Embryo genome profiling by single-cell sequencing for successful preimplantation genetic diagnosis in a family harboring COL4A1 c.1537G>A; p.G513S mutation

Nayana H. Patel, Harsha K. Bhadarka, Kruti B. Patel, Salil N. Vaniawala[1], Arpan Acharya[1], Pratap N. Mukhopadhyaya[1], Nilofar R. Sodagar

Department of IVF, Akanksha Hospital and Research Institute, Near Shrushti English Medium School, Lambhvel-387310, Anand, Gujarat, [1]SN Gene Lab, Floor, A, Above Kansaar Hotel, President Plaza, Ring Rd, Aveerbhav Society 1, Nanpura, Surat, Gujarat, India

Address for correspondence:
Nilofar R. Sodagar, Clinical Research Associate, Akanksha Hospital and Research Institute, Nr. Shrushti English Medium School, Lambhvel - 387 310, Anand, Gujarat, India.
E-mail: nilofar137@gmail.com

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ABSTRACT

CONTEXT: Genetic profiling of embryos (also known as preimplantation genetic diagnosis) before implantation has dramatically enhanced the success quotient of *in vitro* fertilization (IVF) in recent times. The technology helps in avoiding selective pregnancy termination since the baby is likely to be free of the disease under consideration. **AIM:** Screening of embryos free from c.1537G>A; p.G513S mutation within the COL4A1 gene for which the father was known in before be in heterozygous condition. **SUBJECTS AND METHODS:** Processing of trophectoderm biopsies was done from twelve embryos for c.1537G>A; p.G513S mutation within the COL4A1 gene. DNA extracted from isolated cells were subjected to whole genome amplification using an isothermal amplification and strand displacement technology. Oligonucleotide primers bracketing the mutation were synthesized and used to amplify 162 base pairs (bp) polymerase chain reaction amplicons originating from each embryo which were subsequently sequenced to detect the presence or absence of the single base polymorphism. **RESULTS:** Three out of 12 embryos interrogated in this study were found to be normal while 9 were found to harbor the mutation in heterozygous condition. Implantation of one of the normal embryos following by chorionic villus sampling at 11th week of pregnancy indicated that the baby was free from c.1537G>A; p.G513S mutation within the COL4A1 gene. **CONCLUSIONS:** Single-cell sequencing is a helpful tool for preimplantation embryo profiling. This is the first report from India describing the birth of a normal child through IVF procedure where a potential pathogenic COL4A1 allele was avoided using this technology.

KEY WORDS: COL4A1, embryo, *in vitro* fertilization, single cell sequencing, surrogate

INTRODUCTION

There has been a dramatic development in recent times in the area of single cell genome analysis that now arms investigators to interrogate a variety of genetic heterogeneity within cellular populations. A plethora of biological phenomena is now accessible for investigation with single cell sequencing technology that includes clonal diversity within cancer,[1] the role of genetic mosaicism in the biology of multicellular organisms,[2] the genomic variation in gamete cells and embryos,[3] and the metabolism of as-yet-uncultivable microbes.[4]

The development of single cell genomics has revolutionized reproductive genetics in a significant way.[5] This technology, commonly known as single-cell whole genome amplification (WGA), generates enough replicates of genomic DNA to allow several and repeated sequencing of specific

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regions of target genes. It can be broadly divided into two categories; one that relies on thermal cycling as the primary tool for amplifying the target while the other that relies on nonpolymerase chain reaction (PCR)-based isothermal technologies.[6] The former rely on ligation of a common primer sequence to sheared and shortened DNA or on the use of degenerate oligonucleotides for priming. The PicoPLEX Single Cell WGA Kit (New England Biolabs, UK) is one such reagent set that excels in WGA.[1]

Evolving single cell genomics technology and its marriage with reproductive genetics has paved the way for preimplantation genetic diagnosis (PGD) testing, often referred to as genetic profiling of embryos before implantation. When exploited to screen for specific diseases, the primary advantage of this technology is that it helps in avoiding selective pregnancy termination since the technology ensures that the baby is free from the disease under consideration. Hence, this method may be seen as an adjunct to assisted reproductive technology, and hence requires *in vitro* fertilization (IVF) to obtain embryos for evaluation.

In this report, we describe profiling of a set of embryos to identify those which are free from pathogenic genetic mutation(s) in the COL4A1 gene. This gene codes for Type IV collagen alpha 1 in humans and is located on the long (*q*) arm of chromosome 13 at position 34, from 110, 148, 958 base pair (bp) to 110, 307, 157 bp.[7] Dominant mutations within this gene are responsible for highly penetrant cerebrovascular diseases including intracerebral hemorrhages.[8,9] Diseases associated with aberrations within this gene also include hereditary angiopathy with nephropathy, aneurysms, and muscle cramps (HANAC) syndrome that are usually characterized by asymptomatic small-vessel brain disease, cerebral large vessel involvement (i.e., aneurysms), and systemic findings involving the kidney, muscle, and small vessels of the eye.[10]

SUBJECTS AND METHODS

Clinical samples

A couple with a history of HANAC syndrome within the family of the male partner requested consultation from IVF Department of our hospital, to ensure a child-free from the genetic disorder. Following consultation and discussion on all relevant aspects pertaining to PGD, the couple signed an informed consent form to undertake PGD testing with an aim of bearing a child free from HANAC syndrome.

The sources of DNA used in this study were of two categories. (1) Blood DNA from father who was a suspected carrier for mutation within the COL4A1 gene (c.1537G>A; p.G513S) and (2) cells originating from embryos which were in need for genetic profiling for the COL4A1 mutation in question. By means of laser-assisted trophectoderm biopsy [Figure 1], 5–6 cells were obtained from trophectoderm of each embryo while not disturbing the inner call mass from which the fetus develops.

Extraction of DNA and whole genome amplification

DNA was extracted from blood samples using a QIAamp DNA Blood Mini Kit (Germany) and from biopsied samples using an REPLI-g Mini Kit from Qiagen, Germany. WGA was performed using a PicoPLEX™ WGA Kit (New England Biolabs, UK). Sample amplification efficiency was analyzed using a real-time quantitative PCR instrument by adding SYBR Green I Dye at ×0.125 final concentration in the amplification cocktail.

Polymerase chain reaction and nucleotide sequencing

Gene-specific oligonucleotide primers (COL4NF: 5′-TAAAACTCACCAGGCTCCC-3′ and COL4NR: 5′-GACCATTTTCAAGAAGG-3′) were designed to amplify a 162 bp PCR amplicon harboring position c.1537G>A of the COL4A1 gene. The thermal cycling conditions were as follows: A hold of 94°C for 2 min followed by 35 cycles, each comprising of 94°C for 30 s and 58°C for 1 min. This was followed by a hold of 72°C 30 s.

Amplified products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany), sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and resolved on a 3130xl Genetic Analyzer (Thermo Fisher Scientific, USA).

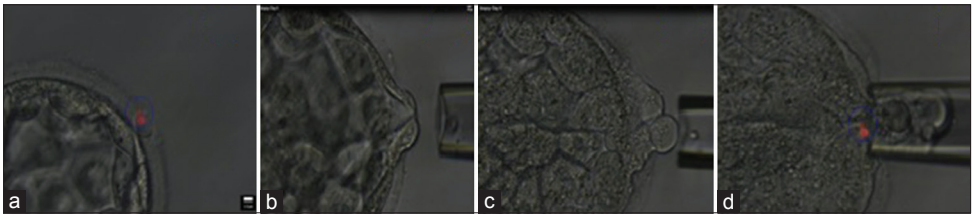


Figure 1: Laser-assisted trophectoderm biopsy: (a) Partial zona pellucida dissection with laser beam, (b) suction of cell of trophectoderm by vacuum created in pipette, (c) suction of cells of trophectoderm, (d) laser-assisted separation of trophectoderm cells from the mass

In vitro fertilization procedure

IVF was carried out as per standard procedure.[11] Very poor quality of oocytes due to higher age of the patient indicated use of donor oocytes. Following evaluation and assessment of semen, the technique of intracytoplasmic sperm injection (ICSI)[12] was adopted.

RESULTS

The family history of the couple in the present study indicated a child, from the family of the male partner, suffering from the HANAC syndrome by acquiring a pathogenic allele of COL4A1 gene (c.1537G>A; p.G513S). As expected, DNA from the male partner indicated a heterozygous condition for the c.1537G>A mutation within COL4A1 gene.

In the first cycle, ICSI was performed on 14 donor eggs, and 3 embryos reached the blastocyst stage [Table 1, 1st IVF cycle]. On day 6, trophectoderm biopsies were taken from all the three embryos and subjected to embryo genome profiling for detection of the target mutation within the COL4A1 gene. The whole genome amplified DNA was used as template to amplify a 162 bp region of the COL4A1 gene with possibility of harboring the c.1537G>A mutation in any sample. The PCR amplicons, represented by a sharp band on an agarose gel, indicated satisfactory functioned of the COL4A1 gene specific oligonucleotide primers [Figure 2]. These oligonucleotide primers were also found to generate clear nucleotide sequencing peaks when subjected to capillary sequencing [Figure 3]. Unfortunately, single cell sequencing data indicated that all the three embryos carried the pathogenic COL4A1 allele thus prompting us to undertake further screening.

By keeping in mind, the possibility of abnormal embryos, in the second cycle, a total of 23 eggs originating from two different donors were used for fertilization, but only 12 embryos made it to the blastocyst stage [Table 1, 2nd IVF cycle]. Trophectoderm biopsies from all these 12 embryos were again subjected to single cell sequencing to interrogate for the c.1537G>A; p.G513S mutation within the COL4A1 gene. Results indicated that out of 12, 9 were heterozygous while 3 were normal or wild type with regard to the COL4A1 mutation [Figure 4]. Out of these three normal embryos, one was arbitrarily chosen and implanted into a surrogate mother, which resulted in successful pregnancy. Chorionic villus sampling at 11th week of pregnancy confirmed the absence of the COL4A1 mutation and eventually a healthy baby was born. The inheritance pattern of the mutant COL4A allele is indicated in Figure 5.

DISCUSSION

The phenotypes resulting from COL4A1 mutations are genetically complex, pleiotropic, and involve other organ systems. Its link with a spectrum of cerebrovascular diseases is well documented. Pathogenic alleles of this gene cause the well-known HANAC syndrome and are associated with milder cerebrovascular diseases also.[13-15]

In standard IVF event, all the functions of the sperm are vital for successful fertilization. This includes its binding to zona pellucida (ZP), undergo the acrosome reaction and penetrate the ZP to fuse with the oolemma before fertilization takes place. However, in contrast, most sperm

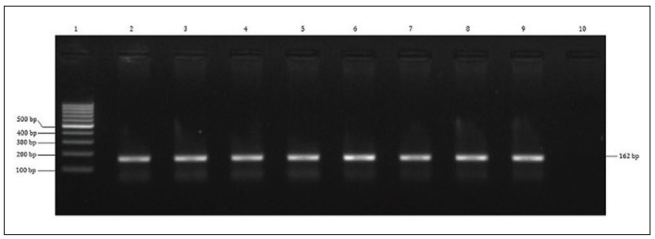


Figure 2: Polymerase chain reaction amplicons of size 162 base pairs (bp) generated from COL4A1 gene and harboring the c.1537G>A mutation. Lane 1: 100 bp DNA marker; Lane 2–8: Amplicons generated from whole genome amplified DNA originating from cells of seven different representative embryos screened in this study; Lane 9: Amplicons generated from blood DNA of father who is heterozygous for the pathogenic COL4A1 gene allele; Lane 10: No template control

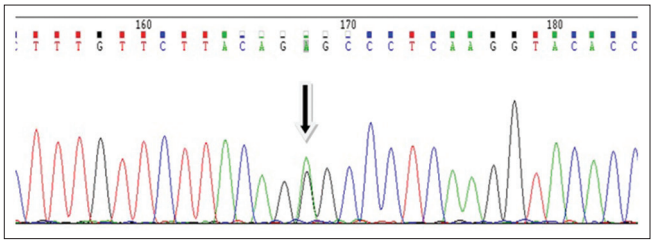


Figure 3: A representative image of COL4A1 normal and mutant alleles (c.1537G>A) coexisting in heterozygous condition. The black arrow indicates a green color peak superimposed on a black one indicating presence of G (guanidine) base in the hotspot region in one copy of the gene while A (adenine) base in the other

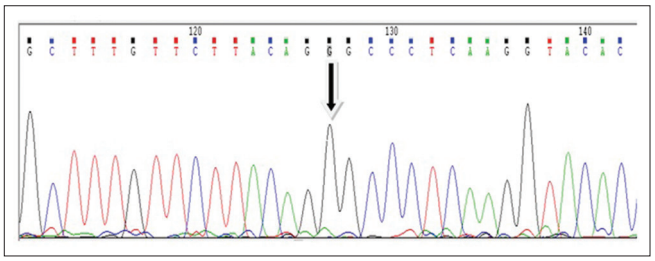
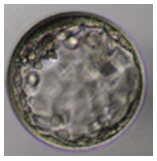
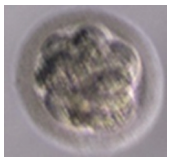
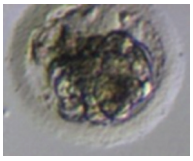
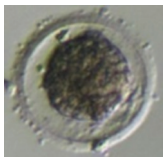
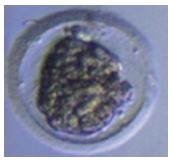
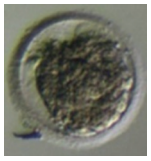


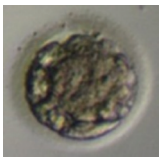
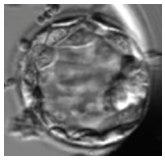
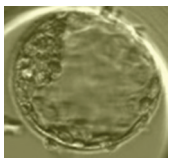
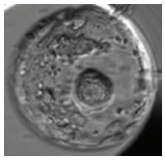
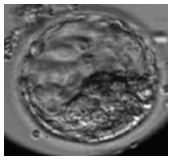
Figure 4: A representative image of COL4A1 wild type or normal allele bearing G (guanidine) at position c.1537G (black arrow). Embryos with this nucleotide sequencing profile were categorized as normal and qualified for potential implantation in surrogate mother

Table 1: Detail characteristics of biopsied embryos with outcome

Embryo number/code	Image	Characteristic	PGS report	Fate of embryo	Pregnancy outcome
First IVF cycle					
NR-1		Early blastocyst with cavity less than half of the volume of embryo Inner cell mass: Sparse with few cells and loosely organized Trophectoderm: Low cell number, stretched appearance	Abnormal	Not transferred	
NR-2		Fully expanded blastocyst Inner cell mass: Defined, discrete cell mass Trophectoderm: High cell number and well organized	Abnormal	Not Transferred	
NR-3		Morula stage embryo with clear cells without fragmentation	Abnormal	Not Transferred	
Second IVF cycle					
NR-1		Hatching stage embryo Inner cell mass: Sparse with few cells and loosely organized Trophectoderm: Low cell number, stretched appearance	Abnormal	Not transferred	
NR-2		Early blastocyst with cavity less than half of the volume of embryo Inner cell mass: Sparse with few cells and loosely organized Trophectoderm: Low cell number, stretched appearance	Normal	Transferred	Positive: Healthy baby was born
NR-3		Early blastocyst Inner cell mass: Absent Trophectoderm: Absent/not visible	Abnormal	Not transferred	
NR-4		Early blastocyst Inner cell mass: Absent Trophectoderm: Absent/not visible	Normal	Froze	
NR-5		Early blastocyst Inner cell mass: Absent Trophectoderm: Absent/not visible	Abnormal	Not transferred	
NR-6		Early blastocyst Inner cell mass: Absent Trophectoderm: Absent/not visible	Abnormal	Not transferred	

Contd...

Table 1: Contd...

Embryo number/code	Image	Characteristic	PGS report	Fate of embryo	Pregnancy outcome
NR-7		Early blastocyst Inner cell mass: Absent Trophectoderm: Absent/not visible	Abnormal	Not transferred	
NR-8		Fully expanded blastocyst Inner cell mass: Defined, discrete cell mass Trophectoderm: High cell number and well organized	Abnormal	Not transferred	
NR-9		Fully expanded blastocyst Inner cell mass: Defined, discrete cell mass Trophectoderm: High cell number and well organized	Abnormal	Not transferred	
NR-10		Fully expanded blastocyst Inner cell mass: Defined, discrete cell mass Trophectoderm: High cell number and well organized	Abnormal	Not transferred	
NR-11		Fully expanded blastocyst Inner cell mass: Defined, discrete cell mass Trophectoderm: High cell number and well organized	Abnormal	Not transferred	
NR-12		Fully expanded blastocyst Inner cell mass: Defined, discrete cell mass Trophectoderm: High cell number and well organized	Normal	Froze	

IVF=*In vitro* fertilization, PGS=Preimplantation genetic screening

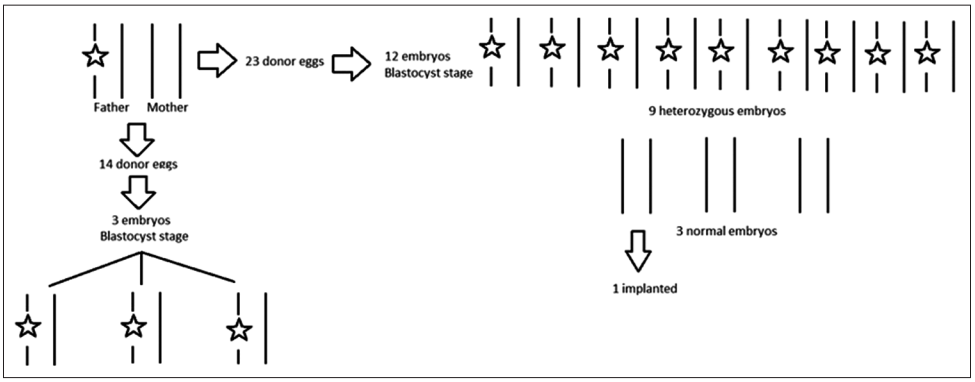


Figure 5: Mode of inheritance of the COL4A1 pathogenic allele. Black line indicates COL4A1 wild type allele while the line with a black star in the middle indicates allele with c1537G>A mutation. In the first cycle, 3 embryos were generated from 14 donor eggs, and all were found to harbor the pathogenic COL4A1 allele. In the second cycle, 23 donor eggs generated 12 embryos where 9 were found to harbor the pathogenic COL4A1 allele while 3 harboring only wild-type alleles. One of these three embryos with a pair of normal, wild type alleles for COL4A1 was identified for implantation. As is evident, there is no scope of any embryo which is homozygous for the pathogenic COL4A1 allele since the mother does not harbor this allele

functions can be ignored while using the ICSI technique since through injection a single sperm directly enters into cytoplasm of oocyte.[12] In this study, we found this step as critical in view of the health of the sperm available for IVF.

Published papers in the public domain indicate that PCR and fluorescent *in situ* hybridization are the two most commonly used techniques in genetic profiling of embryos for a large number of IVF/ICSI laboratories.[16] However, one of the major limitations for these methods is restricted the amount of DNA fragments or genetic information which can never meet the requirements of whole-genome research.[17,18]

This limitation has brought forth new methods of single cell analysis along with improvements in existing protocols for PCR. WGA is one such technology that has potential to circumvent the problem of limited DNA from a single cell.[19] This technique amplifies the entire genome thus providing sufficient DNA for multi-loci, multigene genome research.

In this study, the WGA took 2.9 h of time to complete and generated 4.3 μg of DNA. This was found to be satisfactory since a rapid turnaround time is essential to avoid loss of viability of the genetically healthy embryo if present, for growth in the surrogate mother. Further, due to the availability of large amount of DNA, multiple confirmatory tests can be undertaken from the whole genome amplified DNA leaving scope for interrogating several other genes if necessary.

PGD has dramatic potential to improve chances of successful pregnancy. The appearance of embryos under the microscope is the general tool to determine a "good" or "high-quality" embryo from those of lesser quality. However, these criteria may not always be useful in identifying deeply rooted genetic lesions that are pathogenic in nature. This is the reason why genetic testing before implantation is of great significance to shun away pathogenic alleles that can lead to mortality and/or morbidity.

Counseling plays a critical role in implementing PGD in couples. Before a decision is taken by the couple to undergo PGD treatment, a counselor is required to assess their situation, discuss their genetic risk and the process of PGD including the advantages and disadvantages while taking into account the emotional, practical and financial issues. This would allow the couple to make an informed decision about whether PGD is the most appropriate treatment for them. This practice is required both during and after a treatment cycle ensuring that couples are supported and fully aware of the implications of decisions that need to be made at each stage of the process.

In this program, the father was tested positive for COL4A1 during a referred assay from a medical institution and further, all genetic PGD-related genetic analysis were conducted at the collaborating molecular medicine facility of SN GeneLab at Surat, India.

To our knowledge, this is the first report of embryo genome profiling for a COL4A1 pathogenic allele using single cell sequencing technology in IVF in India. We demonstrated the robustness, speed, and accuracy of the technology and also the need for adopting such recent methods for screening out a potential pathogenic gene allele from embryos and for superior IVF success despite poor sperm and/or egg quality.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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