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A Proposed Model to Establish the PGD Technique for Carriers of BRCA1/2 Gene Mutations in a Diagnostic Laboratory

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ABSTRACT

Background: Pre-implantation Genetic Diagnosis (PGD) has recently been introduced as a reproductive choice for individuals who carry a disease-causing BRCA1/2 mutation. Since this technology has not yet been launched for patients at the Cancer Institute of Imam Khomeini Hospital harboring gene mutations that predispose patients to breast cancer, this study aimed to introduce a PGD-based model using a single cell lymphocyte instead of an embryonic blastomere.

Methods: Two affected and unrelated women with a known mutation in BRCA1/2 were enrolled in this study. Each patient (together with her siblings) was considered as an embryo derived from a hypothetical couple. Blood samples were collected from these individuals as well as their parents. Linkage analysis was performed. Following this process, a mutation-free individual and a mutation carrier was selected from the first and second family, respectively. A single lymphocyte was then extracted from their freshly taken peripheral blood, and afterwards Nested Multiplex PCR was performed.

Results: PGD confirmed that the individual from the first family is free of a mutation and the second one is a pathogenic mutation carrier.

Conclusion: Our results suggested that PGD is a viable choice to offer to families with "Hereditary Breast Cancer Syndrome", who have been diagnosed with a known pathogenic mutation. Our introduced model can be used as a possible option by other laboratories that are planning to launch this technology.

Keywords: Hereditary Breast Cancer Syndrome, BRCA1/2, Pre-implantation Genetic Diagnosis

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INTRODUCTION:

Hereditary Breast and Ovarian Cancer (HBOC) is a well-characterized syndrome which predisposes individuals to a higher lifetime risk of developing breast, ovarian, or other types of cancer compared to the normal population[1]. The main cause of this disease has been attributed to mutations in the BRCA1 (17q21.3) or BRCA2 (13q13.1) genes. BRCA1 and BRCA2 are tumor suppressor genes that encode proteins that (alongside several other proteins) are involved in the repair and correcting of breaks in the double-stranded DNA and thus contribute to the stability of the genetic material inside of the cells[2]. The occurrence of deleterious mutations in BRCA1/2 genes can cause failure in the performance of the BRCA1/2 proteins and may lead to the accumulation of genetic changes that can eventually lead to cancer.

Mutations in BRCA1 and BRCA2 genes are responsible for 20-25% of familial breast cancer[3] and account for 5-10% of all known types of breast cancer[4]. In addition, mutations in these genes are known to cause 15% of ovarian cancers[5].

In a normal population, a woman's chance of developing breast cancer during her lifetime is approximately 12%. However, according to recent statistics, the chance of developing breast cancer due to mutations in BRCA1 and BRCA2 genes is 55-65% and 45%, respectively. The lifetime risk of ovarian cancer for a female individual is about 1.3% whereas the risk of developing ovarian cancer in BRCA1 and BRCA2 mutation carriers by the age of 70 years is 39% and 11-17%, respectively[6,7]. Germline mutations in BRCA1/2 genes can be transmitted from one generation to the next following an autosomal dominant pattern[8-11]. This means that there is a 50% chance of transmission of the destructive mutation to a child[12]. The possibility of transferring the risk to the next generation is a cause for concern for families with a BRCA gene mutation, which may lead to them refraining from having children. Today, with the help of genetically developed Assisted Reproductive Technologies (ART) it is possible to prevent the birth of a child who carries a BRCA1/2 mutation[12,13].

Pre-implantation Genetic Diagnosis (PGD) is a new reproductive technique that has been introduced as an alternative method for Prenatal Diagnosis (PND)- which is done by sampling a pregnant woman's chorionic villus or amniotic fluid. PGD has been developed in the past two decades by pioneers Yury Verlinsky and Alan Handysideto, to help couples with a heritable genetic aberration avoid having diseased children[12,14-16]. In this method, in vitro fertilization (IVF) is carried out, and afterwards a biopsy of 1 or 2 blastomeres at the 6-8 cell stage of the embryo is followed by genetic testing either using fluorescent in situ hybridization or polymerase chain reaction at the single cell level[17]. This process consequently allows the implantation of the genetic-disorder-free embryo to the mother's uterus[18,19]. More importantly, PGD is the only non-invasive and possible method for couples who are at risk of having children with genetic disorders and are unable to use abortion due to legal, religious or moral reasons. Nowadays, PGD is commonly accessible for various genetic disorders and there is an increase in the number of genes that can be studied as well as clinical diagnostic centers in which PGD is done[20].

Even though PGD is widely available and can be used for patients suffering from a number of genetic diseases, so far very few cases of PGD for individuals carrying BRCA1/2 mutations have been reported worldwide[9,20-23]. This technology has not yet been launched for patients harboring BRCA1/2 mutations in Iran; therefore, this study aimed to bring forward a BRCA1/2 PGD protocol using a single cell lymphocyte instead of an embryonic blastomere to assess the accuracy of PGD for mutations in BRCA1/2 and to help launch the PGD technique in the Cancer Institute of Iran.

METHODS:

1. Genetic Counseling and family selection

This study included 2 breast cancer survivors and their families. Both patients had previously been admitted to the Cancer Institute, Imam Khomeini hospital and had been newly identified as BRCA1/2 mutation carriers (**Table 1**).

Genetic testing had been recommended to both patients due to their presenting with one or more typical features of "Familial Breast and Ovarian Cancer Syndrome"; (1) Breast cancer diagnosis at an early age; (2) Triple negative breast cancer ≤60 yrs.; (3) More than 1 primary breast tumor in one individual; (4) Breast cancer patient with at least one close relative with a breast cancer diagnosis ≤50 yrs.; (5) Breast cancer patient with at least one close relative with invasive ovarian cancer irrespective of age at diagnosis; (6) Breast cancer patient with at least 2 close relatives with breast cancer and/or pancreatic cancer irrespective of age at diagnosis. Immediate family individuals were invited to a genetic counseling session and informed about PGD as a reproductive option. A detailed description of the procedure was discussed with every participant and a complete pedigree was recorded for each family. This study was approved by the ethical committee of Tehran University of Medical Sciences. Informed consent was obtained from all individual participants included in the study.

DNA extraction and mutation analysis

In order to check the presence of the mutations in each individual, 10 ml of peripheral blood was obtained in a tube containing EDTA. DNA was extracted using salting-out method[24]. Polymerase Chain Reaction (PCR) was performed in a final volume of 20 µl using 1 µl of genomic DNA (contained 50-200 ng of DNA), 1 µl of each of the Forward and Reverse primers (**Table 2**), 7 µl of Taq DNA Polymerase 2x Master Mix Red (Amplicon, Denmark) and finally 10 µl dH2O.

Table 1. PIdentified mutations of the 2 patients who participated in this study, using Next Generation Sequencing (NGS)

No.	Classification	Gene	Transcript	Exon	HGVS Coding	HGVS Protein
1	Stop gain	BRCA2	NM-000059	20	c.8611G>T	P.Glu2871*
2	Stop gain	BRCA1	NM_007294	10	c.3607C>T	p.Arg1203*

Table 2. Primer sequences of the BRCA1 and BRCA2 genes

Primer Name	Primer Sequence
BRCA1 Forward	5'AGCCGTAATAACATTACAGA3'
BRCA1 Reverse	5'AAGTGTTGGAAGCACGGAAG3'
BRCA2 Forward	5'CTCAGCCTCCCAAAGTTCTG3'
BRCA2 Reverse	5'TGTCCCTTGTTGCTATTCTT3'

The PCR condition consists of initial denaturation at 94°C for 4 min and was followed by 30 cycles at 95°C for 30 sec, 60°C for 45 sec and 72°C for 45 sec. The final extension was at 72°C for 7 min. The PCR products were then sequenced by the DNA sequencer ABI Prism 3130 (Applied Biosystems, Warrington, UK) and analyzed using Genotyper software.

2. Haplotyping

To determine the haplotype of each individual KBC BRCA1 Hap ™ Kit and KBC BRCA2 Hap ™ Kit (Kawsar Biotech Company, Tehran, Iran) were used according to the manufacturer's instructions (**Table 3**). PCR was done in a multi-block system thermocycler (ABI, US). For the BRCA1 gene, 3 downstream and 4 upstream informative markers were selected. There were 2 upstream and 2 downstream markers for the BRCA2 gene.

Fragment analysis was then performed using ABI Genetic Analyzer 3130XL and the obtained results were further analyzed using Gene Mapper® software v1.1 (Applied Biosystems, Warrington, UK). Haplotype was drawn to track disease-causing alleles through the pedigree.

3. Isolation of a single cell lymphocyte

In order to perform IVF/PGD cycle modeling and evaluate the reliability and reproducibility of our technique, each family was considered as a couple and each child (along with their brothers or sisters) was considered as an embryo derived from this hypothetical couple.

To isolate a single lymphocyte, 900 µl of RBC lysis solution was added to 300 µl of peripheral blood. The mixture was left for 10 min at room temperature (15-25°C) and then centrifuged at around 4000 rpm for 5 min. This step was repeated 2 or 3 times until a white cell pellet was obtained. At this stage, the cells were washed with 500 µl of PBS solution and serially diluted in a Petri dish in which a few 5µl-droplets of medium (without Ca2+ and Mg2+) were placed. This step was

Table 3. STR markers in KBC BRCA1 Hap ™ Kit and KBC BRCA2 Hap ™ Kit Locations of the Short Tandem Repeat (STR) markers linked to BRCA1 and BRCA2 genes designed by Kawsar Biotech Company. STR markers in the KBC BRCA1 Hap and KBC BRCA2 Hap kits are four-repeat sequences.

Kit Name	STR maker	Chromosome Position (NC_000017.11)	Heterozygosity%	Distance from the gene (bp) Heterozygosity%
KBC BRCA1 Hap ™ Kit	BRCA1D1	chr17:45730459-45730718	64.7	2.604.976
	BRCA1D2	chr17:41490749-41490938	85.71	1.553.546
	BRCA1D3	chr17:43867454-43867781	80	741.971
	BRCA1U4	chr17:41147726-41148041	41.17	1.896.569
	BRCA1U5	chr17:42238060-42238289	64.7	806.232
	BRCA1U6	chr17:41736930-41737165	70.58	1.307.365
	BRCA1U7	chr17:41158434-41158705	41.66	1.885.861
KBC BRCA2 Hap ™ Kit	BRCA2U1	chr13:31016913+31017120	64.7	1.298.567
	BRCA2U2	chr13:31759316+31759489	23.07	556.164
	BRCA2D3	chr13:32570409+32570690	35.29	171.018
	BRCA2D4	chr13:33434842+33434985	71.42	1.035.170

repeated until only 10-20 cells were left. Afterwards, a single cell was isolated from the last droplet using the cell microinjection system (RI, INTEGRA-T1) situated in the ART center of Imam Khomeini Hospital. Using a biopsy pipette a single cell was immobilized and further isolated with aspiration (**Figure 1**).

Seven single cell lymphocytes were prepared separately and each of them was washed in another droplet before being transferred to a PCR tube containing cell lysis solution (Alkaline Lysis Buffer, Kawsar Biotech Company, Tehran, Iran). The single lymphocyte cell biopsies were then anonymously sent to Kawsar PGD Laboratory.

4. Nested- multiplex PCR

Each tube was incubated at 45°C for 15 min and then lysed in 96°C for 20 min. Following these steps, nested PCR was performed. In the first round, the external primers were used for STR amplification of genes associated with BRCA1/2. PCR was performed with an initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 63°C for 1 min and 30 seconds and 72°C for 2 min and the final extension at 72°C for 15 min. In the second round, 0.5 µl of the first stage PCR product and the internal primers were used for amplification of the target sequence. The PCR with initial denaturation at 95°C for 5 min was followed by 28 cycles at 95°C for 1 min, 63°C for 1 min and 30 seconds and 72°C for 2 min and the final extension was at 72°C for 15 min. After the second round PCR, STR markers were amplified using PCR primers labeled with a fluorochrome. PCR conditions were as follows: initial denaturation at 95°C for 5 min followed by 28 cycles at 95°C for 1 min, 55°C for 1 min and 30 seconds and 72°C for 2 minutes and the final extension at 72°C for 15 min.

RESULTS:

Patient 1 and Family

The first proband was a 44 year old breast cancer survivor. She was identified as a carrier mutation of c.8611G>T, p.E2871X in the BRCA2 gene. In addition, she had a sister who had been diagnosed with breast cancer at the age of 41 years old and a brother who had died of leukemia at the age of 18 years. To identify suspicious carriers of the known mutation present in this family, her mother, 2 sisters and 2 brothers volunteered to participate in this study. PCR and the subsequent sequencing of exon 20 of BRCA2 gene showed that her mother, her breast cancer survivor sister, as well as her two brothers were all carriers of this mutation. Haplotyping results based on four STR markers also confirmed this issue and found that the mutation had been passed on from the mother to the

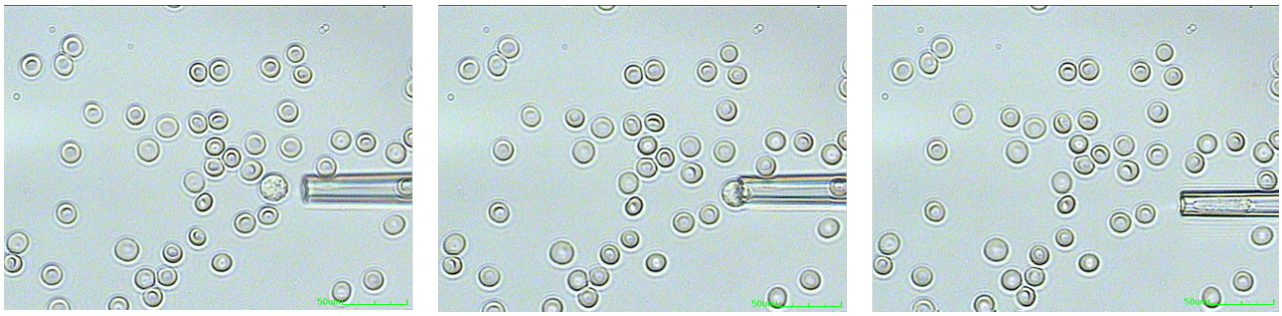

Figure 1. **A Removal of a single cell lymphocyte from peripheral blood**

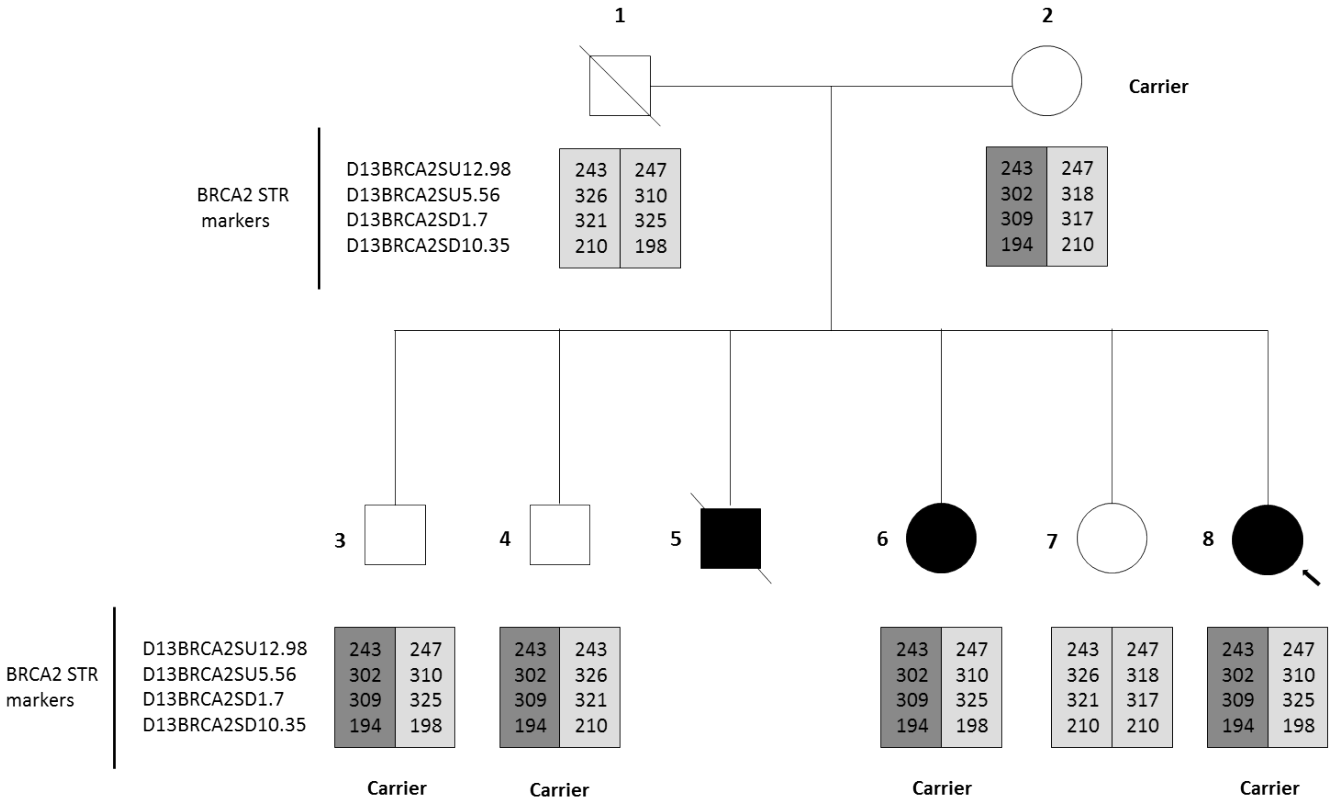

Figure 2. **ALinkage analysis of a family with a history of a pathogenic mutation in the BRCA2 gene using STR markers.** The mutant allele has been depicted in a darker color. Following linkage analysis of the biopsied samples of 5 individuals, we found that 4 affected individuals (3, 4, 6, and 8) inherited a mutant allele from their mother and a wild-type allele from their father while 1 unaffected participant (7) inherited a wild-type allele from her mother and a wild-type allele from her father.

children (**Figure 2**).

Following the isolation of a single cell lymphocyte from a healthy sister (mutation-free) of the family which was anonymously sent to Kawsar PGD laboratory, the person was found to be without mutation as expected.

Patient 2 and family

The second proband was a 34 year old woman with a c.3607C> T (p.R1203X) mutation in the BRCA1 gene. She was selected for genetic testing due to her early onset breast cancer. Following blood collection from 5 healthy individuals from this family – the father, mother, 2 sisters and 1 brother - PCR and sequencing for exon 10 of the BRCA1 gene was performed. The results showed that her mother, one of her sisters and one brother were carriers of the pathogenic mutation. Haplotyping confirmed that the patient's siblings who carried the destructive mutation had inherited the mutation in the BRCA1 gene from their mother (**Figure 3**). PGD process following a single lymphocyte biopsy

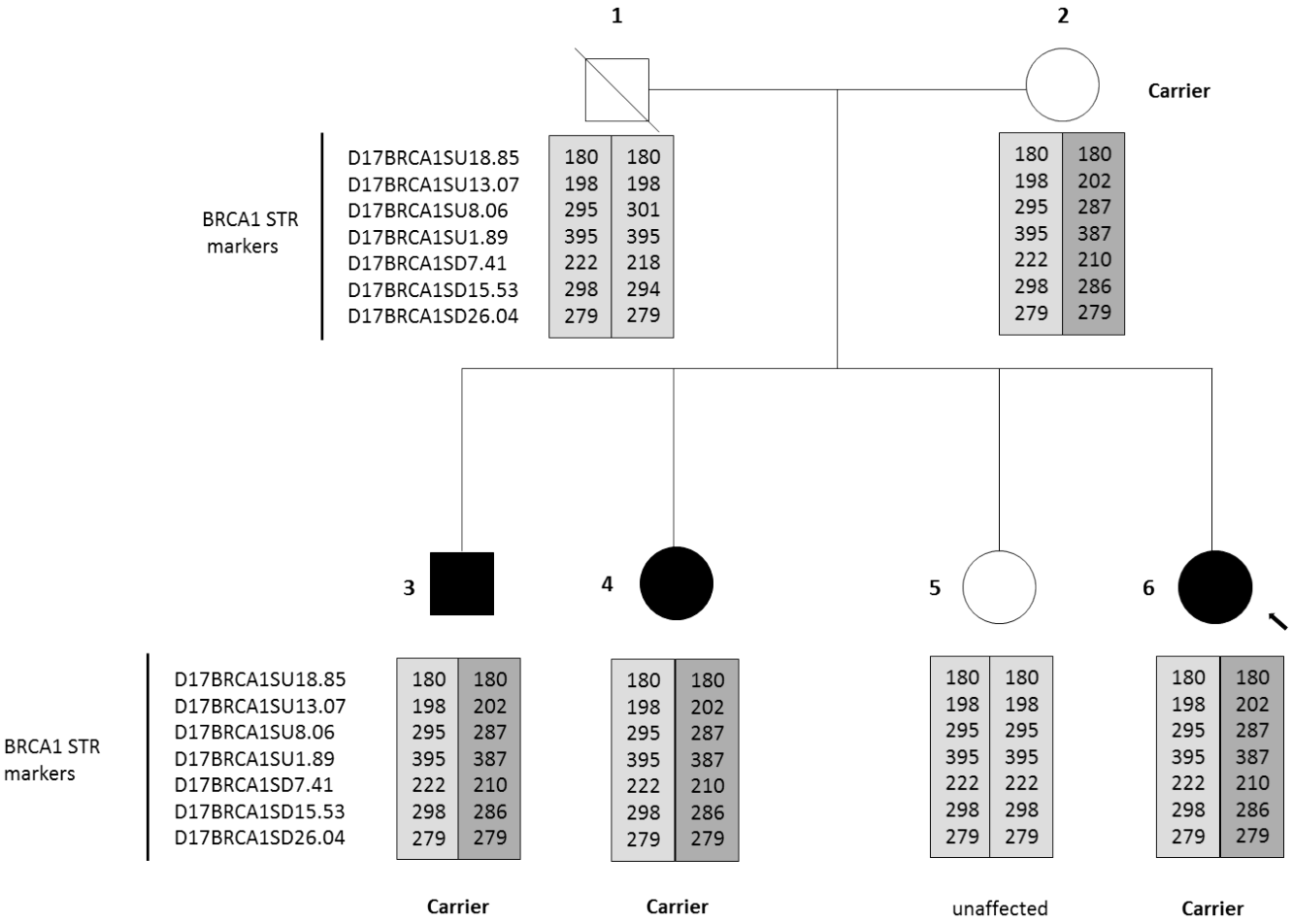

Figure 3. **STR markers in KBC BRCA1 Hap ™ Kit and KBC BRCA2 Hap ™ Kit Locations of the Short Tandem Repeat (STR) markers linked to BRCA1 and BRCA2 genes designed by Kawsar Biotech Company.** STR markers in the KBC BRCA1 Hap and KBC BRCA2 Hap kits are four-repeat sequences.

from the peripheral blood of a mutation-carrier-sister confirmed that this individual is indeed a pathogenic mutation carrier.

DISCUSSION:

In this study, in an effort to establish PGD for couples with a predisposing mutation in cancer susceptibility genes, we invited 2 high-risk families with a pathogenic mutation in their BRCA1/2 genes to participate. The parents of each family were considered a couple, and their children represented their biopsied blastomeres. Pre-implantation Genetic Haplotyping (PGH) using 4 informative markers for BRCA2 and 7 informative markers for BRCA1 gene was performed to identify the haplotype of each individual, followed by Nested Multiplex PCR for 2 volunteers on a single cell lymphocyte. Following this procedure, a volunteer from the BRCA2 family was said to be free of mutation

and the second volunteer was diagnosed as a BRCA1 mutation carrier. Our results showed that linkage analysis and multiplex PCR are useful approaches to properly distinguish mutation carrier cells from mutation-free cells. Using informative STR markers linked to BRCA1 and BRCA2 genes helped us to determine each participant's haplotype and to avoid any misdiagnosis due to allele-drop out (ADO) phenomenon. It also helped us to determine the possible recombination position relative to the gene. These methods, alongside indirect methods such as using STR markers confirmed the results of Sanger sequencing as a direct method. Another point to consider is that Multiplex PCR not only saves time and reagents but also compensates the problem of insufficiency of blastomere DNA.

Today, there is a growing number of individuals who survive malignancies before or during their fertility period, an event that consequently may interfere with their decision to have offspring[25]. PGD, as a reproductive technique, has been suggested as a desirable option in certifying the establishment of a pregnancy that is free of a genetic disorder[19,26-28]. Currently, PGD is commonly applied for different disorders including X-linked disorders, abnormalities due to changes in chromosome structure, monogenic disorders, mitochondrial abnormalities and complex disorders[26,29]. However, in regards to inheritable cancers, there are have been few published reports.

The first report of a successful PGD goes back to 1980. Ao and colleagues applied PGD for Familial Adenomatous Polyposis Coli (FAPC) as a hereditary cancer. They indicated that PGD is a feasible technique for detecting mutations in cancer predisposing genes at the single cell level[30]. In 2001, Verlinskey et al. applied the first PGD for mutations in the p53 tumor suppressor gene which led to the birth of a disease free child[31]. In the same year, PGD was applied for a couple with a predisposing mutation in the FANCC gene. This was the first report of PGD that was combined with HLA antigen testing[32]. After that, in 2002 PGD was used for 6 inheritable genetic disorders including familial adenomatous polyposis coli (FAP), Von Hippel-Lindau syndrome (VHL), retinoblastoma, Li-Fraumeni syndrome, neurofibromatosis types I/II and familial posterior fossa brain tumour (hSNF5). The result was the birth of 4 healthy children, indicating that PGD is a useful technique for high-risk couples who wish to have healthy children[22,33]. The first PGD with the help of informative microsatellite markers was performed for hereditary retinoblastoma by Girardet et al.[34]. In 2004, PGD was done for a couple at risk of familial retinoblastoma. This was the first report of the live birth of a child without retinoblastoma[35]. PGD for neurofibromatosis type 2, a dominantly inherited cancer predisposition syndrome, has also been reported in different studies. Abou-sleiman et al. were the first group who used PGD for couples carrying an NF2-mutation, however, no pregnancy occurred following this attempt[36]. In 2005, Spits et al. reported PGD for an NF1-carrier mutation who eventually delivered a healthy baby[37]. In 2007 Spits et al. reported the feasible use of PGD for APC/NF2/BRCA1 gene mutations[22]. The acceptability of performing PGD for late-onset disease including HBOC was first stated in 2003 by the Ethics Taskforce of the European Society of Human Reproduction and Embryology (ESHRE)[13,38]. Jasper et al. was the first group who did PGD for a 31 year old women with a 6 kb duplication in her BRCA1 gene, which resulted in the live birth of a disease-free child[21]. Recently, Sagi et al. carried out PGD for 3 couples with known pathogenic mutations in BRCA1/2. All three patients had successful pregnancies; two of them gave birth to healthy babies and the last one had an ongoing pregnancy at that time[20]. In 2013, Drusedau and the coworkers established a universal multiplex PCR using 6 informative markers for BRCA1 and 8 for BRCA2. They applied their technique to 30 couples that led to 8 mutation-free babies[39].

Up to now, more than 5000 babies have been born using the PGD technique and based on published studies, no adverse outcomes/major malfunctions have been reported. The rate of misdiagnosis is also very low according to the ESHRE consortium (0.16%)[40]. However, the attitudes of high-risk individuals towards PGD show great variability. This requires raising awareness regarding issues such as cancer prevention, fertility preservation and psychosocial support by healthcare professionals. In this regard, a reproductive decisional aid is needed for use by healthcare professionals such as genetic counselors and psychologists[41,42].

Financial issues are another cause for concern for couples who want to go through the IVF/PGD process. Costs for IVF and PGD can range from US$10,000 to US$20,000 per cycle[43]. In Iran, on average, IVF cycles cost approximately US$500-$800 per cycle, while PGD costs an additional US$2,500–3000 per cycle. Since the IVF/PGD cycle has an approximate rate of success of 30%, these costs may add up[44]. PGD has been suggested as a preventive method in a large scale program through which costs for certain diseases can be reduced[45]. This is especially important in the context of familial cancer, since this disease is very costly and prevention of disease can stop subsequent expenses in the future.

Even though the two individuals who collaborated with us in this study were breast cancer survivors with a BRCA1/2 gene mutation, in the future, our target audience will be all those individuals who may benefit from this technique, including unaffected people with a known mutation who are not willing to transfer this risk to their offspring. Our findings, though preliminary, have shown that PGD at a single cell level can discriminate the mutation carrier cell from the normal cells of applicants with a known cancer predisposition mutation who wish to have an unaffected child. Furthermore, given the experience of dozens of different families with PGD as well as the results of this study, it can be claimed that this center is ready to offer genetic counseling and genetic testing alongside IVF to families with HBOC syndrome who have been diagnosed with a known pathogenic mutation.

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CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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