blastomeres were successfully amplified and none of the blank controls signalled positive. Embryos number 1, 2 and 9 were heterozygous, embryos number 4, 6 and 10 were homozygously mutant for the mutation and the rest of the 4 embryos were healthy for EB-PA. Among these 4 healthy embryos; embryos number 3 and 8 were cryopreserved and number 5 and 7 were transferred. A singleton pregnancy was achieved and a healthy baby was born.

Discussion

Preimplantation genetic diagnosis for couples at risk of pregnancies with genetic disorders is firmly established as an important reproductive option for couples after appropriate genetic counselling. The present study examined a family with a previous history of 3 deceased babies due to EBPA.

Like most of the inherited diseases EBPA is non treatable. Since this is an autosomal recessive disorder, each parent of an affected child is a carrier due to having a single copy of the altered gene. At each conception the possiblity of having an affected child is 25% where as the chance of having a normal or carrier child is 25% and 50% respectively. For this reason PGD at IVF cycle and/or prenatal diagnosis of the pregnant women at risk seems to be the only way to prevent the risk for recurrence.

Prenatal diagnosis of this disease-based on either conformation-sensitive gel electrophoresis (CSGE) or denaturing high-performance liquid chromatography (dHPLC) scanning analysis, followed by nucleotide sequencing, has also been previously described [24]. Likewise in the study of Stoevesandt J et al. (2011), prenatal diagnosis was performed for a case of lethal EB-PA (junctional type) with negative immunoreactivity to integrin $\alpha 6$ and integrin $\beta 4$. Identification of compound heterozygosity for two novel ITGB4 mutations; c.600dupC/p.F201fsX14 and c.2533 C>T/p.Q845X in the affected preterm infant allowed prenatal diagnosis and finally birth of a healthy sibling [25]. Natsuga K et al. (2011) demonstrated that recurrent c.1938delC in ITGB4 is a founder mutation in JEB-PA patients, and that genotyping of the mutation can be utilized for prenatal diagnosis of JEB-PA [26]. In our study to avoid abortion of possibly affected pregnancy, we have offered diagnosis at the embryonic stage before implantation. Embryo biopsy of one blastomere from each embryo obtained via ICSI-PGD cycle allows early detection of the mutation. We previously reported the first case of PGD for Leigh syndrome resulting in delivery of a healthy newborn and declared that for autosomal recessive or dominant disorders PGD can be safely used [27].

To the best of our knowledge, we have for the first time performed IVF-PGD cycle for a couple at risk of having children with EBPA and reported a successful pregnancy followed by a birth of a healthy baby. In literature there are various studies focusing on preimplantation diagnosis of other types of epidermolysis bullosa. Vendrell X et al. reported a pregnancy [1]. Furthermore, Cserhalmi-Friedman PB et al. performed PGD for Herlitz type of junctional epidermolysis bullosa and carried out mutation screening using heteroduplex analysis and direct sequencing of the PCR products [28]. Likewise, Fassihi H et al. (2006; 2010) described an alternative approach of preimplantation genetic haplotyping (PGH) in which whole genome amplification followed by haplotyping of multiple polymorphic markers using standard DNA-based polymerase chain reaction (PCR) assays were carried out [29, 30]. Pregnancy was achieved in the PGD cycle and a healthy child was born.

The forms of EBPA with the severest cutaneous manifestations are caused as a result of mutations causing premature termination codons on both alleles. Also many amino acid substitutions result in severe phenotypes [17]. In this study, we report the successful PGD and delivery of a healthy baby in a couple at risk of transmitting the EBPA disease as a result of a homozygous 4 bp insertion mutation in exon 34 of *ITGB4* of the proband designated c.4505-4508insACTC (p.H1169fsX68). This mutation is novel and is expected to result in a frameshift with a downstream premature termination codon. Both parents are heterozygous carriers of this mutation. According to sequence analysis 4 out of 10 embryos were found to be normal while 3 of them were mutant and the rest 3 were heterozygous. Among the 4 healthy embryos, two healthy embryos were transfered and the others were cryopreserved for a future embryo transfer. A singleton pregnancy was resulted, declining confirmatory prenatal diagnosis a healthy baby was subsequently delivered at term.

Under clinical PGD circumstances, single-cell PCR requires an extremely sensitive protocol. The adaptation of PCR protocols to single cell conditions should be a well-optimised, rapid and safe approach. The preliminary work up with genomic DNA of the parents is very important to determine the best PCR conditions not only for the amplification of mutation sites but also for STR fragments. To identify the informativity of the STR markers is necessary. The informative ones will be used as a support for direct genotyping of the embryos, a strong indicator revealing ADO rates and a validator of PCR efficiency [31].

The single-cell PCR assay involving nested PCR reactions described in this paper has shown a satisfactory level of amplification efficiency for which all of the embryos analyzed could be amplified and diagnosed. Inclusion of STR markers reduces the risk of misdiagnosis due to ADO by allowing the discrimination of maternal and paternal alleles and significantly improves the diagnostic accuracy [32]. As a result of the

increased sensitivity of fragment analysis, cases of true ADO and extreme preferential amplification could be distinguished.

In conclusion, PGD for each single gene disorder can be based on robust techniques of mutation detection in single cells. The use of PGD has come into prominence especially for the detection of lethal genetic diseases. On the other hand, for the mendelian inherited diseases, PGD is a very effective application with respect to the prenatal diagnostic tests such as amniocentesis or chorionic villus sampling (CVS). The major benefit of PGD is the elimination of requirement of medical abortion. The inclusion of more optimised and accurate PCR strategies will allow to improve the reliability of PGD. The experience provided by this study encourages the development of standardized molecular PGD protocols for many rare diseases.

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