DEVELOPMENT OF SINGLE CELL GENETIC TESTING STRATEGIES: ASSESSMENT OF MHC COMPATIBILITY, MEIOTIC RECOMBINATIONS AND BETA-THALASSEMIA IN HUMAN EMBRYOS

by

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Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Graduate Program in Molecular Biology and Genetics
Boğaziçi University
2011

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my thesis supervisor and mentor Assoc. Prof. Dr. Ender Altıok for his continuous guidance, endless support and valuable criticism throughout the study.

I would like to thank my thesis co-supervisor Prof. Neş'e Bilgin for her guidance and support.

I would like to express my special thanks to the members of my thesis committee, Dr. Kuyaş Buğra, Dr. Ayşe Özer, Dr. Esra Battaloğlu and Dr. Tamer Yağcı for allocating their time to evaluate this work.

I would like to thank Asena Selcen Yüzbaşıoğlu, Deniz Çınar Gül, Derya Özciğer, Gülşah Demirkeser, Güven Yenmiş, Melike Aydın Tekgüç, Şirin Yüksel and Tolga Sütlü for technical assistance in the laboratory.

I would like to thank my family for their endless love and support throughout my life.

I would like to thank Genetika Genetic Diagnosis Center, Istanbul for supporting my laboratory work throughout my thesis.

ABSTRACT

DEVELOPMENT OF SINGLE CELL GENETIC TESTING STRATEGIES: ASSESSMENT OF MHC COMPATIBILITY, MEIOTIC RECOMBINATIONS AND BETA-THALASSEMIA IN HUMAN EMBRYOS

Single cell molecular biology, a relatively new scientific branch, is promising to study unique questions and leading to novel applications in biology and medicine. Single cell studies have been challenged by difficulties in selection and isolation of appropriate cells, low amplification efficiencies, allele drop outs, PCR contaminations and inefficiency of conventional analysis strategies. This study has explored the possibilities of analyzing multiple genetic conditions particularly concerning the beta globin and the HLA regions in human embryos. The HLA genes, beta-globin gene, and the associated microsatellites have been amplified simultaneously by multiplex PCR. DNA sequencing has been optimized for high resolution genotyping. The real-time PCR and melting curve analysis have been adapted for the first time for rapid and reliable analysis of the HLA compatibility. Use of microsatellites of the extended HLA locus has enabled more accurate and efficient detection of the allele drop outs, contaminations and recombinations. Amplification and informative detection have been obtained for 1012 blastomeres out of 1180 human embryos used in this study, giving a detection rate of 86%. A total of 122 (13%) embryos were found unaffected from beta thalassemia and had identical genotype at ten HLA regions. Transfer of 94 embryos that have resulted into 16 pregnancies with 14 healthy offsprings indicates the feasibility of the single cell applications for preventive medical approaches. Microsatellite typing of the extended HLA locus has enabled to study the characteristics of the meiotic recombinations in human embryos. The recombination rate was determined as 0.44 cM/Mb, 2.1 fold less compared to the general genomic recombination rate of 0.92 cM/Mb. It was 3.83 fold higher in the maternal MHC regions compared to the paternal MHC regions. Upto 14 fold difference was observed among individuals. Breakpoints of recombinations in the class II region were clustered between the DRB1 and DPB1 genes covering the TAP1 and TAP2 genes.

ÖZET

TEK HÜCREDE GENETİK TEST YÖNTEMLERİNİN GELİŞTİRİLMESİ: İNSAN EMBRİYOLARINDA MHC UYUMU, MAYOTİK REKOMBİNASYON VE BETA-TALASEMİ İNCELEMESİ

Tek hücre moleküler biyolojisinin, tıpta ve biyolojide yeni uygulamalara yol açması beklenmektedir. Uygun hücrelerin seçimi ve elde edilmesindeki zorluklar, DNA'nın çoğaltılmasındaki yetersizlikler, alel okuyamama (ADO), polimeraz zincir reaksiyonu (PZR) kirlenmeleri ve mevcut inceleme yöntemlerinin yetersizlikleri tek hücre çalışmalarını kısıtlamaktadır. Bu çalışmada, insan embriyolarında beta globin geni ve HLA bölgelerinin incelenmesi hedeflenmiştir. HLA genleri, beta globin geni ve ilişkili mikrosatelitleri kapsayan genetik bölgeler PZR ile aynı anda çoğaltılmıştır. Yüksek çözünürlük genotipleme için DNA dizi incelemesi yapılmıştır. Gerçek zamanlı PZR ve ergime eğrisi kullanılarak HLA doku uyumu ve tek hücrede beta-globin mutasyonları incelenmiştir. Genişletilmiş HLA lokusunda bulunan mikrosatelitler kullanılarak alel okuyamama, DNA kirlenmesi ve rekombinasyonlar daha doğru ve hızlı belirlenmiştir. Sonuç olarak 1180 insan embriyosunun 1012 tanesinde %86 başarı ile PZR yapılabilmiştir. Ayrıca 122 adet (%13) embriyonun Akdeniz anemisinden etkilenmediği ve HLA uyumlu olduğu saptanmıştır. Toplam 94 adet embriyo transferi sonrasında 16 adet gebelik elde edilerek 14 sağlıklı bebek doğmuştur. Bu sonuç, tek hücre uygulamalarının koruyucu tıp için uygulanabilir bir seçenek olduğu göstermektedir. Genişletilmiş HLA lokusunun mikrosatelit incelemesi, insan embriyolarında mayotik rekombinasyonların özelliklerinin değerlendirilmesine olanak sağlamıştır. Bu bölge için rekombinasyon oranı ortalama 0,44 cM/Mb saptanmış olup genel genomik orandan (0,92 cM/Mb) 2,1 kez daha azdır. Ayrıca, anneye ait MHC bölgelerinde babaya ait MHC bölgelerine oranla 3,83 kat daha fazla rekombinasyon saptanmıştır. Kişiler arasında rekombinasyon oranı 14 kata kadar değişim göstermiştir. HLA sınıf II bölgesindeki rekombinasyon kırılma noktalarının TAP1 ve TAP2 genlerini kapsayan DRB1 ve DPB1 genleri arasında kümelendiği gösterilmiştir.

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LIST OF SYMBOLS / ABBREVIATIONS

α	alpha
β	beta
µl	Microliter (10^{-6} L)
µM	Micromolar (10^{-6} M)
A	Adenine
C	Cytosine
F	Forward
G	Guanine
R	Reverse
s	Second
T	Thymine
U	Unit
ADO	Allele drop out
bp	Base pair
CD	Cluster of Differentiation
cDNA	Complementary DNA
CEPH	Centre d'Etude du Polymorphisme Humain
CGH	Comparative genomic hybridization
cM	CentiMorgan
CTL	Cytolytic T lymphocyte
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DOP-PCR	Degenerate oligonucleotide primed PCR
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylenediaminetetraacetatic acid

FAM	6 - Carboxyfluorescein
FISH	Fluorescence *in situ* hybridization
GAB	Genetic Association Database
GVHD	Graft versus host disease
HBV	Hepatitis B virus
hCG	Human chorionic gonadotropins
HEX	6 - carboxy - 2',4,4',5',7,7' - hexachlorofluorescein
HIV-1	Human immunodeficiency virus 1
HLA	Human Leukocyte Antigen
ICSI	Intracytoplasmic sperm injection
IVF	*in vitro* fertilization
IVS	Intervening sequence
Kb	Kilobase
kD	KiloDalton
kV	Kilovolt
Mb	Megabase
MDA	Multiple displacement amplification
$MgCl_2$	Magnesium Chloride
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter (10^{-3} L)
mM	Millimolar (10^{-3} M)
mRNA	Messenger RNA
ng	Nanogram (10^{-9} g)
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase chain reaction
pg	Picogram (10^{-12} g)
PEP	Primer extension preamplification
PGD	Preimplantation genetic diagnosis
PGS	Preimplantation genetic screening
pmol	Picomole (10^{-12} mole)
Pr	Probe
RFLP	Restriction fragment length polymorphism

RNA	Ribonucleic acid
SBT	Sequence based typing
SDS	Sodiumdodecylsulphate
SSCP	Single-strand conformation polymorphism
SSOP	Sequence-specific oligonucleotide probe
SSP	Sequence specific primer
STR	Short tandem repeat
TAMRA	Carboxytetramethylrhodamine
Taq	*Thermus aquaticus*
TBE	Tris – Boric acid – EDTA
TE	Tris – EDTA
Tm	Melting temperature
Tris	Tris(hydroxylmethyl)aminomethane
tRNA	Transfer ribonucleic acid
UTR	Untranslated region
WGA	Whole genome amplification
xMHC	Extended MHC

1. INTRODUCTION

Single-cell molecular-biology is a relatively new scientific branch in biology. Its applications have expanded rapidly from single cell diagnostics to research in gene expression, proteomics and cellular physiology. In terms of its potential, the field has focused initially on the analysis of blastomeres biopsied from the embryos for preimplantation genetic diagnosis. Its applications in research and medicine have been limited by the inefficiencies of nucleic acid amplification and detection techniques. The future of the single cell molecular biology is expected to go beyond the preimplantation genetic diagnosis and become a routine diagnostic analysis of diseases such as cancer in cases where the cancerous tissue available for analysis is very limited to few cells.

1.1. Applications of Single Cell Genetic Testing

Single cell analysis has potential applications from embryo molecular biology to cancer cell diagnostics.

1.1.1. Fetal Cells in Maternal Body Fluids

Prenatal diagnosis of fetal chromosomal abnormalities or genetic disorders involve invasive procedures such as amniocentesis or chorionic villus sampling. Although sufficient amount of fetal cells are obtained via these methods, a fetal loss rate of 1.9% and 2% have been reported for amniocentesis and chorionic villus sampling, respectively (Mujezinovic and Alfirevic, 2007). Bidirectional blood flow between the fetus and the mother during pregnancy allowed detection of intact fetal cells and cell free fetal nucleic acids in the maternal bloodstream (Lo *et al.,* 1996). It has been reported that some fetal cell types persist in the maternal bloodstream for many years after pregnancy (Bianchi *et al.,* 1996), which may indeed interfere with the subsequent pregnancies. On the other hand, the life span of nucleated fetal erythrocytes is relatively short (Lurie and Mamet, 2000). The frequency of fetal cells has been reported as rare as one in a million maternal cells (Hahn *et al.,* 2000). Since the amount of the fetal cells are very limited in maternal bloodstream,

various methods have been applied and developed for enrichment of fetal cells (Sekizawa *et al.,* 2007).

The presence of fetal cells circulating in the maternal bloodstream has been first demonstrated on non-enriched blood taken from pregnant women by amplification of Y chromosome specific PCR (Lo *et al.,* 1989, Lo *et al.,* 1993a). Shortly after, amplification of allele inherited from the paternal genome to the fetus but not present in the maternal genome was achieved to determine the fetal RhD status of the fetus (Lo *et al.,* 1993b). However, determination of mutations carried by both parents requires isolation of fetal cells according to their morphology by micromanipulation under microscope. Afterwards, the DNA of the isolated single cell is amplified by nested PCR strategy to determine the genotype (Hahn *et al.,* 1998). In this respect, Duchenne muscular dystrophy was the first sex-linked single gene disorder which has been diagnosed after micromanipulation of the fetal nucleated red blood cells (Sekizawa *et al.,* 1996). Prenatal diagnosis of different genetic conditions such as ornithine transcarbamylase deficiency, spinal muscular atrophy, beta thalassemia and sickle cell anemia have been performed by using single fetal cells in maternal blood (Watanabe *et al.,* 1998, Chan *et al.,* 1998, Cheung *et al.,* 1996).

The possibility of capturing fetal cells from the maternal reproductive tract permits a minimally invasive prenatal diagnosis. The technique involves cervical mucus aspiration, cervical swabbing and cervical/intrauterine lavage. The trophoblast cells during the first trimester are retrieved by either methods for prenatal genetic analysis. FISH and quantitative fluorescent PCR are sensitive to detect the sex and aneuploidy screening of the fetus (reviewed in Imudia *et al.,* 2010).

1.1.2. Pathology

Laser microdissection is an advanced technique which enables isolation of specific single cells from stained sections of formalin-fixed, paraffin-embedded, frozen tissues and from cell cultures. The technique allows the isolation of the desired cells individually. This technique, combined with single cell genetic analysis techniques promise precise molecular genetic fingerprinting of tumor tissues (reviewed by Esposito, 2007). Analysis at the single cell level may assist determination of clonal composition of normal and

malignant cells within a tissue. Gene expression profile of single cells may help tracking the tissue of origin within metastatic foci. Recently developing microfluidic platforms are capable of quantitative proteomic analysis of signaling molecules in the single cells. Such improvements in molecular diagnosis of clinical oncology will finally enable a predictive, preventive and personalized medicine (Sun *et al.,* 2010). Molecular analysis of the single cells isolated from pathological samples is a promising application for exploring heterogeneity within a population of cells and their clonality.

1.1.3. Developmental Biology

The preimplantation stage is one of the most exciting stages in mammalian development (Watson, 1992, Watson and Barcroft, 2001). The embryo development is characterized by several morphological and physiological transitions. The embryos undergo several processes such as genome activation, compaction, cavitation, and cellular differentiation. The embryonic genome is functionally active and gene expression is extremely intensive. The variations in gene expression are largely responsible from the control of these processes. The expression in early developmental stages is extremely dynamic (Scultz, 2005, Duranthon *et al.*, 2008).

Gene expression studies in single cells from different embryological stages will provide enormous information about the control of development and differentiation. Specific subsets of rare cells play essential roles during development (Kurimoto *et al.,* 2006). Gene expression studies presented an average result obtained from a population of cells. However, the expression profile of each member of a cell population may show variability at a certain time point (Levsky and Singer, 2003). Therefore, novel reverse-transcription quantitative real time PCR strategies have been developing to detect the differences with a high sensitivity in expression levels (Ståhlberg and Bengtsson, 2010). Expression profiling of microRNAs (miRNAs), on the other side, has been focused on single cells in order to elucidate diverse action of miRNAs in various biological processes during development (Tang *et al.,* 2006).

1.1.4. Preimplantation Genetic Diagnosis (PGD)

In early 1980s, *in vitro* fertilization (IVF) techniques in assisted reproduction and polymerase chain reaction (PCR) in molecular biology were developed contemporaneously (Steptoe and Edwars, 1978, Mullis *et al.,* 1986). In the following years, these techniques were combined in preimplantation genetic diagnosis (PGD) which allowed diagnosis and selection of disease free embryos before they were transferred back into uterus for implantation (Coutelle *et al.,* 1989; Holding and Monk, 1989; Handyside *et al.,* 1990). The embryos for PGD are conventionally obtained in IVF laboratory by embryologists. After ovulation induction and oocyte retrieval by the IVF specialist physician, collected oocytes are fertilized with sperms.

In the first PGD cases, the method was applied commonly for sex determination for couples carrying X-linked disorders. The first reports of the preimplantation genetic diagnosis were based on polymerase chain reaction. The early trials of embryo genetic selection were performed for two couples at risk of transmitting adrenoleukodystrophy and X-linked mental retardation (Handyside *et al.,* 1990). The studies were based on PCR amplification of Y specific regions to determine the sex of the embryos. In these studies, since both of them were X-linked disorders, female embryos were selected and transfered. Twin pregnancies were obtained. Pre-conception genetic analysis has been performed for alpha-1-antitrypsin deficiency in first polar bodies removed from the oocytes by micromanipulation (Verlinsky *et al.,* 1990). The genetic analysis was limited to the detection of the maternal alleles. A conclusive result was obtained for five of the seven polar bodies. Although two unaffected embryos were transferred, pregnancy was not established. Nearly 600 instances of preimplantation genetic diagnosis have been applied for various X-linked, autosomal recessive and autosomal dominant disorders in the following years (Handyside, 1998). As the number of loci to be analyzed increased, the multiplex amplification strategy has been developed. Birth of a healthy girl after the preimplantation genetic diagnosis for cystic fibrosis deltaF508 mutation was one of the first cases reported for single gene disorders (Handyside *et al.,* 1992). The technique has been applied extensively for the monogenic disorders by PCR and for chromosomal abnormalities by FISH. It has been applied for late-onset diseases with genetic predisposition (Schulman *et al.,* 1996). Its applications have been extended to aneuploidy

screening of embryos to raise the implantation rate in assisted reproduction (reviewed in Wilton, 2002). The most extreme application of PGD has been the selection of embryos according to the human leukocyte antigen (HLA) matching which is not an indication for the prenatal diagnosis (Verlinsky *et al.,* 2001).

1.1.5. PGD for Chromosomal Abnormalities

Risk of aneuploidy in the offspring increases as the maternal age increases since the chromosomes in oocytes are less likely to divide properly. The most common aneuploidies can be listed as trisomy 21, 18 and 13 which lead to Down syndrome, Edwards syndrome and Patau syndrome, respectively. The risk of having a child with Down syndrome at maternal age of forty is as high as 1/75 (Hook *et al.,* 1983). Use of PGD for common aneuploidies increases the chance of healthy pregnancies and prevents termination of pregnancies due to aneuploidies (reviewed in Wilton, 2002 and Munné *et. al.,* 2002).

Another important application of PGD is the selection of embryos for chromosomal rearrangements such as reciprocal and robertsonian translocations, inversions and deletions (Munné *et al.,* 2000). The chromosomal abnormalities are commonly detected by FISH in PGD instead of PCR. The couples with chromosomal abnormalities have difficulties to have a baby and pregnancies are generally resulted in spontaneous miscarriages due to imbalanced chromosomes. The main reason to undergo PGD for the chromosomal abnormalities is to increase implantation rate and to decrease the number of spontaneous abortions (Gianoroli *et al.,* 1999, Munné *et al.,* 2005). Polymerase chain reaction based preimplantation genetic diagnosis of the chromosome translocations has been developed recently which overcomes some technical limitations of the fluorescence *in situ* hybridization (Fiorentino *et al.,* 2010, Traversa *et al.,* 2010). This strategy benefits from the microsatellites scattered through the translocated region of the chromosomes.

1.1.6. PGD for Monogenic and Sex-Linked Disorders

The initial preimplantation genetic diagnosis applications have been started with sexing by DNA amplification of a Y chromosome-specific repeat sequence without any need for testing of the mutated gene (Handyside *et al.,* 1990). Fluorescence *in situ*

hybridization has been used as an alternative tool in order to select the female embryos for X-linked recessive diseases (Munné *et al.,*1993). PGD has been widely applied for X-linked disorders such as hemophilia A (Gigarel *et al.,* 2004), fragile-X (Apessos *et al.,* 2001, Sermon *et al.,* 1999a), and neuromuscular dystrophies (Liu *et al.,* 1995).

The preimplantation genetic diagnosis for single gene disorders in couples with a high risk of transmitting a genetic condition helps to prevent the birth of an affected child or pregnancy termination due to the prenatal diagnosis of an affected fetus. PGD allows the selection of mutation free embryos. The most commonly diagnosed autosomal recessive disorders are beta-thalassemia, sickle cell disease, cystic fibrosis and spinal muscular atrophy type one (Goossens *et al.,* 2009). The most common X-linked disorders which are diagnosed with PGD are Duchenne's muscular dystrophy, hemophilia A, and Fragile X syndrome (Harper *et al.,* 2010). The preimplantation genetic diagnosis for autosomal dominant disorders are commonly applied for myotonic dystrophy type one, Huntington's disease, neurofibromatosis type one and Charcot-Marie-Tooth disease type 1A (Harper *et al.,* 2010). The preimplantation genetic diagnosis is available for more than two hundred single gene disorders. The list of common single gene disorders which are widely diagnosed by PGD is given in table 1.1.

Table 1.1. List of single gene disorders which are commonly diagnosed by PGD.

Disease	***Gene***
Beta- Thalassemia	HBB
Charcot- Marie-Tooth type X	CMTX
Congenital Adrenal Hyperplasia	CYP21A2
Cystic Fibrosis	CFTR
Duchenne Muscular Dystrophy	DMD
Familial Mediterrenan Fever	MEFV
Haemophilia A	F8
Haemophilia B	F9
Huntington's Disease	HD
Myotonic Dystrophy type 1	DMPK
Neurofibromatosis type 1	NF1
Retinoblastoma	RB1
Sickle Cell Anemia	HBB
Spinal Muscular Atrophy	SMN1
Wiskott-Aldrich Syndrome	WAS

1.1.6.1. PGD for Beta-Thalassemia and Sickle Cell Anemia. Beta-thalassemia is a common autosomal recessive blood disorder which is caused by either complete absence or reduced synthesis of the beta globin chains (Rund and Rachmilewitz, 2005). The prevalance of beta-thalassemia reaches its highest levels in the Mediterrenean countries, Middle East and Asia and the carrier rate was reported as 4.83% for the world population (Angastiniotis and Modell, 1998, Urbinati *et al.,* 2006). The gene frequency was estimated to be 2.1% for Turkey, but it shows regional variations and reaches to 10% in south coasts of Turkey (Arcasoy, *et al.,* 2003).

The beta-globin (HBB) gene is located at the short arm of chromosome 11 (11p15.5). More than 200 point mutations and – infrequently - deletions were associated with beta-thalassemia phenotype (Urbinati *et al.,* 2006). The beta-globin gene analysis of more than 1500 patients between 1987 and 2006 was demonstrated that IVS I:110 (G>A) is the most common mutation in the Turkish population with a frequency of 39.2% (Başak, 2007). This study has reported the frequencies of other common beta-thalassemia mutations in the Turkish population which are listed in table 1.2.

Table 1.2. Most common beta-globin mutations and their frequencies in Turkish population (Başak, 2007).

Mutation	***Frequency (%)***
IVS I:110 (G>A)	39.2
IVS I:6 (T>C)	9.5
Codon 8 (-AA)	6.1
IVS I:1 (G>A)	5.5
IVS II:1 (G>A)	5.4
IVS II:745 (C>G)	4.6
Codon 39 (C>T)	3.8
-30 (T>A)	3.1
Codon 5 (-CT)	2.2
Codon 8/9 (+G)	1.5

Since the disease is inherited in autosomal recessive manner, the carriers of mutation do not show disease phenotype. The patients carrying two mutations are diagnosed as either beta thalassemia intermedia or beta thalassemia major. Mild to moderate decrease in the beta globin synthesis generally result in variability of the severity of symptoms. Severe

decrease in the beta globin synthesis cause severe symptoms such as splenomegaly, growth retardation, overstimulation of the bone marrow, skeletal abnormalities, osteoporosis, jaundice and pallor. These patients take blood transfusions regularly. The complication of multiple blood transfusions is iron overload in the body. Therefore, iron chelation therapy is performed in order to remove the excess iron from the body and prevent the related organ damage (Muncie and Campbell, 2009). Bone marrow transplantation from HLA compatible donors is the only curative therapy in the childhood. The stem cell therapy is succesful in patients that has not severe disease damage (Rund and Rachmilewitz, 2005).

Sickle cell disease which is inherited in an autosomal recessive manner is caused by a point mutation at codon position six in the beta globin gene (Frenette and Atweh, 2007). The mutation causes aggregation of hemoglobin which results in the distortion of the erythrocytes and reduction of the cell elasticity. The cells have sickle shape. In low oxygen conditions, the cells are sickled and become rigid. As a result, they clog the narrow vessels and lead to vessel occlusion and ischemia (Vekilov, 2007). The disease shows various complications such as hemolytic anemia, vasoocclusion episodes, autosplenectomy, stroke, jaundice and pulmonary hypertension (Madigan and Malik, 2006). The carrier rate for sickle cell mutation is higher in the geography where the malaria is common. The carriers are asymptomatic and protected from the malaria infection. The treatment involves the management of the symptoms and use of penicillin in order to protect the patients from encapsulated microorganisms since they have immature immune system. The bone marrow transplantation from HLA matched donors is a promising option to cure the disease (Sonati and Costa, 2008).

The beta-globin mutations for beta-thalassemia and sickle cell disease were previously analyzed in single cells (Varawalla *et al.,* 1991, Pickering *et al.,* 1992). A fragment of the beta globin gene has been amplified in preimplantation embryos at various stages. Detection of five beta-thalassemia mutations has been achieved by allele specific priming of the PCR by using nested PCR strategy in samples containing 10 pg of DNA of individuals containing mutations. The optimization studies have been continued with the single cells obtained from buccal cells containing sickle cell mutation (Monk *et al.,* 1993). One of the first applications of the preimplantation genetic diagnosis for beta-thalassemia

has been used the strategy of introducing a restriction site in one of the primers to identify the wild type and the mutant allele in the single cells (Ray *et al.,* 1996a).

Previously reported PCR success in the single cells to detect the beta-globin mutations varied from 87% to 99% in different PCR and mutation detection strategies (Varawalla *et al.,* 1991; Ray *et al.,* 1996b; El-Hashemite *et al.,* 1997; Vrettou *et al.,* 1999; De Rycke *et al.,* 2001; Hussey *et al.,* 2002).

1.1.7. PGD for HLA Compatible and Disease Free Embryos: "Saviour Siblings" for Stem Cell Therapy

Hematopoietic stem cell transplantation from an HLA-matched donor is an advanced option to cure various inherited genetic diseases completely. Therefore, families with an existing affected child request not only an unaffected child but also an HLA compatible donor. More than fifty disorders could be treated with the stem cell transplantation from an HLA compatible donor. The most common diseases treated with the stem cell transplantation are fanconi anemia, beta-thalassemia, sickle cell anemia, wiskott-aldrich syndrome, hyperimmunoglobulin M syndrome, hypohidrotic ectodermal dysplasia with immune deficiency, X-linked adrenoleukodystrophy, aplastic anemia, diamond-blackfan anemia, osteopetrosis, leukemias and lymphomas.

The most extreme application of PGD is selection of an HLA-compatible and healthy embryo so that the baby will be a stem cell donor for the affected child of the family. The first case of PGD for fanconi anemia in combination with the HLA typing was reported by Verlinsky and his colleagues in 2001, ten years after the first PGD cases (Verlinsky *et al.,* 2001). The preimplantation HLA typing has been combined with various single gene disorders such as beta thalassemia (Van de Velde *et al.,* 2004), Fanconi anemia (Verlinsky *et al.,* 2001), wiskott-aldrich syndrome (Fiorentino *et al.,* 2004). The most common application of PGD with HLA typing has been performed for beta-thalassemia in Mediterranean countries. Since the HLA typing is not an indication for prenatal diagnosis, there is an increasing demand for the HLA typing of embryos to provide a stem cell donor for the affected child.

The Major Histocompatibility Complex (MHC) is a cluster of genes that encode a group of immune related and non immune related molecules in various species (Trowsdale, 1995). The MHC in humans is called Human Leukocyte Antigen (HLA). The human MHC extends 3.6 Mb on the short arm of chromosome six (6p21.3). It is composed of three main regions which are called class I, class II and class III region. The extended MHC region is about 7.6 Mb DNA sequence which covers the extended class I and class II regions (Stephens *et al.,* 1999, Totaro *et al.,* 1996) and includes 421 genes of which 60% are considered to be expressed (Horton *et al.,* 2004).

The class I region is 1.9 Mb long with eighteen HLA class I genes. HLA-A, HLA-B and HLA-C are the three classical HLA class I genes which encode heavy chains of class I molecules. (Shiina *et al.,* 2004). The class II gene region extends about 0.7 Mb and contains nineteen class II genes. The classical HLA class II genes are HLA-DP (DPA1 and DPB1), HLA-DQ (DQA1 and DQB1), and HLA-DR (DRA and DRB1 to DRB9). The class III gene region is located between the class I and the class II region. Sixty-two loci were found within 0.9 Mb and fifty-eight of them are expressed. This region does not contain the HLA genes, however, contains the cytokine genes, complement factor genes and many other genes related with inflammation and immune function (Xie *et al.,* 2003, Shiina *et al.,* 2004, Beck and Trowsdale, 2000) which play role in transcription regulation, biosynthesis, electron transport, housekeeping, chaperone function and signalling, inflammation and heat shock response (figure 1.1).

The extended MHC (xMHC) contains cluster of genes for histones and tRNA which are expressed at high levels. One hundred and fifty-seven tRNA genes and sixty-six histone genes are associated with the MHC. The genomic structure of MHC locus points out that this gene region is an expression hotspot of the human genome (Trowsdale, 2005).

Another feature of the MHC region is its enormous polymorphic sequence. The total number of the class I and the class II alleles were reported as 3249 and 1198, respectively (Robinson *et al.,* 2003). The most polymorphic sites in the class I genes are localized to exon two and exon three. For the class II genes, exon two has the most polymorphic sequence. The polymorphisms result in aminoacid changes of antigen binding domains of

HLA molecules. The point mutations, gene conversions and the recombinations generate excessive polymorphisms (Little *et al.,* 1999).

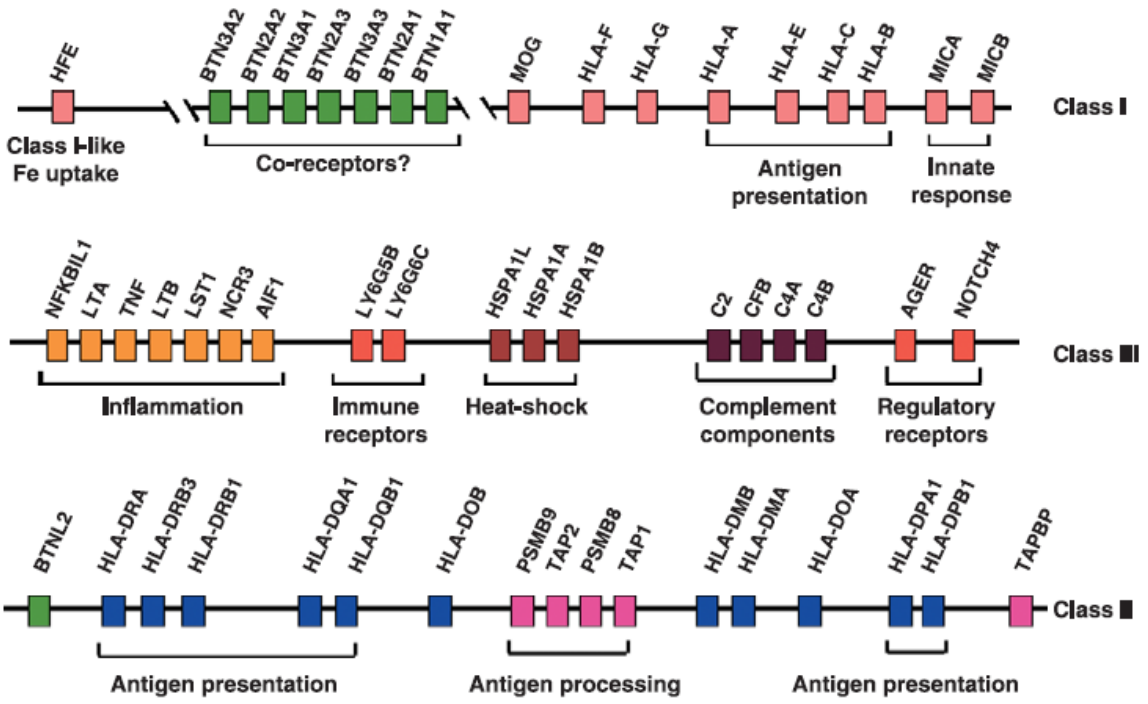

Figure 1.1. A simple drawing of the MHC map demonstrating the immune system genes (Traherne, 2008).

The MHC locus is also rich in microsatellites. Six hundred and sixty-four microsatellites were mapped to the MHC locus (Gourraud *et al.,* 2007). Moreover, this region comprises large genomic variations such as copy number variants, duplications and inversions.

The MHC genes are codominantly expressed in the cells. Therefore, heterozygosity of this locus is of utmost importance in order to maximize the variability of the MHC molecules in an individual, suggesting that numerous peptides can be recognized efficiently.

The MHC encodes glycoproteins that function on the cell surface in order to present antigenic peptides to the T lymphocytes. The class I and the class II genes encode two groups of homologous proteins which are structurally distinct. The HLA-A, HLA-B and

HLA-C genes encode the heavy chains of the class I molecules. Therefore, the class I MHC molecules consists of a heavy 44-47 kD alpha chain and an invariant 12 kD β_2 microglobulin. These polypeptide chains are covalently linked to each other. The class I heavy chain is formed from three extracellular domain (α1, α2 and α3), a transmembrane region and an intracellular cytoplasmic domain. The α1 and α2 domains of the α chain consist of the polymorphic residues and form the peptide binding cleft. Antigenic specifities of the class I MHC molecules are determined by the variable amino acid residues located in the peptide binding cleft. The peptides of eight-eleven amino acids can be bound to this cleft and presented to the CD8+ cytolytic T lymphocytes (Chelvanayagam *et al.,* 1997).

The class II genes encode the α and β chains of the class II molecules. The class II molecules are composed of one 32-34 kD α chain and one 29-32 kD β chain which are noncovalently linked glycosylated polypeptide chains. They have a transmembrane domain, two extracellular domains (for α chain: α1 and α2 domains; for β chain: β1 and β2 domains) and a cytoplasmic domain. The polymorphic amino acid residues reside at the α1 and β1 domains which determine the antigen specificity of class II molecules. The α1 and β1 domains of the polypeptide chains form the peptide binding cleft which has capacity to bind 10-30 amino acid long peptides (Brown *et al.,* 1993).

The major function of T lymphocytes is to recognize foreign antigens that are presented on the cell surface of an infected cell. The antigen receptors of T lymphocytes can only recognize non-self peptides that are bound to MHC molecules on the cell surface.

The class I MHC molecules present eight-eleven amino acid long peptides to CD8+ cytolytic T lymphocytes (CTLs) (Pamer, *et al.* 1998). The class II MHC molecules present 10-30 amino acid long peptides to the CD4+ helper T lymphocytes. The class I molecules are expressed in almost all cells. On the other hand, the class II molecules are presented generally on the surface of dendritic cells, B lymphocytes, macrophages and a few other cell types. The function of CD8+ T cells is to kill infected cells. Since the viruses can infect almost any type of cell, the receptors of CD8+ T lymphocytes could bind to its ligand on any type of cell. Therefore, the class I MHC molecules need to be expressed in all the cells. The naïve CD4+ T cells recognize the antigens presented on the dendritic cells

in the peripheral lymphoid organs. The differentiated CD4+ T cells induce antibody production and activate macrophages in order to eliminate extracellular microbes. In contrast to the class I molecules, the class II molecules display peptides derived from extracellular microbes and proteins (Meyer and Thomson, 2001).

Molecular HLA typing is performed for different purposes. Since the HLA is associated with transplantation, immmunotolerance, autoimmune diseases, and immunity to other conditions, different molecular typing methods have been developed. Typing of the HLA alleles in individuals is of utmost importance since the transplantation could be achieved after determination of the HLA match between the donor and the recipient. Moreover, determination of the HLA alleles is used to diagnose certain disorders or to determine susceptibility to a specific condition in individuals. Therefore, the polymerase chain reaction (PCR) based technologies has been developed for HLA typing. Three methods are commonly used. The first method uses sequence-specific oligonucleotide probes (SSOP). In this method, the most polymorphic exon is amplified and allele typing is achieved with the hybridization of a variable of synthetic oligonucleotide probes specific to the polymorphic sequences of the HLA alleles. The second method utilizes the sequence specific primers (SSP) which are designed from the most polymorphic sequences of alleles. Thus, the primers can only bind to allele sequences complementary to the primer sequence and amplify the alleles selectively. The third method is called sequence based typing (SBT) which is based on amplification and sequencing analysis of exon two and three of the HLA alleles. The allele is determined by comparison with the sequences found in the HLA databases (Choo, 2007).

In recent years, analysis of microsatellite markers scattered through the HLA locus has become an option in order to screen the HLA match indirectly between the donor candidates and the recipient in the case when the donors and the recipient are related. Since it does not require any allele specific amplification, the microsatellite analysis is preferred in single cell analysis to determine the HLA compatible embryos (Van de Velde *et al.,* 2004, Fiorentino et al., 2004). Instead of amplifying each HLA allele, the microsatellites throughout the HLA locus are amplified and the allele sizes are determined by capillary electrophoresis. The HLA haplotypes are established according to the segregation of the

alleles. Then, the HLA haplotypes of the donor candidates are compared with that of the recipient.

Accumulation of thousands of HLA alleles required special nomenclature for naming of the alleles. The naming is basically formed from two parts. The first part is the locus name which is separated from the second part by an asteriks (*). The second part contains the allele number. The first two digit indicate the serological equivalent and it is generally used for low resolution HLA typing. For high resolution, the allele number consists of four digits. (Choo, 2007).

1.1.8. Meiotic Recombinations at Major Histocompatibility Complex

Meiosis is a special nuclear division which takes place in germ cells in order to reduce the number of chromosomes into half. The maternal and the paternal chromosomes are homologs of autosomal chromosome pairs. Meiosis involves two consecutive cell division in order to produce cells with haploid genome after an initial duplication of the homologous chromosomes in interphase. The chromosomal cross-over, exchange of part of homologous chromosomes, takes place during prophase I. DNA double helix of both maternal and paternal homologs are broken and the genetic material is exchanged reciprocally between two nonsister chromatids by genetic recombination. Both copies of the homologous chromosomes pair with each other and are physically connected at certain points called chiasma (plural chiasmata) where crossover occurs between two nonsister chromatids. The main role of chismata is to hold the homolog chromosomes together until they are separated at anaphase I. After segregation of the homologous chromosomes into separate daughter cells at telophase I, the process continues with a second cell division without any duplication of the genetic material to produce four haploid gametes (Zickler and Kleckner, 1999, Smith and Nicolas, 1998).

The rearrangement of DNA sequences from generation to generation in order to evolve and give a better response to altering environmental conditions is called genetic recombination. The genetic exchange between the homologous DNA sequences are generally known as homologous recombination. It is essential for every proliferating cell in order to repair DNA replication mistakes and breaks. Moreover, it is crucial for proper

chromosome segregation during meiosis. Bacterial RecA protein and its eukaryotic homologs direct single stranded DNA to pair with its homologous region at the double stranded DNA. These interactions are formed in a special structure, known as Holliday junction which is a four stranded structure. The structure is then cut and form two repaired double stranded DNA helices. According to the pattern of the cut, either two repaired chromosomes or two recombined chromosomes are formed (Smith and Nicolas, 1998 and Kowalczykowski, 2000).

Several autoimmune diseases, susceptibility to a condition or protection from a pathogen have been associated with certain HLA haplotypes (reviewed in Shiina *et al.,* 2004). These associations are generally result of the strong linkage disequilibrium within the extended HLA locus. Determining the recombination rate across the HLA locus is of significance in order to understand the disease association of certain HLA haplotypes since various HLA alleles have been closely associated with distinct conditions. The HLA haplotypes which show beneficial interactions are protected through the evolution. They are closely linked to each other and segregate together through the generations in population. A selective pressure is expected to preserve certain alleles in combination in certain haplotypes as they show the best immune response to common pathogens. Novel haplotypes that could be efficient against novel pathogens could be arisen by recombinations. If they were not beneficial, their frequency would be limited in the population. Therefore, in order to understand the recombination at HLA locus, large pedigrees and single sperms from several donors have been investigated (Martin *et al.,* 1995, Cullen *et al.,* 1995, Yu *et al.,* 1996, Cullen *et al,* 1997, Jeffreys *et al.,* 2000, Cullen *et al.,* 2002).

Several autoimmune diseases such as type I diabetes, Crohn's disease, multiple sclerosis, rheumatoid arthritis and ankylosing spondylitis have been associated to certain class I, class II alleles or haplotypes. According to Genetic Association Database (GAB) (Becker *et al.,* 2004), five hundred records of disease phenotype have been found in relation to the HLA alleles. At least 100 dieases have been associated to the genes located at the MHC region. The spectrum of diseases listed in the database varies from immune, metabolic, dermatological, infectious, neurodegenerative, cardiovascular to oncologic disorders. Moreover, the MHC alleles are linked to predisposition to or resistant to some

infectious diseases, such as HIV-1 and HBV. Although certain alleles have been associated with some disease phenotypes, the underlying mechanism is not clear. There are non-HLA genes in the MHC locus which have been determined as the main cause of some genetic disorders. One of the best known non-HLA gene is the CYP21A2 gene of which mutations cause 21-hydroxylase deficiency and congenital adrenal hyperplasia (OMIM 201910) (Shiina *et al.,* 2004).

The MHC region is extremely dense for microsatellites and single nucleotide polymorphisms which make possible determination of haplotypes, recombination rate and fine-mapping of recombination hotspots within the MHC locus. Since generation of polymorphisms includes insertions, deletions and recombinations, the MHC region is one of the first gene regions in the human genome to be analyzed for the recombination hotspots (reviewed by Vandiedonck and Knight, 2009). Several autoimmune diseases have been associated with particular HLA loci. These associations are generally result of strong linkage disequilibrium within the extended HLA locus.

Studies in the mouse MHC constitute much of the knowledge about the mammalian recombination. These studies used limited number of inbred strains with definite H2 haplotypes which have revealed several properties of the mammalian recombination, including sequence motifs that determine the initiation of recombination, effects of haplotypes on recombination rate, location of recombination site, differences in recombination rate between females and males (Steinmetz *et al.,* 1986, reviewed in Lindahl, 1991 and Shiroishi *et al.,* 1995). The analysis is much more complicated in humans due to great variability of the haplotypes. The haplotypes and the recombination rate were determined initially in family studies. The HLA haplotypes and the linkage disequilibrium values were observed in forty CEPH pedigrees (Carrington *et al.,* 1994). The DRB1, DQA1 and DQB1 loci were found in very strong linkage disequilibrium according to the molecular typing. Whereas the DPB1 loci which is located at the proximal end of the class II region showed a much lower disequilibrium, indicating that a region between them has a role in recombination. According to the study of eleven recombinant chromosomes, at least three regions were found to be responsible from recombination at the class II region: from DQB1 to DOB, the second intron of the TAP2 and from intron three of TAP1 to DPB1 (Cullen *et al.,* 1995). The initial findings directed the studies to

concentrate on the characterization of recombination in the HLA class II region since a lack of association between alleles of the TAP1 and the TAP2 gene has been observed inspite of a 15 kb physical separation (Cullen, *et al.* 1997).

Twenty-seven HLA class II recombinant families and 59 CEPH pedigrees were studied to define where the recombination occurred in the class II region. Additionally, the recombination at the maternal chromosomes were observed in twenty-one of the thirty recombinant chromosomes which was coherent with the observation of a higher recombination frequency in females than in males (Thomsen, *et al.,* 1989, Thomas and Rothstein, 1991, Cullen, *et al.* 1997). These studies showed poor recombination events and strong linkage disequilibrium between the HLA-B and the HLA-C genes and between the HLA-DQA1 and the HLA-DRB1 genes. The recombination rate in the class II and the class III regions were identified as 0.74 and 0.94 cM/Mb, respectively. However, it was determined as 0.31% in the class I region (Martin *et al.,* 1995). These results were concordant with the presence of recombination hotspots between the TAP1 and the TAP2 genes and it was mapped to the second intron of the TAP2 gene by using SSCP analysis (Cullen *et al.,* 1995). Due to limited number of progenies in the selected families, these studies could not define the properties of recombination between the individuals.

Sperm typing allows the analysis of thousands of meioses from a single sperm donor. One of the first studies determined the recombination rates in five sperm donors in a 20-25 Mb region comprising the extended HLA locus. The recombination rates varied between 5.1% and 11.2% among donors (Yu, *et al.,* 1996). Another study used MOG-CA and D6S439/ D6S291 markers which flank approximately seven megabase region comprising the HLA locus. According to this study, the recombination rate varied between 1.66% and 6.5% among five donors (reviewed in Carrington, 1999). The analysis of 20031 single sperm from twelve sperm donors revealed a high resolution recombination map of 3.3 Mb region encompassing HLA locus. An average of recombination rate was estimated 0.49 cM/Mb since 325 recombinations were localized to 3.3 Mb region (Cullen, *et al.,* 2002). The male specific recombination rate was estimated 0.92 cM/Mb and 0.71 cM/Mb for the whole genome and chromosome six, respectively (Broman *et al.,* 1998). Cullen's study exhibited some critical observations about the recombination characteristics of the HLA locus. The recombination rates and the location of recombinations show variations between

individuals substantially. Intense hotspots could be found in every 0.8 Mb, but they do not have to be regulary spaced. Specific sequence motifs were found to be related substantially with the recombination events (Cullen, *et al.,* 2002).

A putative recombination hotspot in the TAP2 gene which is localized to the class II region has been characterized by haplotype analysis and direct detection of crossovers in the single sperms (Jeffreys *et al.,* 2000). The hotspot was localized to the second intron of the TAP2 gene. Allele specific PCR primers were used to amplify selectively the recombined TAP2 genes. The study revealed a 1.2 kb long recombination hotspot which is rich in the sequence polymorphisms and flanked by DNA which is less active in the recombination. The hotspot was found to be more active in female meiosis than in male meiosis (Jeffreys *et al.,* 2000), indicating the recombination rate differences between the females and the males.

1.2. Strategies Used for Single Cell Genetic Testing

The amplification of DNA to detect mutations and polymorphisms in the single cells can be achieved by either polymerase chain reaction (PCR) or whole genome amplification (WGA).

1.2.1. Polymerase Chain Reaction (PCR)

The first preimplantation genetic diagnosis was based on amplification of Y chromosome specific regions to select female embryos (Handyside *et al.,* 1990). The analysis of single gene disorders are based on polymerase chain reaction of the target region in single cells. As the single cells contain only five-ten pg of DNA, PCR has some technical limitations in single cells. Since the DNA content is too minute, large number of amplification cycles are needed to obtain a detectable amplification product. Therefore, a nested or heminested PCR strategy is followed. However, this fact brings the high risk of contamination (reviwed in Wells, 2004). One of the major problems in the single cell PCR is the amplification of only one of the two alleles in a single cell. That's why, a heterozygous genotype could be erroneously detected as homozygous which leads to misdiagnosis. This phenomenon is called allele drop out (ADO). The allele drop out rate

varies to more than 20% of all amplifications (Findlay *et al.,* 1995, Ray *et al.,* 1996b, Rechitsky *et al.,* 1998, Piyamongkol *et al.,* 2003). As the number of target sequences to be analyzed increased due to addition of linked microsatellites in order to exclude the allele drop out or analysis of the HLA alleles coupled with monogenic disorder, the multiplex PCR strategy has been developed accordingly (reviwed in Wells, 2004).

1.2.2. Whole Genome Amplification (WGA)

Whole genome amplification method allows amplification of whole DNA up to microgram quantities (Paunio *et al.,* 1996). Primer extension preamplification (PEP) (Zhang *et al.,* 1992) and degenerate oligonucleotide primed PCR (DOP-PCR) (Telenius *et al.,* 1992) are two common methods used in the whole genome amplification. Additionally, the multiple displacement amplification (MDA) is a recently developed whole genome amplification method (Lizardi *et al.,* 1998, Dean *et al.,* 2002).

Primer extension preamplification (PEP) was used initially for single sperm cell haplotyping (Zhang *et al.,* 1992). In PEP method, a mixture of 15 base oligonucleotides and T*aq* polymerase are used. The template is denatured at 92^{o}C and primers are allowed to anneal at 37^{o}C. The temperature is raised to 55^{o}C slowly and the reaction is incubated at this temperature for four minutes for extension. This protocol is cycled for 50 times. Approximately 78% of the genomic sequences can be copied in PEP method and this amplification is believed to produce approximately 1000 copies (Paunio *et al.,* 1996). PEP was previously tried in prenatal and preimplantation genetic diagnosis. Moreover, PEP can be used in tumors, malignant cells and limited tissues (Peng *et al.,* 2007). Although it has an extensive success in the amplification of DNA from single cells, it has some pitfalls such as that it may introduce new mutations, it may create shorter amplified products due to degenerate primers and allele sizes may change in the repeat regions due to GC content. Imbalanced amplification of highly polymorphic microsatellite markers has been observed. PEP produces fragment sizes of around 450 bp to 1500 bp. This WGA method has been used in PGD cycles for Tay-Sachs disease (Gibbons *et al.,* 1995), cystic fibrosis (Xu *et al.,* 1993), duchenne muscular dystrophy (Kristjansson *et al.,* 1994), beta-thalassemia (Jiao *et al.,* 2003).

The second common whole genome amplification method is degenerate oligonucleotide primed PCR. The method has been developed by Telenius *et al.* (1992). This method uses partially degenerate primers. At the beginning, primers are allowed to anneal to the genomic DNA at very low stringent temperatures. After several cycles of annealing, the temperature is increased to allow specific binding of primers to previously annealed primers that have tag sequences. Then, an exponential increase of the product is obtained. The DOP-PCR provides sufficient amplification with a high efficiency for the single cells. The starting template DNA of 15 ng could be amplified to as much as 400 ng (Peng *et al.,* 2007). The DOP-PCR is preferred to use in the single cells for comparative genomic hybridization (CGH) (Wells *et al.,* 1999). The DOP-PCR has been used in limited tumor cells from formalin-fixed and paraffin embedded samples. Since the DOP-PCR can be complemented with the CGH, it has been previously used in PGD of aneuploidy screening, chromosomal translocations, gross deletions and insertions (Voullaire *et al.,* 1999). The average size of the products is 500 bp but it can be increased more than 10 kb with a few modifications. The DOP-PCR produces more amplification products compared to the PEP.

A more recent whole genome amplification method is multiple displacement amplification (MDA) (Dean *et al.,* 2002). This method uses random exonuclease-resistant hexamer and bactoriophage Phi29 DNA polymerase for double-stranded DNA displacement and primer extension. The amount of the starting template has no effect in the amplification efficiency. The polymerase used in this method has a high proofreading activity with a very low error rate. Therefore, analysis of single nucleotide polymorphisms is more reliable. The MDA is performed at 30 ^{o}C and it does not require cycling. The average product length is more than 10 kb. As a whole genome amplification method, the MDA has the highest accuracy rate in single cells. The MDA has been commonly used for allele amplification, sequence variations, RFLP analysis, chromosome translocations and mutation detection (Murthy *et al.,* 2005).

In single cell analysis, the MDA enables sufficient amplification of DNA from single blastomeres and single lymphocytes (Handyside *et al.,* 2004, Hellani *et al.,* 2004). The MDA allows analysis of more than twenty different loci in single cells. Analysis of several microsatellites was achieved by using MDA products of single cells (Handyside *et al.,*

2004, Hellani *et al.,* 2005). Although it provides a good yield of DNA, preferential amplification and allele drop out are still the major problems in single cell DNA amplification. The ADO rates for MDA were reported between 10-31% in single cells which is higher compared to the ADO rate of the genomic DNA (Handyside *et al.,* 2004, Hellani *et al.,* 2005, Spits *et al.,* 2006, Renwick *et al.,* 2006). The MDA was used for PGD of cystic fibrosis and beta-thalassemia initially. The MDA products were used to amplify the mutation region and sixteen microsatellites simultaneously in PGD cycles (Hellani *et al.,* 2005). Preimplantation genetic haplotyping was performed for single gene disorders to select healthy embryos by using MDA and linked microsatellites (Renwick *et al.,* 2006). The MDA was also used for PGD of fragile X syndrome with a successful diagnosis rate of 86% (Burlet *et al.,* 2006).

1.2.3. Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism has been used for diagnosis of different genetic disorders in the single cells in early years. It has been widely used for diagnosis of point mutations (Kuliev *et al.,* 1998). The final products are digested by a restriction enzyme and separated according to their lengths. One of the major pitfalls of the PCR-RFLP technique is the risk of incomplete restriction enzyme digestion of the PCR products which results in false results. It has been used earlier for PGD of cystic fibrosis, sickle cell anemia and beta-thalassemia (Xu *et al.,* 1999, Goossens *et al.,* 2000, Kuliev *et al.,* 1998). Due to the technological improvements in the mutation detection, PCR-RFLP is not preferred in the single cell genetic testing any more.

1.2.4. Allele Specific Amplification

Allele specific amplification is established on the annealing of sequence specific primers and it is widely known as amplification refractory mutation system (Newton *et al.,* 1989). The sequence variations are detected by the presence or absence of the amplified product on the agarose gel. Two forward primers are designed for the polymorphic sequence. One of them anneals to the wild type allele, and the other anneals to the mutant allele. So, the amplification is performed in two tubes. The method has been applied to the

single cells to detect the most common five cystic fibrosis mutations after an initial amplification (Scobie *et al.,* 1996).

1.2.5. DNA Sequencing

Direct sequencing of the PCR products is a gold standard method. It is reliable and accurate. It is commonly used to detect the single nucleotide polymorphisms, small insertions and deletions. In the single cell diagnostics, a small region containing the mutation of interest is usually amplified and sequenced. To increase the accuracy rate in the single cell strategies, the amplified product is sequenced by both forward and reverse primers (reviewed in Thornhill and Snow, 2002).

1.2.6. Minisequencing

Minisequencing is a novel detection method that allows detection of specific mutations without sequencing the whole PCR product (Pastinen *et al.,* 1997, Syvanen*,* 1999). The method is based on single dideoxynucleotide extension of unlabelled oligonucleotide primers (Sokolov*,* 1990), which enables detection of known single nucleotide polymorphisms. This technique allows identification of mutations without sequencing the entire PCR product. It has been applied for preimplantation genetic diagnosis of beta-thalassemia, cystic fibrosis, sickle cell anemia, hemophilia A, retinoblastoma, spinal muscular atrophy, Charcot-Marie-Tooth type X (Fiorentino *et al.,* 2003, Fiorentino *et al.,* 2006). The method was well-adapted for the single cell genetic testing due to its sensitivity, reliability, possibility of multiplexing and easy data interpretation. Minisequencing was performed on 887 blastomeres from 55 PGD cases of different genetic conditions with an efficiency of 96.5% (Fiorentino *et al.,* 2003).

1.2.7. Linkage Analysis

Linkage analysis is relied on the PCR amplification of polymorphic short tandem repeats (STRs). Fluorescently labelled primers are often preferred for the amplification in single cells. The amplified products are electrophoresed on automatic genetic analyzers. The linked markers in close proximity to the mutated gene are selected. The association of

linked markers with the mutated allele is determined by studying family members. The most informative markers are selected for the single cell diagnostics. Since the linkage analysis does not require the detection of mutation, this method has an increasing popularity in the single cell diagnostics. The method can be applied to diagnose several mutation types, including triplet repeat expansions, large deletions and insertions. The linkage analysis has been applied for several genetic diseases in the single cells such as Fragile X syndrome (Apessos *et al.,* 2001), Marfan syndrome (Sermon *et al.,* 1999b), Charcot-Marie-Tooth disease (De Vos *et al.,* 1998) and cystic fibrosis (Eftedal *et al.,* 2001). The linked microsatellites for beta-thalassemia, myotonic dystrophy, hemophili A, and Wiskott-Aldrich syndrome has been used in the single cells (Fiorentino *et al.,* 2006) Quantitative fluorescent PCR is based on the amplification of microsatellites to establish the number of specific chromosomes present in a cell (Pertl *et al.,* 1996). Since aneuploidy of chromosomes X, Y, 13, 18 and 21 are very common; STRs on these chromosomes are amplified in the single cells in order to detect the aneuploidy of these chromosomes. Moreover, STRs located in and around the HLA locus are used to determine indirectly the HLA haplotype of the single cell, without the need for further HLA typing (Fiorentino *et al.,* 2004). Before studying on the single cell, microsatellites are studied on the family members to determine the informative ones and HLA haplotypes.

1.2.8. Real-Time PCR

Real-time PCR is used to detect single nucleotide polymorphisms and gene dosage changes (deletions and duplications) (Wilhelm and Pingoud, 2003.). It is a rapid and advanced technique which eliminates the post-PCR processing, such as agarose gel electrophoresis and sequencing for known mutations. Because it is a closed tube system, it eliminates the post PCR contamination.

This method uses fluorescently labeled probes and primers. Different probe types can be used with different real-time PCR platforms. In general, the probes bind to the region that covers the mutation site and are used to monitor emitted fluorescence during amplification and melting curve analysis in order to detect the genotype or quantify the gene dosage.

The single nucleotide changes are analyzed by melting curve analysis. After the amplification of target region, the hybridization probes are annealed to the amplified product. During the melting curve analysis, as the temperature increases, loss of fluorescence is detected due to the melting off the probe with lower melting temperature (Tm). A single base mismatch under the probe with lower Tm causes a Tm shift of 5- 10^{o}C which enables simple detection of wild type and mutant alleles, separately. The use of different dyes as probe label and probes with different melting temperatures allow the real-time PCR to screen more than one mutation in a single reaction (Vrettou *et al.,* 2003).

The real-time PCR assays for single cell analysis have been developed for a Y chromosome specific marker (Pierce *et al.,* 2000), a common Tay-Sachs disease mutation (Rice *et al.,* 2002), the most common cystic fibrosis mutations (Pierce *et al.,* 2003), beta-thalassemia and sickle cell disease (Vrettou *et al.,* 2004).

The quantitative real-time PCR has been used for different applications, including determination of gene copy number, DNA methylation status, viral load and gene-expression level. The most common application of the quantitative real-time PCR is quantifying RNA levels inside cells (Higuchi *et al.,* 1993). Transcripts of different genes vary widely according to the cell type, environment, tissue and differentiation. The mRNA is converted into the cDNA by reverse transcription. Then, it is amplified by real-time PCR. Therefore, it is the simplest way of monitoring gene expression in special cells or tissues.

1.2.9. Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* hybridization is a technique which is applicable to the single cell diagnostics. A fluorescently labelled DNA fragment is used as a probe which hybridizes to its complementary sequence on the chromosome. It is used to detect chromosomal imbalances, microdeletions, Robertsonian translocations, reciprocal translocations, inversions and other complex rearrangements. This method is initially used to select female embryos in families with X-linked disorders in the preimplantation genetic diagnosis (Griffin *et al.,* 1994). It is commonly used for aneuploidy screening in the

preimplantation genetic diagnosis when the indication is either advanced maternal age, recurrent IVF trials or low implantation rate (reviewed in Harper and Wells, 1999).

1.2.10. Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) is a molecular cytogenetic method which has been developed for the analysis of chromosomal abnormalities in tumors (Kallionemi *et al.,* 1993). The technique is based on simultaneous hybridization of two differentially labelled DNA samples to normal metaphase chromosomes. One of the DNA sample belongs to the subject and the other is wild type control DNA. Both DNA samples are mixed and allowed to hybridize the metaphase chromosomes. The final signals give information about the genetic abnormality in the subject sample. The application of CGH in single cells requires the whole genome amplification (WGA) (Wells *et al.,* 1999). This method has been used to identify aneuploidies in the single fibroblasts, amniocytes and blastomeres (Voullaire *et al.,* 1999).

1.2.11. Microarray

Microarray analysis is a developing technology in molecular biology. A microarray is basically a collection of microscopic sequences, arrayed on a special surface. The subject material is applied to the array for hybridization. A detection system collects the data and it is evaluated according to the hybridization signals. Microarray technology has a diverse number of applications in the single cells, including expression profiling (Ginsberg *et al.,* 2004), analysis of gene dosage (Hu *et al.,* 2004), and diagnosis of sequence variations (Kim *et al.,* 2003). The DNA microarrays provide screening of several genetic mutations simultaneously. A whole genome amplification or multiplex PCR is commonly performed prior to the microarray analysis for mutation detection in the single cells as the amount of the DNA is very limited. The CGH microarrays have been developed to determine chromosome copy numbers (Pinkel *et. al.,* 1998). The microarrays have not been yet established for single cell diagnostic human applications.

1.3. Gene Expression in Single Cells

Studying gene expression in the single cell is one of the ultimate goals of researchers to understand the molecular mechanisms. The gene transcription is the most significant regulatory mechanism by which a cell determines its fate. The quantitation of gene expression at the mRNA level for specific cell types gives numerous information about the changes in cell physiology due to the alterations in the environment, differentiation and apoptotic signals (Elowitz *et al.,* 2002, Raj *et al.,* 2006). The cells in a population are found in various conditions and states. Therefore, gene expression data from a population of cells present an average of whole cells.

Since the single cell has limited RNA content, most of the classical methods for RNA analysis such as Northern blot and dot-blot are not suitable. The single cells are isolated from their surrounding tissues for analysis of expression profile. For tumor tissues, the single cells are isolated commonly via laser capture microdissection. For studying embryonic gene expression profiles, the single blastomeres are generally isolated by sucking after laser zona drilling. Since the quantity of total RNA is as low as 0.1-1.0 picograms in a single mammalian cell, mRNA extraction is of utmost importance for analysis (Kacharmina *et al.,* 1999). New methods with high precision and reliable measurements are required to characterize cell to cell variations. However, the single cell gene expression studies have been conducted for a few years and limited to few studies. These studies include optimization of RNA isolation from the single cells, development of a sensitive and reliable amplification methods (Hartshorn *et al.,* 2005, Bengtsson *et al.,* 2008). Hartshorn *et al.* (2005) developed a method that includes single tube cell lysis, reverse transcription and RNA quantification. The female and male blastocysts were used in this study to quantify Xist RNA content, and copy numbers of Xist and Sry genes. Noise in RT-qPCR measurements in the single cells increases with initial low copy numbers and interfere with the accuracy of the measurements. The single cell RT-qPCRs were optimized in order to minimize the technical noise (Bengtsson *et al.,* 2008). A multiplex PCR method has been developed in order to quantify different transcripts independent from their relative abundance in a single cell (Hartshorn et al., 2007). The studies of gene expression in single cells are generally concentrated on development of reliable methods.

Prior to microarray analysis, the whole genome reverse transcription - polymerase chain reaction is performed in order to synthesize double stranded cDNA. In summary, studying gene expression in the single cells is of utmost importance to understand the disease mechanisms and developmental stages at the molecular level.

2. PURPOSE

This thesis firstly aims

- to develop strategies to detect disease associated DNA variations and haplotypes in single cells. Polymerase chain reaction and mutation detection strategies will be used in the isolated single embryo blastomeres.
- to simplify differential diagnosis at the single cell level.
- to develop new therapeutic potentials by selecting disease free and HLA compatible embryos for the most serious diseases requiring stem cell transplantation.
- to optimize and validate diagnosis in human blastomeres to prevent the hereditary diseases.

The second aim of this thesis is

- to investigate characteristics (frequency, gender difference, individual variations, patterns, breakpoints) of the meiotic recombinations at the human MHC loci in embryos.

The single cell genetics may open up new possibilities for economic and efficient prenatal screening of the genetic diseases. Applications of this strategy may contribute to the hereditary disease prevention because of their ethically more acceptable approaches in Turkey. Preimplantation genetic diagnosis (PGD) is an application of the single cell genetic diagnosis used for prevention of the genetic disease inheritance by selection of the unaffected embryos. The PGD is also used as a means of therapy by selection of the HLA matched "saviour siblings" for transplantation of stem cells to an affected child in the family. The PGD for HLA matching is expected to increase the number of available donors for the affected family member awaiting the stem cell transplantation. The single cell studies can be extended as a research model to characterize the gene expression and cell fate which will bring potential applications in the medicine. The significance of this project lies in its contribution to adapt methods and develop new strategies for applications to the genetic investigations in various fields of basic and applied biological sciences.

3. MATERIALS

3.1. Subjects

Forty-two couples were taken into an *in vitro* fertilization program. A total of 1180 embryos which were obtained from 107 *in vitro* fertilization cycles were biopsied and genetically analyzed. Peripheral blood samples were collected from each couple and their children in order to identify the most informative microsatellites of HLA complex, HLA alleles and disease causing mutations. Signed informed consents were obtained from all patients at IVF and Genetic centers after appropriate counselling.

3.2. Chemicals

All chemicals used throughout the study were molecular biology grade and purchased from Merck (Germany), Applichem (Germany) or Sigma (USA).

3.3. Buffers and Solutions

3.3.1. Stock Solutions for Blastomere Lysis

The stock solutions and their compositions for blastomere lysis were listed in table 3.1.

Table 3.1. List of solutions and their compositions for blastomere lysis.

Solution	***Composition***
Proteinase K	14-22 mg/ml Proteinase K in 10 mM Tris - HCl, pH 7.5 (Roche Diagnostics GmbH)
Sodiumdodecylsulphate (SDS)	10% SDS (w/v) in dH_2O
Blastomere Lysis Buffer	125 µg/ml Proteinase K 17 µM Sodium Dodecyl Sulphate (SDS)

3.3.2. Stock Solutions for Polymerase Chain Reaction (PCR)

Stock solutions and buffers which were used for polymerase chain reaction were listed in table 3.2.

Table 3.2. List of stock solutions for polymerase chain reaction (PCR).

Solution	***Composition***
5X Colorless GoTaq Flexi Buffer	Proprietary formulation of Promega at pH 8.5
GeneAmp 10X PCR Buffer II	100 mM Tris-HCl (pH 8.3), 500 mM KCl. (Applied Biosystems, USA)
10X Taq Buffer with KCl	100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40. (Fermentas, Lithuania)
$MgCl_2$	25 mM $MgCl_2$
Deoxyribonucleotides (dNTPs)	10 mM of each dATP, dGTP, dCTP, dTTP (Promega) in TE
TE Buffer	10 mM Tris-HCl (pH 8.0), 1 mM EDTA

3.3.3. Stock Solutions for Agarose Gel Electrophoresis

Stock solutions for agarose gel electrophoresis were given with their composition in table 3.3.

Table 3.3. List of stock solutions for agarose gel electrophoresis.

Solution	***Composition***
5X TBE buffer	54 g/l Tris (Base), 27.5 g/l Boric acid, 20 ml of 0.5 M EDTA (pH 8.0).
6X Loading buffer	0.03% Bromophenol Blue (BPB), 0.03% xylene cyanol FF, 10 mM Tris-HCl (pH 7.6), 60 mM EDTA, 60% Glycerol
2 % Agarose gel	2% (w/v) agarose in 0.5X TBE buffer, containing 0.5µg/ml ethidium bromide (Promega)

3.4. Kits

The list of commercial kits used in this study is given in table 3.4. A brief description of the kit and the manufacturer company is also provided in the table.

Table 3.4. List of kits used in this study.

Kit Name	*Description*	*Manufacturer*
High Pure PCR Template Preparation Kit	It is used for genomic DNA isolation from anticoaggulated blood	Roche Applied Sciences, Germany
MagNA Pure LC DNA Isolation Kit I	It is used for genomic DNA isolation from anticoaggulated blood by MagNA Pure LC Instrument	Roche Applied Sciences, Germany
LightCycler® DNA Master HybProbe	It is used to perform quantitative PCR, SNP and mutation detection.	Roche Applied Sciences, Germany
AxyPrep™ PCR Cleanup Kit	It is used to purify PCR products.	Axygen Biosciences, USA
Montage SEQ_{96} Sequencing Reaction Cleanup Kit	It is used to purify sequencing reactions.	Millipore, USA
DYEnamic ET Terminator Cycle Sequencing Kit	Ready to use reaction mix contains Thermo Sequenase ™ II DNA polymerase which provides a convenient and flexible dye-terminator format for high throughput sequencing.	GE Healthcare, USA

3.5. Enzymes

The list of enzymes used in this study were given in table 3.5.

Table 3.5. List of enzymes used in this study.

Enzyme	*Manufacturer*
Go Taq Flexi DNA Polymerase	Promega, USA
Taq DNA Polymerase	Fermentas, Lithuania

Table 3.5. List of enzymes used in this study (continued).

Enzyme	***Manufacturer***
AmpliTaq Gold Polymerase	Applied Biosystems, USA
ExoSAP-IT (Exonuclease I, Shrimp Alkaline Phosphatase)	Usb Corporation, USA

3.6. Oligonucleotide Primers and Probes

The oligonucleotide primers and the hybridization probes used in this study were purchased from TIB Molbiol (Germany). The lyophilized primers and probes were dissolved in TE in order to obtain 100 μM and 10 μM stock solutions, respectively.

In house primers and hybridization probes were designed by using Primer3 web interface (http://primer3.sourceforge.net/webif.php). The references for published primers and probes were given in the relevant tables.

Table 3.6. List of external oligonucleotide primers designed for amplification of beta-globin (HBB) gene in single cells.

Primer Sequence (5'→3')	***Size (bp)***	***Annealing Temperature (°C)***
F: TAAGCCAGTGCCAGAAGAGCC	771	55 or 60
R: CAATCATTCGTCTGTTTCCCATTC		
F: AATCTCTTTCTTTCAGGGCAAT	245	55 or 60
R: GGCCTAGCTTGGACTCAGAA		

Table 3.7. List of inner oligonucleotide primers used for nested PCR and DNA sequencing of the beta-globin (HBB) gene in single cells

Primer Sequence (5'→3')	***Size (bp)***	***Annealing Temperature (°C)***
F: GCTGTCATCACTTAGACCTCA	586	60
R: CAAGTGCAGCTCACTCAG		

Table 3.7. List of the inner oligonucleotide primers used for nested PCR and DNA sequencing of beta-globin (HBB) gene in single cells (continued).

Primer Sequence (5'→3')	***Size (bp)***	***Annealing Temperature (oC)***
F: TGACTCTCTCTGCCTATTGGTC	380	55
R: CAATCATTCGTCTGTTTCCCATTC		
F: TATCATGCCTCTTTGCACCA	201	55 or 60
R: GGCCTAGCTTGGACTCAGAA		

Table 3.8. List of oligonucleotide primers used for amplification and DNA sequencing of beta-globin (HBB) gene to identify mutations in beta-thalassemia patients and carriers.

Primer Sequence (5'→3')	***Size (bp)***	***Annealing Temperature (^{o}C)***
F: CCAAGCTGTGATTCCAAATA	806	59
R: ATCAAGGGTCCCATAGACTCA		
F: TGAGCTGCACTGTGACAAG	854	59
R: GGCCTAGCTTGGACTCAGAA		
F: TCTGGGTTAAGGCAATAGCA	665	59
R: GAATAAGGCATAGGCATCAGG		
F: TGACTCTCTCTGCCTATTGGTC	406	60
R: ACGATCCTGAGACTTCCACACT		

Table 3.9. List of inner oligonucleotide primers specific for beta-globin gene designed for real-time PCR by Vrettou *et al.,* 2003.

Primer Sequence (5'→3')	***Size (bp)***	***Annealing Temperature (^{o}C)***
F: GCTGTCATCACTTAGACCTCA	586	58
R: CACAGTGCAGCTCACTCAG		

Table 3.10. List of oligonucleotide primers and hybridization probes to detect beta-globin gene mutations by real-time PCR.

Mutation	***Sequence (5'→ 3')***	***Label***	***Annealing Temperature (°C)***
IVS I:110 (G>A)	F: GGTTTAAGGAGACCAATAGA		50
	R: AAAGGACTCAAAGAACCTC		
	PR: TTCTGATAGGCACTGACTCTCTCTG	3' FITC	
	PR: CTATTGGTCTATTTTCCCACC	5' LC RED 640	
IVS II:1 (G>A)	F: ATCTGTCCACTCCTGATGCT		55
	R: CCCCTTCCTATGACATGAAC		
	PR: AACTTCAGGGTGAGTCTATGGGA	5' LC RED 640	
	PR: CAAGCTGCACGTGGATCCTGA	3' FITC	
Codon 8/9 +G	F: GCAACCTCAAACAGACACCA		56
	R: TTGGTCTCCTTAAACCTGTCTTG		
	PR: CTGCCCTGTGGGGCAAGGTGAA	5' LC RED 705	
	PR: TCCTGAGGAGAAGTCTGCCGTT	3' FITC	
IVS II:745 (C>G)	F: TTGCACCATTCTAAAGAATAAC		51
	R: ACTCAGAATAATCCAGCCTTAT		
	PR: CTGATGTAAGAGGTTTCATATTGCTAA TAGCAG	3' FITC	
	PR: TACAATCCAGCTACCATTCTGC	5' LC RED 640	

Table 3.11. List of oligonucleotide hybridization probes to detect beta-globin gene mutations by real-time PCR (Vrettou *et al.,* 2003).

Mutation	***Sequences of hybridization probes (5'→ 3')***	***Labels***
Codon 39 (C>T)	PR: ACCCTTGGACCCAGAGGTTCTT	5' LC RED 705
	PR: CCCTTAGGCTGCTGGTGGTC	3' FITC
Codon 5 (-CT), Codon 6 (-A), Codon 6 (A>T) Codon 8 (-AA), Codon 8/9 (+G)	PR: GACTCCTGAGGAGAAGTCTGC	5' LC RED 640
	PR: CCTCAAACAGACACCATGGTGCACC	3' FITC
IVSI:1 (G>A), IVSI:5 (G>T), IVSI:6 (T>C)	PR: TGTAACCTTGATACCAACCTGCCCA	5' LC RED 705
	PR:TGCCCAGTTTCTATTGGTCTCCTTAAACCTGTC	3' FITC

Table 3.12. List of external oligonucleotide primers for microsatellites to detect allele drop out of HBB gene (Fiorentino, *et al.,* 2006).

Marker Name	***Primer Sequence (5'→3')***
D11S4146	F: AAAAACACGAGGTTAAGCAGAG
	R: CTACCAAACATGATTCCTAGGA
D11S988	F: CACAGAAAATAGTTCAGACCACCAT
	R: CTCATCACAAAAAGTGTCCAGAGAA
D11S4181	F: CTGGGCAACAAGAGTAAGTCTCT
	R: CAACACTAAACATCCAGCTCAAAG
D11S4149	F: TGAATTATACCCCTGACCAATCTG
	R: CCATATATAGAATCACACTGGCCAA
D11S1331	F: TTCCATGAGAGGATACTGACTTTG
	R: CTTCCTTCGTCTTTCTCACTTTTAC
D11S1997	F: TTCCTAAGAAAGATAAAGCACCAG
	R: GGACAAAATAAAGACCAGCTTTAC

Table 3.13. List of the fluorescently labeled inner primers for microsatellites to detect allele drop out of HBB gene (Fiorentino, *et al.,* 2006).

Marker Name	***Primer Sequence (5'→3')***	***5' Fluorescent Dye Label***
D11S4146	F: GGTTAAGCAGAGTTTAATAGGC	6-FAM
	R: CTACCAAACATGATTCCTAGGA	
D11S988	F: CACAGAAAATAGTTCAGACCACCAT	HEX
	R: TGGGACAAGAGAAAGTTGAACATAC	
D11S4181	F: CTGGGCAACAAGAGTAAGTCTCT	TAMRA
	R: CCTTAAGAACTGAGACCAAGAACA	
D11S4149	F: GGCTAAAAAGGCAACAGATAACATC	FAM
	R: CCATATATAGAATCACACTGGCCAA	
D11S1331	F: GATGTTTAGATGCACAAGACACAGA	HEX
	R: CTTCCTTCGTCTTTCTCACTTTTAC	
D11S1997	F: TTCCTAAGAAAGATAAAGCACCAG	TAMRA
	R: CAATTGACAGTGGATTTTTGAC	

Table 3.14. List of the external oligonucleotide primers to amplify exon two of HLA-A and HLA-DRB1 gene in single cells (Verlinsky *et al.,* 2001).

Region	***Primer Sequence (5'→3')***	***Annealing Temperature (°C)***
HLA-A	F: GCCCCGAACCCTCCTTCCTGCTA	55
	R: CCGTGGCCCCTGGTACCCGT	
HLA-A	F: GGCCTCTGTGGGGAGAAGCAA	55
	R: GTCCCAATTGTCTCCCCTCCTT	
HLA-DRB1	F: CCGGATCCTTCGTGTCCCCACAGCACG	55
	R: CCGCTGCACTGTGAAGCTCT	

Table 3.15. List of sequence specific primers to amplify the most common HLA-A alleles in single cells (Browning *et al.,* 1993)

HLA-A Allele	***Primer Sequence (5'→3')***	***Annealing Temperature (°C)***
A*1	F: ACAGACTGACCGAGCGAA	55
	R: CTCCAGGTAGACTCTCCG	
A*2	F: GACGGGGAGACACGGAAA	55
	R: CAAGAGCGCAGGTCCTCT	
A*3	F: CGGAATGTGAAGGCCCAG	55
	R: CACTCCACGCACGTGCCA	
A*26	F: ACAGACTGACCGAGCGAA	55
	R: TGTAATCCTTGCCGTCGTAA	
A*11	F: CGGAATGTGAAGGCCCAG	55
	R: TCTCTGCTGCTCCGCCG	
A*28	F: CGGAATGTGAAGGCCCAG	55
	R: CAAGAGCGCAGGTCCTCT	
A*29	F: ACAGACTGACCGAGCGAA	55
	R: GCGCAGGTCCTCGTTCAA	
A*30	F: CGGAATGTGAAGGCCCAG	55
	R: TCTCAACTGCTCCGCCCA	
A*32	F: CCTGCGGATCGCGCTCC	55
	R: GCGCAGGTCCTCGTTCAA	

Table 3.16. List of sequence specific primers to amplify HLA-DRB1 alleles in single cells (Gillespie *et al.,* 2000 and Voorter, C. and E. van den Berg-Loonen, 2000).

HLA-DRB1 Alelle	***Primer Sequence (5'→3')***	***Annealing Temperature (^oC)***
DRB1*01	F: TTGTGGCAGCTTAAGTTTGAAT	62
	R: CTCGCCGCTGCACTGTGAAG	
DRB1*03	F: GTTTCTTGGAGTACTCTACGTC	62
	R: CTCGCCGCTGCACTGTGAAG	
DRB1*04	F: GTTTCTTGGAGCAGGTTAAACA	62
	R: CTCGCCGCTGCACTGTGAAG	
DRB1*07	F: CCTGTGGCAGGGTAAGTATA	62
	R: CTCGCCGCTGCACTGTGAAG	
DRB1*11	F: GTTTCTTGGAGTACTCTACGTC	62
	R: CTCGCCGCTGCACTGTGAAG	
DRB1*12	F: AGTACTCTACGGGTGAGTGTT	62
	R: CTCGCCGCTGCACTGTGAAG	
DRB1*13	F: GTTTCTTGGAGTACTCTACGTC	62
	R: CTCGCCGCTGCACTGTGAAG	
DRB1*14	F: GTTTCTTGGAGTACTCTACGTC	62
	R: CTCGCCGCTGCACTGTGAAG	
DRB1*15	F: TTCCTGTGGCAGCCTAAGAGG	62
	R: CTCGCCGCTGCACTGTGAAG	
DRB1*16	F: TTCCTGTGGCAGCCTAAGAGG	62
	R: CTCGCCGCTGCACTGTGAAG	

Table 3.17. List of sequence specific hybridization probes to detect the most common HLA-A alleles in single cells at real-time PCR platform.

HLA-A Allele	***Sequences of hybridization probes (5'→3')***	***Fluorescent Label***	***Tm ^oC***
A*01	PR: GACGGGCGCTTCCTCCGCG	3'FITC	74
	PR: GTACCGGCAGGACGCCTACGAC	5' LC RED 640	69
A*02	PR: AACCAGAGCGAGGCCGGTTCTCA	3'FITC	72
	PR: ACCGTCCAGAGGATGTATGGCT	5' LC RED 705	64
A*30	PR: TCCGCGGGTATGAACAGCAC	3'FITC	66
	PR: CCTACGACGGCAAGGATTACATCGCCCTGAA	5' LC RED 640	77

Table 3.17. List of sequence specific hybridization probes to detect the most common HLA-A alleles in single cells at real-time PCR platform (continued).

HLA-A Allele	*Sequences of hybridization probes (5'→ 3')*	*Fluorescent Label*	*Tm ^{o}C*
A*03 & A*11	PR: CCTACGACGGCAAGGATTACATCGCCCTGAA	5' LC RED 640	77
	PR: CCGCGGGTACCGGCAGGAC	3'FITC	72
A*26	PR: GACGGTTCTCACACCATCCAGA	3'FITC	64
	PR: GATGTATGGCTGCGACGTGGG	5' LC RED 705	68
A*32	PR: ATGTATGGCTGCGACGTGGGGCC	3'FITC	74
	PR: GACGGGCGCCTCCTCCGCGGGTA	5' LC RED 640	80

Table 3.18. List of sequence specific hybridization probes to detect the most common HLA-DRB1 alleles in single cells at real-time PCR platform.

HLA-DRB1 Alelle	*Sequences of hybridization probes (5'→ 3')*	*Fluorescent Label*	*Tm ^{o}C*
DRB1*11	PR: TCTATAACCAAGAGGAGTACGTGCGCT	3'FITC	66
	PR: CGACAGCGACGTGGGGGAGTTCCG	5' LC RED 640	77
DRB1*03	PR: CGACAGCGACGTGGGGGAGTTCCG	5' LC RED 640	77
	PR: TAACCAGGAGGAGAACGTGCGCT	3'FITC	68
DRB1*14	PR: GGTGACGGAGCTGGGGCGGC	3'FITC	75
	PR: TGCTGCGGAGCACTGGAACA	5' LC RED 705	68
DRB1*15	PR: CGGCCTGACGCTGAGTACTGGAACAGC	3'FITC	74
	PR: AGAAGGACATCCTGGAGCAGGC	5' LC RED 705	66
DRB1*07	PR: AGTCCTGGAACAGCCAGAAGGACAT	3'FITC	67
	PR: CTGGAGGACAGGCGGGGCCAGGT	5' LC RED 640	77
DRB1*04	PR: AGCGCCGAGTACTGGAACAGCCA	3'FITC	71
	PR: AAGGACCTCCTGGAGCAGAGGCGGGC	5' LC RED 705	77
DRB1*01	PR: AGCCAGAAGGACCTCCTGGAGCA	3'FITC	69
	PR: AGGCGGGCCGCGGTGGACA	5' LC RED 640	77

Table 3.19. List of external oligonucleotide primers for microsatellites located in HLA locus (Fiorentino, *et al.,* 2004).

Marker Name	***Primer Sequence (5'→3')***
D6S276	F: GTGTACACATCAATCAAATCATC
	R: CCTCTTCAGTAGTTTTGCTACAG
D6S291	F: CTCAGAGGATGCCATGTCTAAAATA
	R: GGGGATGACGAATTATTCACTAACT
D6S265	F: CCTTCTATCTGACTGTACGTTCGT
	R: AGCTTTAAAAGGTTGATCTAATCG
D6S2792 (TNFa)	F: ATGCAAGAAGGGTAAAGCCTCTAG
	R: TTTTGAGGTTGCAGTGGGCTAT
D6S2874 (TAP1CA)	F: CTGTTCATATCCTCATACATCTGCT
	R: GTTTTTCTTAAGGTAAGGAGGACAA
D6S510	F: AACACACTGATTTCCATAGCAGGT
	R: CTGCAATGGGCTACTACTTCACA
82-1	F: ATTACAAGGGCTTTAGGAGGCTG
	R: AGGTCAAAGCTGCAGTAGCCAT
D6S2876 (G51152)	F: AGACAGCTCTTCTTAACCTGCC
	R: GGTAAAATTCCTGACTGGCCT
LH-1	F: GAACAGAGACAATGCCTAGAACC
	R: ACCTTACTGGGCACAAATTCAC
D6S2972 (MOG-CA)	F: ACCAGGCTAGGATCTGTCACA
	R: TTGACCATGGGTAACTGAAGC
D6S2883 (DRA-CA)	F: ACTTTCCTAATTCTCCTCCTTC
	R: CATCCATGTAAAACAGAGACC
D6S439	F: GGCTTCACAACTTTGGCACTAC
	R: CAGCCTCAGGGAAGACACATT
D6S2810 (MIB)	F: ATCCTGCCTCAGAATTAGAACATC
	R: TCTAGAACCACTCTTCGTACCACA
D6S2811 (HLABC-CA)	F: GTCAAGCATATCTGCCATTTGG
	R: GCCCAGTAGTAAGCCAGAAGCTA

Table 3.20. List of fluorescently labeled inner primers for microsatellites located in HLA locus (Fiorentino, *et al.,* 2004).

Marker	***Primer Sequence (5'→3')***	***5' Fluorescent Dye Label***	***Size range (bp)***
D6S276	F: TCAATCAAATCATCCCCAGAAG	HEX	190-220
	R: GGGTGCAACTTGTTCCTCCT		
D6S291	F: GTCTAAAATATCCATCCGGCAT	6-FAM	156-166
	R: TTAATTGTGGTGATGGTTTCAC		
D6S265	F: TCGTACCCATTAACCTACCTCTCT	6-FAM	110-125
	R: TCGAGGTAAACAGCAGAAAGATAG		
D6S2792	F: GCCTCTAGATTTCATCCAGCCAC	6-FAM	97-121
	R: CCTCTCTCCCCTGCAACACA		
D6S2874	F: TCATACATCTGCTTTGATCTCCC	TAMRA	195-215
	R: GGACAATATTTTGCTCCTGAGG		
D6S510	F: TTTGTCTTTCCCAATGTACTACAC	HEX	140-155
	R: GCTACTACTTCACACCAATTAGGA		
82-1	F: GAGCCAGGATGGAGACCAAA	6-FAM	100-122
	R: CCTGGATAACAGAACGAGACCC		
D6S2876	F: GGAAAAGAGCTCACGCACAT	TAMRA	145-158
	R: CCTGCCATCATGACTTCAAG		
LH-1	F: GCTAGTCTGTGCCAAGGAACTC	6-FAM	126-160
	R: ACCTTACTGGGCACAAATTCAC		
D6S2972	F: AGATCACCTCGAGTGAGTCTCTT	6-FAM	205-235
	R: TTGACCATGGGTAACTGAAGC		
D6S2883	F: ACTTTCCTAATTCTCCTCCTTC	HEX	122-140
	R: GCATGAGTAAACTATGGAATCTC		
D6S439	F: CCCCTATTCTCCACCCACTAGA	TAMRA	116-130
	R: CAGCCTCAGGGAAGACACATT		
D6S2810	F: CGTTTTCAGCCTGCTAGCTTAT	HEX	155-186
	R: CCACAGTCTCTATCAGTCCAGATTC		
D6S2811	F: GTCAAGCATATCTGCCATTTGG	HEX	113-144
	R: ACTTGGGCAATGAGTCCTATGA		

3.7. DNA Molecular Weight Markers and Size Standards

GeneRuler™ 100 bp DNA ladder was purchased from Fermentas, Canada. GS500 ROX size standard was purchased from Applied Biosystems, USA.

3.8. Equipment

All the equipments used throughout this study was given with their models and manufacturers in table 3.21.

Table 3.21. List of equipments used in this study.

Equipment	***Model***	***Manufacturer***
Autoclave	Harvey SterileMax™ Steam Sterilizers	Barnstead International, USA
Balance	Adventurer Pro	Ohaus, Switzerland
Centrifuge	MiniSpin Plus	Eppendorf, Germany
Centrifuge	Labofuge 400R Function Line	Heraeus, Germany
Centrifuge	Biofuge pico	Heraeus, Germany
Centrifuge	Biofuge fresco	Heraeus, Germany
Centrifuge for plates	Allegra X-22	Beckman Coulter, USA
Cooler	Sample Cooler SC-2	Biosan, Latvia
Deep Freezer	-20^{o}C deep freezer	Arçelik, Turkey
Deep Freezer	-80^{o}C VIP PLUS MDF-C8V1	SANYO, USA
DNA Isolation Instrument	MagNA Pure LC Instrument	Roche, Germany
Documentation System	GelDoc Documentation System	Molargen
Electrophoretic Equipment	Horizon 11.14 Electrophoresis tank	Whatmann, UK
Heater	Thermoblock TDB-120	Biosan, Latvia

Table 3.21. List of equipments used in this study (continued).

Equipment	***Model***	***Manufacturer***
Heater	ECHO Therm MODEL IC20	Torrey Pines Scientific, USA
Magnetic Stirrer	MSH 300	Biosan, Latvia
Magnetic Stirrer	ARED	VELP Scientifica, Italy
Micropipettes	Pipetman Ultra	Gilson, France
Microwave Oven	MD1500	BEKO, Turkey
pH Meter	Inolab pH Level 1	WTW, Germany
Power Supply	Electrophoresis Power Supply Model 250 EX	GIBCO BRL Life Technologies, USA
Refrigerator	+ 4^{o}C refrigerator	Arçelik, Turkey
Sequencer	ABI PRISM ABI 3100 Genetic Analyzer	Applied Biosystems, USA
Sequencer	MegaBACE 1000	Amersham Biosciences, USA
Shaker	Maxi-Mix III™ Type 65800	Barnstead/Thermolyne, USA
Spectrophotometer	Ultraspec 3100 pro UV/Visible spectrophotometer	Amersham Biosciences, USA
Thermal Cycler	DNAEngine	Bio-Rad, USA
Thermal Cycler	GeneAmp PCR System 9700	Applied Biosystems, USA
Thermal Cycler	LightCycler 2.0	Roche, Germany
Thermal Cycler	PTC-200	MJ Research, USA
Thermal Cycler	Veriti	Applied Biosystems, USA
Thermal Cycler	XPCycler	BIOER, China
Vacuum Pump	Vacuum pump	KNF Neuberger, Germany

Table 3.21. List of equipments used in this study (continued).

Equipment	*Model*	*Manufacturer*
Vortex	Bio Vortex V1	Biosan, Latvia
Vortex	Zx^3	VELP Scientifica, Italy
Water Bath	Boekel Grant PB-600	Boekel Scientific, USA

3.9. Electronic Databases

A list of the electronic databases used in this study is given in table 3.22 with a brief description.

Table 3.22. List of electronic databases.

Name	*Description*
NCBI Gene *http://www.ncbi.nlm.nih.gov/gene*	A searchable database of genes from RefSeq genomes
Basic Local Alignment Search Tool *http://blast.ncbi.nlm.nih.gov/Blast.cgi*	It finds regions of local similarity between biological sequences.
dbMHC *http://www.ncbi.nlm.nih.gov/gv/mhc/main.cgi?cmd=init*	It provides a publicly accessible platform for DNA and clinical data related to the human Major Histocompatibility Complex (MHC).
IMGT/HLA Database *http://www.ebi.ac.uk/imgt/hla/*	A specialist database for sequences of the human major histocompatibility complex (HLA).

4. METHOD

4.1. DNA Extraction from Peripheral Blood Samples

The peripheral blood was taken into the tubes containing EDTA which is used as an anticoagulant. The genomic DNA was extracted from at least 200 µl blood using either High Pure PCR Template Preparation Kit with the manual procedure or DNA Isolation Kit I in MagNA Pure LC Instrument, following the manufacturer's instructions. For the manual procedure, the blood cells were lysed in 200 µl guanidine-HCl containing lysis buffer and 40 µl proteinase K at 70^{o}C for ten minutes. Afterwards, the column-bound DNA was washed with ethanol containing the washing buffers in order to remove impurities by centrifugation. Finally, the purified DNA was eluted from the column using 200 µl of the provided elution buffer.

The concentration of the genomic DNA was determined spectrophotometrically. The average DNA concentration was determined in the range of 30-100 ng/µl. DNA samples were stored at +4 °C until further use.

4.2. ICSI, Embryo Biopsy Procedure and Cell Lysis

After oocyte retrieval, intracytoplasmic sperm injection (ICSI) was carried out by embryologist in the embryology unit as previously described (Joris *et al.,* 1998). ICSI was preferred to avoid contamination with the sperms on the fertilized egg and to increase the fertilization rate. At the third day of fertilization, one blastomere was removed from each of the embryos with six-nine cells and transferred into a sterile well of a PCR plate containing ten microliters of the blastomere lysis buffer. Two negative controls were prepared from the wash drops in order to control contamination. Proteinase K / SDS lysis took place at 65 °C for 35 minutes followed by denaturation at 95 °C for 35 minutes to prevent the enzyme activity in future analysis.

4.3. Analysis by Agarose Gel Electrophoresis

The size of the DNA samples or the PCR products were analyzed electrophoretically by running on 2% agarose gels prepared in 0.5X TBE (Tris-Boric acid-EDTA) buffer. TBE buffer (300 ml) containing six gram agarose was melted in the microwave oven. For the detection of bands under ultraviolet (UV) light, ethidium bromide was added to a final concentration of 0.5 μg/ml. The solution was poured onto the electrophoresis plate and left to polymerize at room temperature. After polymerization, the gel plate was placed into an electrophoresis chamber containing 0.5X TBE buffer. Ten microlitres of the PCR products were loaded on the gel after mixing with two microlitres of 6X loading dye. The samples were run at 100V, for 30-40 minutes. The gels were, then, analyzed under UV_{254nm} light and the images were saved to the computer.

4.4. Multiplex PCR to Amplify Different Regions Simultaneously in Single Cells

Different genetic regions could be amplified in the single cells by multiplex PCR. The multiplex PCR contain primer pairs for more than two target regions. Therefore, the second exon of the HLA-A gene, the second exon of the HLA-DRB1 gene and the mutation sites of the HBB gene were selected to amplify in the multiplex PCR. The reaction was carried out by using the primer pairs given in table 3.14. and table 3.6., respectively. A 37 μl aliquot of mastermix containing 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P40, 2 mM $MgCl_2$, 200 μM of dNTP mix, 20 pmol of the outer primers specific to the beta globin gene, the second exon of the HLA-DRB1 and the HLA-A genes. 2.5U T*aq* DNA Polymerase (Fermentas) and PCR grade water to a volume of 50 μl was added into wells containing the lysed single cells. The PCR plate was placed into a PTC-200 DNA engine thermal cycler and it was cycled as given in table 4.1.

After the initial amplification, the reaction was followed with several nested amplifications for the detection of each of the maternal and the paternal HLA alleles and further amplification of the beta-globin gene for mutation analysis.

Table 4.1. Conditions of initial multiplex amplification in single cells

Step	***Temperature***	***Duration***	***Number of cycles***
Initial denaturation	96^{o}C	3 minutes	1 cycle
Denaturation	94 ^{o}C	30 seconds	43 cycles
Annealing	55 ^{o}C	60 seconds	
Extension	72 ^{o}C	90 seconds	
Final extension	72 ^{o}C	10 minutes	1 cycle

4.5. PCR Amplification of Short Tandem Repeats

The PCR amplification of the short tandem repeats (STRs) in the HLA complex and the linked microsatellites to the HBB gene were designed as multiplex, fluorescent and nested PCR. The microsatellites were selected according to their previously reported heterozygosity rates. The microsatellites distributed through the HLA region and linked to the HBB gene were studied initially in the parents and the children to select the informative microsatellites in order to use in the blastomeres. The heterozygous microsatellites were determined to assess the inheritance of the maternal and the paternal alleles in the single cells. The HLA and the HBB related microsatellites of the couples and their child and/or children were amplified directly with the fluorescently labelled inner primers. However, the successful amplification could be detected only by the nested PCR strategy in the single cells. Therefore, the informative microsatellites selected previously were amplified with the outer primers at first. The outer and the nested primers for the HLA STRs and the HBB linked STRs used in this study were published previously (Fiorentino *et al.,* 2004, Fiorentino *et al.,* 2006). The outer primers for all the microsatellites were listed in tables 3.12. and 3.19. The location of the HLA microsatellites used in this study is shown in figure 4.1.

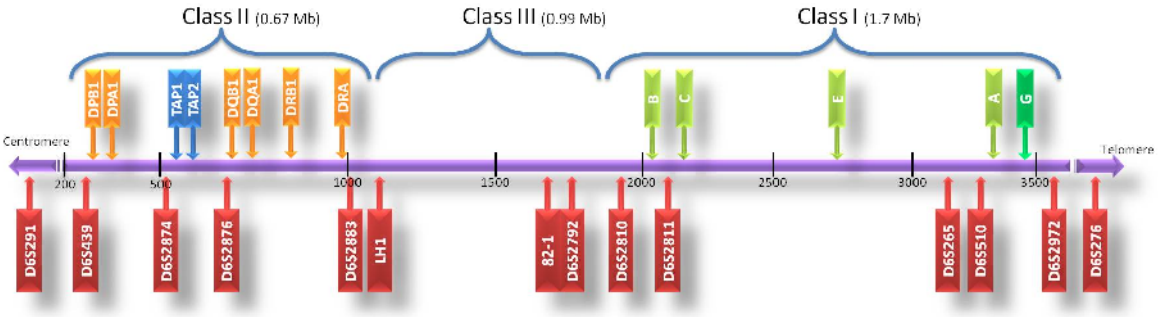

Figure 4.1. The distribution of the microsatellites throughout the HLA region and the location of the HLA class I and class II genes. The class I, the class II genes and microsatellites were represented with green, orange and red arrows, respectively .

After the complete blastomere lysis, a multiplex PCR was done to amplify all the selected loci. The multiplex outer PCR contained the external primers for the selected informative HLA microsatellites, the gene region containing the mutation site and the microsatellites linked to this region to detect the allele drop out (ADO). The outer multiplex PCR reaction mix in 50 µl contained 1X Colorless GoTaq Flexi Buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP); 0.4 - 1 µM of each external primer, and 1.75 U of T*aq* Polymerase (Go Taq Flexi DNA Polymerase). The PCR mix was added into the wells containing ten microlitres lysed single cells. The reaction was covered with the mineral oil. The PCR reactions were performed using DNA Engine PTC-200 Peltier Thermal cycler. The PCR cycles were given in table 4.2.

Table 4.2. Cycling conditions for the multiplex amplification of the target regions with outer primers in the single cells.

Step	***Temperature***	***Duration***	***Number of cycles***
Initial denaturation	94°C	4 minutes	1 cycle
Denaturation	94 °C	30 seconds	35 cycles
Annealing	60 °C	30 seconds	
Extension	72 °C	30 seconds	
Final extension	72 °C	10 minutes	1 cycle

In order to accomplish the amplification and the haplotyping, 5 µl of PCR purified primary PCR products from the single cells or 5 µl of genomic DNA were added to a new well containing 5 µl of 10X PCR Buffer II (500 mmol/l KCl, 100 mmol/l Tris-HCl, pH 8.3; Applied biosystems), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 2.5U AmpliTaq Gold Polymerase (Applied Biosystems), 20 pmol of each inner primers and DNase free distilled sterile water to a total volume of 50µl. The inner STR primers and their specifications were listed in tables 3.13. and 3.20. The forward inner primers in this reaction were labelled with either of the FAM, HEX or TAMRA dyes. Since different dye labels and different size ranges allowed us to amplify and analyze two or more microsatellites simultaneously, the nested PCRs were designed as multiplex PCR. The heminested PCR strategy was used for D11S4146, D11S4149, D11S1331, D6S2883, D6S439, D6S2811, D6S2972 and LH-1 markers. The PCR reactions were carried out using either DNA Engine PTC-200 Peltier Thermal cycler or GeneAmp PCR System 9700. The inner PCR cycles were given in table 4.3.

Table 4.3. Conditions for multiplex, fluorescent and nested amplification of the target regions with the inner primers in the single cells.

Step	***Temperature***	***Duration***	***Number of cycles***
Initial denaturation	95°C	12 minutes	1 cycle
Denaturation	95 °C	35 seconds	35 cycles
Annealing	52 °C	40 seconds	
Extension	65 °C	35 seconds	
Final extension	65 °C	1 hour	1 cycle

The final extension was applied for one hour at 65 °C in order to reduce the plus-A effect which could lead to misgenotyping of the microsatellites.

4.6. Capillary Analysis of Microsatellites

For haplotyping analysis, 0.5 µl of each dye-labelled PCR product was mixed with 0.5 µl of GS500 ROX (Applied Biosystems) size standard and ten microlitres of Hi-Di Formamide (Applied Biosystems) in 96-well plate. The samples were denatured at 95 °C

for three minutes and cooled on ice for one minute. The samples were run on an automated DNA Sequencer ABI Prism 3100 (Applied Biosystems). The samples were injected to the capillaries for 22 seconds under 1kV injection voltage. The samples were run on 36-cm capillaries filled with Performance Optimized Polymer–4 (POP4) (Applied Biosystems). The peak signals were analyzed and interpretted with GeneMapper v3.5 Analysis Software (Applied Biosystems). The fragment sizes were automatically determined by the software.

4.7. Real-Time PCR for Typing of HLA Alleles

The hybridization probes were designed between the sequence specific primers to type the selected common HLA alleles. The sequences of the amplification primers and the hybridization probes were given in tables 3.15., 3.16., 3.17., and 3.18. The sequence specific primers for the HLA-A gene were published previously (Browning *et al.,*1993). The primers for the HLA-DRB1 alleles were selected from the previous publications (Gillespie *et al.,* 2000, Voorter, C. and E. van den Berg-Loonen, 2000). The hybridization probes which were specific to the polymorphic sequences were designed in this study. The real-time PCR was performed in a total volume of 20 µl containing either 1-5 µl of purified amplicon from the initial multiplex PCR. In addition, the reaction mixture contained 2µl of 10X LightCycler® DNA Master HybProbes containing T*aq* DNA polymerase, reaction buffer, dNTP mixture, and 10 mM $MgCl_2$, 2mM $MgCl_2$, 0.5 µM each forward and reverse primers, 0.2 µM hybridization probes. The reaction was performed in the LightCycler® 2.0 instrument and the results were analyzed in the LightCycler Software version 4.0. The temperature transition rate for all the steps of the PCR cycles was 20 ^{o}C / s. The PCR cycles for the HLA-A and the HLA-DRB1 alleles were given in the table 4.4. The annealing temperatures for the HLA-A and the HLA-DRB1 alelles were 55 ^{o}C and 62 ^{o}C, respectively. The amplified PCR fragments were subjected to the melting curve analysis to determine the melting temperature of each allele. The melting conditions were given in table 4.4.

Table 4.4. Amplification and melting curve conditions for detection of HLA alleles.

Step	***Temperature***	***Duration***	***Temrepature transition rate***	***Number of cycles***
Initial denaturation	95^{o}C	45 seconds	20 ^{o}C / s	1 cycle
Denaturation	95 ^{o}C	10 seconds	20 ^{o}C / s	50 cycles
Annealing	55 ^{o}C / 62 ^{o}C	10 seconds	20 ^{o}C / s	
Extension	72 ^{o}C	10 seconds	20 ^{o}C / s	
Denaturation	95 ^{o}C	0 second	20 ^{o}C / s	1 cycle
Annealing	45 ^{o}C	2 minutes	20 ^{o}C / s	
Target Temperature	85 ^{o}C	0 second	0.2 ^{o}C / s	

For the amplification, ‘‘single’’ was selected as fluorescence acquisition mode and for the melting curve analysis, ‘‘continuous’’ was selected. An appropriate detection channel was selected due to the fluorophores of the hybridization probes used in the reaction.

4.8. Real-time PCR for Common Beta-Thalassemia Mutations

The primers and the hybridization probes which had already been designed for the common beta-thalassemia mutations were used (Vrettou at al., 2003). These mutations were codon 5 (-CT), codon 6 (-A), codon 6 (A>T), codon 8 (-AA), codon 8/9 (+G), IVS I:1 (G>A), IVS I:5 (G>T) and IVS I:6 (T>C). The real-time PCR primers and the hybridization probes for the other common mutations in the Turkish population such as IVS I:110 (G>A), IVS II:1 (G>A) and IVS II:745 (C>G) were designed in the scope of this study. The PCR reactions for all the beta-thalassemia mutations listed here were prepared as described in the section 4.8 “Real-time PCR for typing of HLA alleles”.

The cycling and melting curve conditions varied due to the melting temperatures of the primer pairs. The annealing temperature for each primer pair was given in tables 3.14 and 3.15. The temperature transition rate for all steps of the PCR cycles was 20 ^{o}C / s. In general, the cycling conditions were given in table 4.5.

Table 4.5. Amplification and melting curve conditions for detection of common beta-globin mutations.

Step	***Temperature***	***Duration***	***Temrepature transition rate***	***Number of cycles***
Initial denaturation	94^{o}C	16-60 seconds	20 ^{o}C / s	1 cycle
Denaturation	95 ^{o}C	3-10 seconds	20 ^{o}C / s	35 - 50 cycles
Annealing	55 - 58 ^{o}C	5-50 seconds	20 ^{o}C / s	
Extension	72 ^{o}C	10 – 20 seconds	20 ^{o}C / s	
Denaturation	95 ^{o}C	0 second	20 ^{o}C / s	1 cycle
Annealing	45 ^{o}C	2 minutes	20 ^{o}C / s	
Target Temperature	85 ^{o}C	0 second	0.15 – 0.3 ^{o}C / s	

The temperature transition rate from 45 ^{o}C to 85 ^{o}C varied for different mutations. The rates were 0.15 ^{o}C / s, 0.25 ^{o}C / s and 0.3 ^{o}C / s for codon 8/9 (+G), IVS I:110 (G>A), and IVS II:745 (C>G), respectively. For the other mutations, the transition rate was applied as 0.2 ^{o}C / s.

4.9. Nested PCR for Beta-Globin Gene (HBB)

Initially, sequencing of the whole HBB gene of the affected individual (child) was carried out to identify the beta-thalasssemia mutations. Afterwards, the presence of the mutations in the parents were confirmed by the targetted mutation analysis. The primers were designed in order to amplify the HBB gene in four overlapping fragments. These primers and their sequences were given in table 3.12. with the annealing temperatures. In the single cell studies, a 200-500 bp region containing the mutation sites was targetted to amplify rather than amplifying the whole beta globin gene. Therefore, the alternative outer and inner primers were designed for this strategy.

The amplification of the beta-globin gene was performed for the rare mutations of beta-thalassemia in order to analyze the mutations by sequencing. The PCR products were sequenced to determine the genotype of the embryo. Seven microlitres of ExoSAP-IT treated aliquot from the initial multiplex PCR was added to a 43 µl of reaction mix containing 1X Flexi PCR buffer, 1.5 mM $MgCl_2$, 200 µM of dNTP mix, 20 pmol of the inner forward and reverse primers for the beta-globin gene, 1.25 U T*aq* DNA polymerase (Go T*aq* Flexi DNA Polymerase). Although the primer pairs were different, all the PCR reactions were prepared as described here. The PCR cycles for the beta-globin gene were given in table 4.6.

Table 4.6. Amplification conditions for fragments of beta-globin gene.

Step	***Temperature***	***Duration***	***Number of cycles***
Initial denaturation	96^{o}C	2 minutes	1 cycle
Denaturation	94 ^{o}C	30 seconds	35 cycles
Annealing	60 ^{o}C	30 seconds	
Extension	72 ^{o}C	45 seconds	

The PCR products were electrophoresed on ethidium bromide stained 2% agarose gels in 0,5X TBE (Tris, EDTA) in order to qualify the success of the amplification. The amplified products were treated with ExoSAP-IT in order to purify prior to downstream sequencing analysis.

4.10. Conventional Amplification of HLA-A and HLA-DRB1 Alleles

The HLA-A and HLA-DRB1 allele typings were done by using the sequence specific primers listed in table 3.15 and table 3.16, respectively. Five microlitres aliquot from the initial multiplex PCR was added to 45 µl of the reaction mix containing 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P40, 1.5 mM $MgCl_2$, 200 µM of dNTP mix, 20 pmol of the forward and the reverse primers, 2U T*aq* DNA polymerase (Fermentas). The PCR cycles for the HLA-DRB1 and HLA-A allel were given in table 4.7.

Table 4.7. Amplification conditions of HLA-A and HLA-DRB1 alleles.

	Step	***Temperature***	***Duration***	***Number of cycles***
HLA-DRB1	Initial denaturation	96°C	3 minutes	1 cycle
	Denaturation	94 °C	30 seconds	40 cycles
	Annealing	62 °C	60 seconds	
	Extension	72 °C	60 seconds	
HLA-A	Initial denaturation	96°C	2 minutes	1 cycle
	Denaturation	94 °C	30 seconds	40 cycles
	Annealing	55 °C	60 seconds	
	Extension	72 °C	30 seconds	

The PCR products were electrophoresed on ethidium bromide stained 2% agarose gel in 0,5X TBE buffer. The samples with ambiguous or co-amplifying alleles were subjected to the PCR purification and DNA sequencing in order to distinguish the alleles.

4.11. Cyclesequencing of HBB Gene and HLA Alleles

The mutation containing regions of the HBB gene and the second exon of amplified alleles of the HLA-A and HLA-DRB1 genes were cyclesequenced. The cyclesequencing reaction was performed by using DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare). Two microlitres of ExoSAP-IT treated PCR products were added to the reaction mix containing five pmol primers, two microlitres of sequencing reagent premix and two microlitres halfTSII dilution buffer (Genetix, UK) and water to a total reaction volume of 10 µl. The reaction was performed by both the amplification primers, separately in order to obtain bidirectional sequence. The cyclesequencing was performed in XPCycler Thermocycler. The cycling conditions were given in table 4.8.

Table 4.8. Cycling conditions for sequencing reaction.

Step	***Temperature***	***Duration***	***Number of cycles***
Denaturation	95 ^{o}C	20 seconds	25 cycles
Annealing	50 ^{o}C	20 seconds	
Extension	60 ^{o}C	60 seconds	

4.12. Dye Removal from Sequencing Reactions

After the cyclesequencing, the unbound dyes were removed by using Montage SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore). Thirty microlitres of wash buffer was added into each well containing ten microlitres sequencing reaction. The total volume was pipetted into the wells of the Montage SEQ_{96} plate by a multi-channel pipette. Eight hundred mbar pressure was applied for five minutes in order to elute the unbound excess dyes from the sequencing reaction by the vacuum pomping. Thirty microlitres wash buffer was added once more and the vacuum pressure was applied, again. Then, twenty microlitres of injection buffer was added into the wells and mixed for ten minutes by a shaker. Finally, all the purified reaction was taken from the wells of the Montage SEQ_{96} plate and pipetted into the wells of the plate which was loaded into the sequencer.

4.13. Sequencing and Analysis of the Data

The purified products were electrophoresed and analyzed in the MegaBACE 1000 Genetic Analyzer. The samples were injected to the capillaries filled with the LPA matrix at 3 kV for 75 seconds. After the injection, the samples were run on the instrument at 7 kV for 100-140 minutes. The quality of the sequences were assessed initially in MegaBACE Sequence Analyzer, Professional, v4.0 software (Amersham Biosciences). The sequences were analyzed for the detection of mutations by using Lasergene Seqman II version 6.0 software (DNASTAR, Inc). The sequences of HLA alleles were analyzed for typing by NCBI BLAST.

5. RESULTS

5.1. Successful DNA Amplification From Single Cells: Detection of Polymorphic HLA-A and DRB1 Alleles by Multiplex PCR

The single cell genetic testing was applied to the blastomeres, which were biopsied from six-nine cell embryos, in order to determine the HLA-match and the genetic status of the embryo with regard to the mutations related to a certain genetic condition. In our study, beta-thalassemia and sickle cell disease were selected as a model of monogenetic disorders tested in the single cells, since both diseases are results of the mutations in the beta-globin gene (HBB).

Prior to the genetic testing in single cells or blastomeres, the HLA-DRB1 and HLA-A alleles of the parents and the children were amplified with sequence specific primers given in table 3.15 and table 3.16. The single blastomere was taken from each six-nine cell embryo at the day three of the fertilization by the laser assissted embryo biopsy. The HLA-A and HLA-DRB1 SSP typing was applied to the single blastomeres after an initial cell lysis step and a multiplex amplification of the HLA-A and DRB1 alleles as described in section 4.4.

In the initial multiplex PCR, the regions covering exon two of the HLA-A and DRB1 genes were amplified irrespective of the HLA alleles by primers targeting conserved sequences, although each family had distinct combinations of the HLA alleles. The HLA typing was accomplished after the amplification of the PCR product of the initial multiplex PCR by the using sequence specific primers. Since the DRB1 typing primers were working better in blastomeres and the total number of DRB1 alleles are limited when compared to the number of the HLA-A and HLA-B alleles, the DRB1 typing was preferred initially.

The informative DRB1 alleles of the blastomeres, parents and the child(ren) were amplified simultaneously with the sequence specific primers. After the amplification, the PCR products were run on 2% agarose gels. The positive amplification band on the agarose gel indicated that the cell inherited the amplified DRB1 allele. An empty lane

points out that either the cell does not harbor the allele or amplification could not be achieved due to the allele drop out or the amplification failure. Since the alleles were determined due to their amplification status, the recombination and the allele drop out could not be excluded. The results may lead to misdiagnosis or incomplete HLA typing. After determination of the HLA-DRB1 alleles, the informative HLA-A alleles were amplified with the sequence specific primers to confirm and support the HLA-match of the blastomeres and the child. DNA of parents and the children were studied with single blastomeres as positive and negative controls of the amplification of HLA alleles.

The following agarose gel images showed the amplification of distinct HLA-DRB1 and HLA-A alleles in the blastomeres, parents and child(ren). The strong and clear bands indicated sequence specific amplification of the HLA alleles. When the sample did not contain the targetted allele, the primers could not bind to the DNA and PCR amplification could not be obtained. As a result, no band was seen in the corresponding lane.

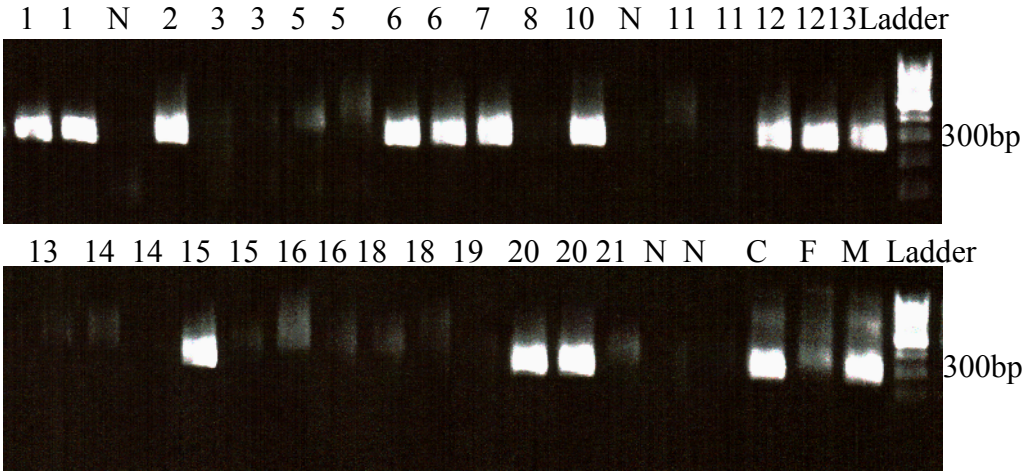

Figure 5.1. Agarose gel electrophoresis of PCR products obtained by using DRB1*15 specific primers. The bands showed the DRB1*15 positive samples. 100 bp DNA ladder was run in the last lanes. The approximate size of the amplified product was 300 bp.

The figure 5.1. presented amplified DRB1*15 allele in single cells, parents (M and F) and child (C). The blastomeres were enumerated according to their embryo number. Some of the embryos were studied in duplicates since two separate blatomeres were biopised from the same embryo. The alphabet “N” was denoted for negative controls.

Mother (M) and child (C) were positive for DRB1*15 allele, however, the father (F) did not carry this allele. Embryos 1, 2, 6, 7, 10, 12, 13, 15 and 20 were detected as DRB1*15 positive. The remaining embryos were found negative for this allele.

The figure 5.2 and the figure 5.3 presented the HLA-DRB1 typing results of the same blastomeres. The figure 5.2. presented amplification of the DRB1*04 allele in blastomeres. Mother and child were DRB1*04 positive and the father was determined as negative. In this case, single blastomeres were studied for each embryo. Blastomeres 3, 4, 6, 8, 9 and 11 were determined as positive for DRB1*04 allele. Blastomeres 1, 2, 5, 7, 12 were found as negative. A weak band was observed in the father. This was a nonspecific amplification product. Since the genomic DNA was used for parents, the sequence specific primers in the second round amplification bind to the sequences with low specificity after the initial amplification due to excess amount of genomic DNA.

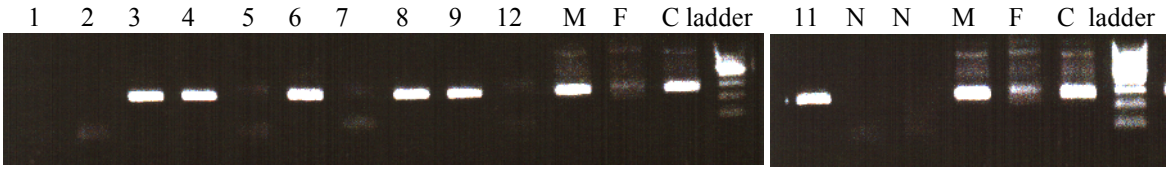

Figure 5.2. PCR products obtained by using DRB1*04 specific primers. Samples were run on 2% agarose gels. The bands showed the DRB1*04 positive samples. 100 bp DNA ladder was run in the last lanes. The approximate size of the amplified product was 300 bp.

The figure 5.3. demonstrated co-amplification of the DRB1*03 and DRB1*11 alleles. Since there is a high sequence homology between the DRB1*03 and DRB1*11 alleles, sequence specific primers could not distinguish these alleles and amplify them simultaneously. Both parents and child were found positive for amplification as seen in the gel image. Actually, father and child were positive for the DRB1*11 allele and mother was positive for the DRB1*03 allele. But the alleles could not be distinguished from the gel image. Except blastomere two, all the blastomeres were amplified simultaneously for either DRB1*03 or DRB1*11 allele. The discrimination of amplified alleles were only achieved by sequencing analysis. The HLA-DRB1 allele typing for co-amplifying alleles could be achieved by sequence based typing which was mentioned in the following section.

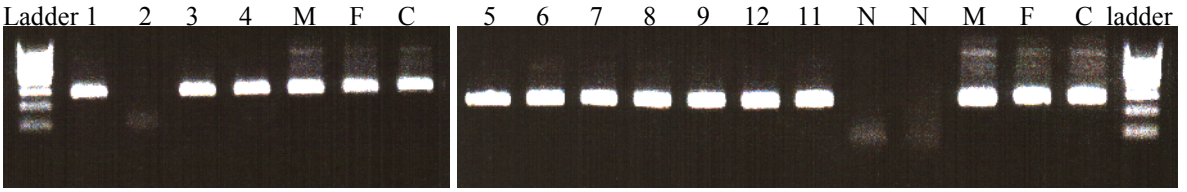

Figure 5.3. Agarose gel electrophoresis of PCR products obtained by using DRB1*03 and DRB1*11 specific primers. The samples were positive for either the DRB1*11 or DRB1*03 allele. The approximate size of the amplified product was 300 bp.

The HLA-A allele typing was applied directly after the HLA-DRB1 allele typing in the blastomeres. The strategy used for the HLA-A typing was similar with that of the HLA-DRB1 typing. The following gel images presented the amplification of the HLA-A alleles in blastomeres by using sequence specific primers.

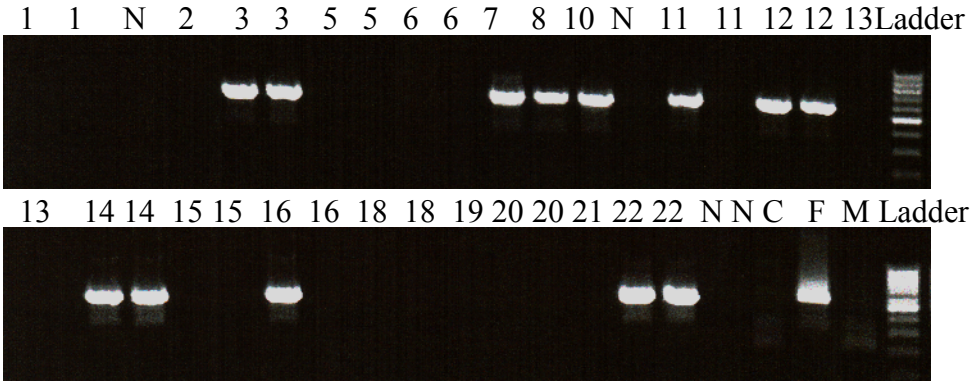

Figure 5.4. Agarose gel electrophoresis of PCR products obtained by using A*30 specific primers. The positive bands indicated specific amplification of the A*30 allele. The approximate size of the amplified product was 600 bp.

The HLA-A*30 allele was amplified in the blastomeres. The gel image for A*30 allele was presented in the figure 5.4. As seen in the image, the father was A*30 positive but the child and the mother were negative for this allele. The embryos 3, 7, 8, 10, 11, 12, 14, 16 and 22 were found positive for A*30 allele. Although the embryos 11 and 16 were studied in duplicates, successful amplification could be obtained from the first blastomeres of these embryos. The second blastomeres were found as negative. Amplification failure,

allele drop out or low nucleus quality of the blastomere could be the reason for the negative PCR result. The other blastomeres were qualified as negative for A*30 allele.

The figure 5.5 demonstrated the A*32 and A*02 alleles in blastomeres, respectively. The first six lanes demonstrated the amplifications of the A*32 allele and the remaining lanes presented the amplifications of the A*02 allele. The mother, the child and the embryo four were found A*32 positive. The father, the embryos two and three were evaluated as A*32 negative since no amplification was observed for these samples. The mother, father and the child were positive for the A*02 allele. The embryos two and four were found A*02 positive but the embryo three was negative for this allele.

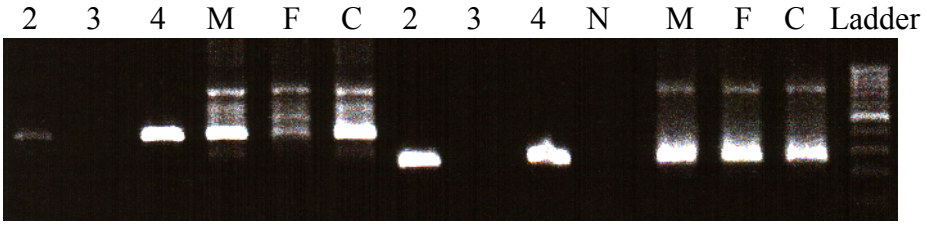

Figure 5.5. Agarose gel electrophoresis of PCR products obtained by using A*32 (the first six lanes) and A*02 (the second six lanes) specific primers. The approximate size of the A*32 and A*02 alleles were 500 and 300 bp, respectively.

The amplification of the HLA-A*01 allele in blastomeres was demonstrated in figure 5.6. The father and the child were A*01 positive. The mother was negative although a strong band was seen in lane M. Since the size of this band is shorter than that of the A*01 allele, it was considered as a non-specific amplification product. The blastomeres 3, 5, 11 and 14 were found A*01 positive. Blastomeres 1, 8, 9, 12 and 13 were negative for A*01 allele. The nonspecific strong band in the mother was caused by the nonspecific binding of the allele specific primers due to excess amount of DNA after an initial amplification of genomic DNA of the mother.

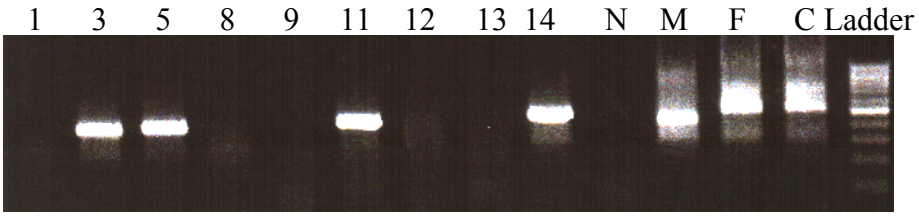

Figure 5.6. PCR products obtained by using A*01 specific primers. Samples were run on 2% agarose gels. The bands indicated A*01 positive samples. 100 bp DNA ladder was run in the last lane. The approximate size of the amplified product was 550 bp.

The HLA-typing of the single cells which were the blastomeres biopsied from the embryos were achieved by using the sequence specific primers. Different primer sets were used to amplify different HLA alleles.

The amplification of the HLA alleles was achieved in single cells which provided information from two genes with eight alleles simultaneously. Due to the time limitation of in vitro fertilization, a more rapid amplification and genotyping strategy has to be developed before embryos stop development in laboratory conditions. Another obstacle of this method was the co-amplification of HLA alleles with high sequence homology. This could be resulted in misdiagnosis or misinterpretation. Therefore, co-amplification of alleles with high sequence homology directed us to sequence the amplified products in order to resolve the ambiguity of the HLA alleles.

5.2. Co-amplifying HLA Alleles Could be Discriminated by High Resolution DNA Sequencing.

The HLA typing of the DRB1 and A genes were achieved predominantly by using sequence specific primers in single cells. However, not all HLA-DRB1 alleles could be distinguished from each other by using sequence specific primers. The DRB1*15 and DRB1*16 or DRB1*03, *11, *13 and *14 alleles could be co-amplified due to the high sequence homology at the primer binding sites of these alleles. Therefore, sequencing of the amplified products has to be done in order to discriminate alleles in cases of co-inheritance of co-amplifying HLA alleles. The sequencing of the HLA-A alleles in single

cells were done only to confirm the ambiguous results obtained from the PCR-SSP and gel electrophoresis.

There are major thirteen DRB1 alleles. The HLA-DRB1 alleles exhibit sequence polymorphisms predominantly in exon two. The primers were usually designed to bind the polymorphic sequences to amplify the alleles selectively. Since the co-amplified alleles could not be distinguished from each other, the allele typing could be accomplished by sequencing analysis of exon two of the DRB1 genes. The sequence differences in exon two of thirteen major HLA-DRB1 alleles were shown in the alignment produced by the MHC database at the NCBI web page (figure 5.7). The sequence differences between the DRB1 alleles were highlighted. The sequence based allele typing can be achieved by utilizing these polymorphic sequences. The BLAST analysis compares the input sequence with the reported HLA-DRB1 alleles in the MHC databases and reports the allele name of the input sequence.

The sequence based HLA-DRB1 typing was carried out in blastomeres in certain cases. The HLA DRB1*03, DRB*11, DRB1*13 and DRB1*14 alleles were amplified with the same primer pairs. Therefore, in cases, when parents inherited two or more of these alleles, the HLA typing in single cells could not be achieved by the sequence specific primers due to co-amplification of the alleles. As a result, the amplified products were sequenced with the conserved sequencing primers in order to distinguish the alleles. Another sequence based typing strategy in single cells was performed to distinguish the HLA-DRB1*15 and DRB1*16 alleles in cases when the parents inherited both alleles since the DRB1*15 and DRB1*16 alleles are the co-amplifying alleles. The other DRB1 alleles were not sequenced in the single cells as they could be distinguished successfully with the sequence specific primers.

The sequence based HLA typing of two different blastomeres from the same couple were presented in the figure 5.8. Both alleles were amplified with the same primer pairs, simultaneously. Then, they were sequenced. The electropherograms showed the sequence differences which were highlighted in red in consensus sequence at the top. The obtained sequences were subjected to the BLAST analysis. This analysis revealed that the top

sequence was belong to the DRB1*11 allele and the bottom sequence was belong to the DRB1*03 allele.

<< <R> >>	[Exon2	Exon2	Exon2	Exon2	Exon2	Exon2	Exon2	Exon2
cDNA/Intr.	110	120 //	130	140 //	150 //	170	180 //	190
DRB1*010101	CACGTTTCTT	GTGGCAGCTT //	AAGTTTGAAT	GTCATTTCTT //	CAATGGGACG //	GGTTGCTGGA	AAGATGCATC //	TATAACCAAG
DRB1*03010101	CACGTTTCTT	GGAGTACTCT //	ACGTCTGAGT	GTCATTTCTT //	CAATGGGACG //	GGTACCTGGA	CAGATACTTC //	CATAACCAGG
DRB1*040101	CACGTTTCTT	GGAGCAGGTT //	AAACATGAGT	GTCATTTCTT //	CAACGGGACG //	GGTTCCTGGA	CAGATACTTC //	TATCACCAAG
DRB1*07010101	CACGTTTCCT	GTGGCAGGGT //	AAGTATAAGT	GTCATTTCTT //	CAACGGGACG //	AGTTCCTGGA	AAGACTCTTC //	TATAACCAGG
DRB1*080101	CACGTTTCTT	GGAGTACTCT //	ACGGGTGAGT	GTTATTTCTT //	CAATGGGACG //	GGTTCCTGGA	CAGATACTTC //	TATAACCAAG
DRB1*090102	CACGTTTCTT	GAAGCAGGAT //	AAGTTTGAGT	GTCATTTCTT //	CAACGGGACG //	GGTATCTGCA	CAGAGGCATC //	TATAACCAAG
DRB1*100101	CACGTTTCTT	GGAGGAGGTT //	AAGTTTGAGT	GTCATTTCTT //	CAACGGGACG //	GGTTGCTGGA	AAGACGCGTC //	CATAACCAAG
DRB1*110101	CACGTTTCTT	GGAGTACTCT //	ACGTCTGAGT	GTCATTTCTT //	CAATGGGACG //	GGTTCCTGGA	CAGATACTTC //	TATAACCAAG
DRB1*120101	CACGTTTCTT	GGAGTACTCT //	ACGGGTGAGT	GTTATTTCTT //	CAATGGGACG //	GGTTACTGGA	GAGACACTTC //	CATAACCAGG
DRB1*130101	CACGTTTCTT	GGAGTACTCT //	ACGTCTGAGT	GTCATTTCTT //	CAATGGGACG //	GGTTCCTGGA	CAGATACTTC //	CATAACCAGG
DRB1*140101	CACGTTTCTT	GGAGTACTCT //	ACGTCTGAGT	GTCATTTCTT //	CAATGGGACG //	GGTTCCTGGA	CAGATACTTC //	CATAACCAGG
DRB1*15010101	CACGTTTCCT	GTGGCAGCCT //	AAGAGGGAGT	GTCATTTCTT //	CAATGGGACG //	GGTTCCTGGA	CAGATACTTC //	TATAACCAGG
DRB1*160101	CACGTTTCCT	GTGGCAGCCT //	AAGAGGGAGT	GTCATTTCTT //	CAATGGGACG //	GGTTCCTGGA	CAGATACTTC //	TATAACCAGG

Figure 5.7. The alignment of the second exons of thirteen major HLA-DRB1 alleles . The sequence differences between the DRB1 alleles were high-lighted with green color.

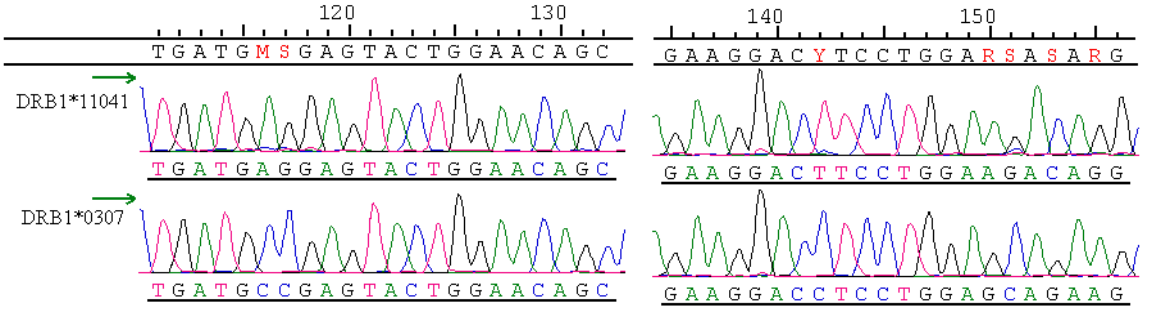

Figure 5.8. The electropherogram of DRB1*11 and DRB1*03 alleles obtained from two different blastomeres. Both alleles of the blastomeres were amplified with the same primer pairs. They were distinguished from each other by sequencing.

Sequencing is a gold standard method. It allows high resolution HLA typing and resolves the allele ambiguities. However, this method requires elaborate work such as PCR purification, cyclesequencing reaction, clean up of sequencing reaction, run on a sequencer and analysis of the sequences in the HLA databases. Since the genetic testing in single cells is time-limited, a simple and rapid typing method should be developed in order to determine the HLA alleles and distinguish the co-amplifying alleles.

5.3. More Rapid Amplification and Genotyping Could be Achieved by Real-Time PCR And Melting Curve Analysis in Single Cells

The PCR with the sequence specific primers and the sequence based typing allowed the HLA typing in single cells as the results presented in the previous sections. Although these methods were well-established in single cells, they required some additional laboratory work such as gel electrophoresis, PCR purification and cyclesequencing. Moreover, PCR-SSP was inconclusive in situations when the parents inherited co-amplifying alleles. Therefore, sequence based typing had to be applied to the single cells which required elaborate work in a limited time period. As a result, an easy and rapid method has been developed to determine the HLA alleles in the single cells and in limited samples such as buccal cheek swabs. Real-time PCR and melting curve analysis is a rapid, simple and efficient method which has been used to detect known SNPs in samples. This method has been applied to the HLA typing of the DRB1 and A alleles. Previously used sequence specific primers for the HLA-DRB1 and HLA-A genes were used to amplify the HLA alleles. The hybridization probes which were designed to bind the polymorphic sequences of the HLA alleles were added into the real-time PCR reaction in order to monitor amplification and to determine the melting temperatures of the amplified alleles. Therefore, the co-amplified alleles could be distinguished from each other by a simple melting curve analysis.

The real-time PCR for HLA typing was established in blood samples initially. After the conditions were optimized, the method was carried to the single cell applications. Figure 5.9 showed the amplification curves versus PCR cycles. The amplification of the HLA alleles was detected cycles between 21 and 28. The amplification could be monitored during the PCR. The samples that did not carry the targetted HLA allele, did not undergo amplification and they were interpreted as negative, amplification failure or allele drop out of the allele. The specificity of the amplification was controlled by the melting curve analysis.

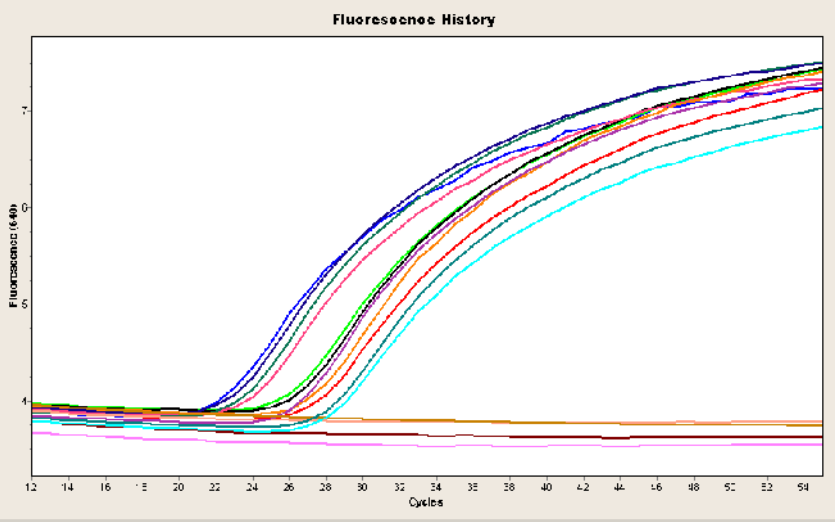

Figure 5.9. Real-time PCR of HLA-DRB1*01 alleles. The amplification curves detected for the samples which were positive for the DRB1*01 allele. The samples that are negative for the DRB1*01 allele did not have any amplification curve.

The specific amplification of the HLA alleles was determined by the melting curve analysis after the PCR. The figure 5.10 demonstrated the melting peaks of the DRB1*01 alleles. The melting temperature for DRB1*01 allele was approximately 70^{o}C.

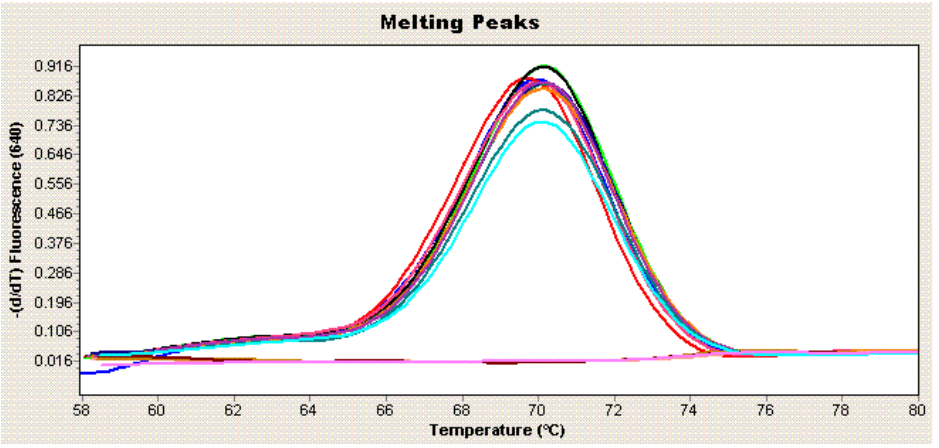

Figure 5.10. Melting peaks of HLA-DRB1*01 alleles. The amplification curves detected for the samples which were positive for the DRB1*01 allele. The samples that are negative for the DRB1*01 allele did not have any amplification curve.

The melting curve analysis after real-time PCR for the HLA-DRB1*03 and DRB1*11 was shown for blastomeres in figure 5.11. Both alleles were co-amplified with the sequence specific primers. The hybridization probes were specific to detect the DRB1*03 allele. Therefore, melting temperature (Tm) for the DRB1*03 allele was determined as 67.1 ^{o}C which was approximately 10 ^{o}C higher than Tm of the DRB1*11 allele. The arrows on the figure showed the melting peaks of the mother, father and the child. The remaining peaks were belong to the blastomeres. The mother and the child were positive for both the DRB1*03 and the DRB1*11 alleles. The father was positive for the DRB1*11 allele. The figure 5.11 showed the single cells positive either for the DRB1*03 or the DRB1*11 allele. The single cells positive for both the DRB1*03 and the DRB1*11 alleles were displayed in figure 5.12. The melting curve analysis has the ability to differentiate the co-amplifying alleles successfully in the single cells.

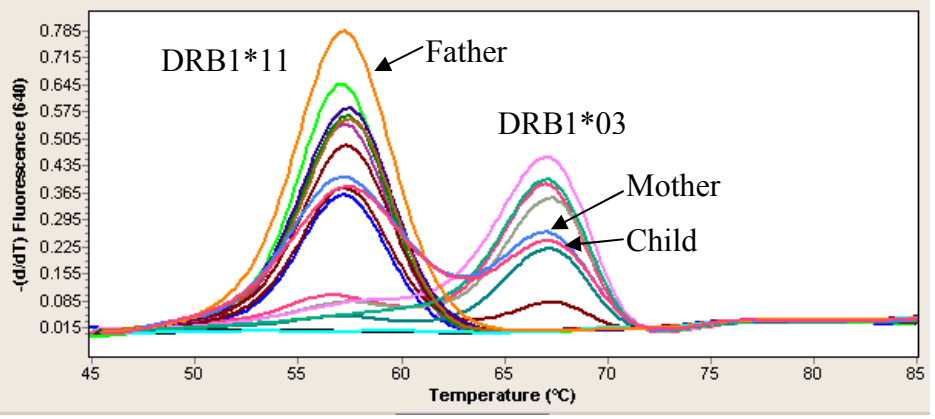

Figure 5.11. Melting curve analysis after real-time PCR for HLA-DRB1*03 and DRB1*11 alleles in single cells. DRB1*03 allele specific probe was used in this reaction to detect amplification curves and melting peaks.

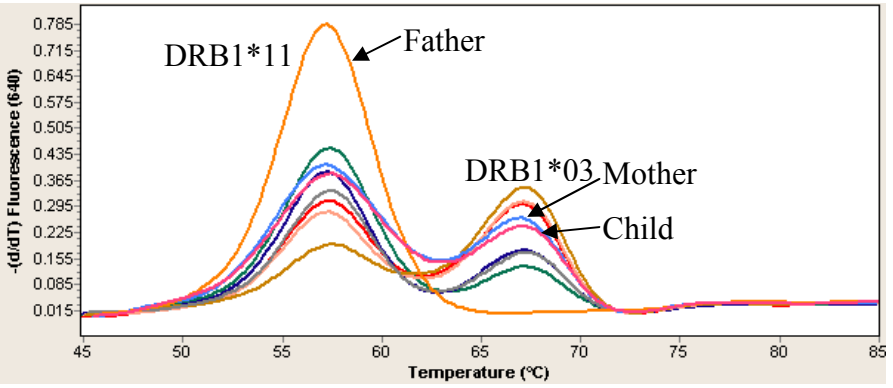

Figure 5.12. Melting curve analysis after real-time PCR for HLA-DRB1*03 and DRB1*11 alleles in single cells. The DRB1*03 allele specific probe was used. In this figure, single cells which were positive for both DRB1*03 and DRB1*11 alleles were shown.

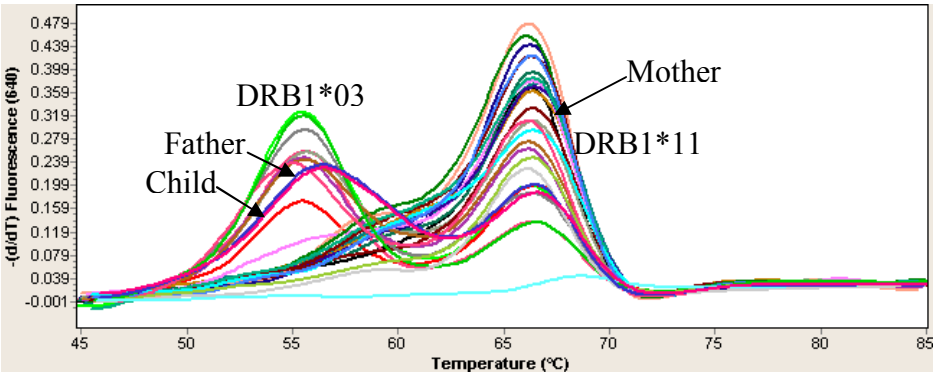

Figure 5.13. Melting curve analysis after real-time PCR for HLA-DRB1*03 and DRB1*11 alleles in single cells. DRB1*11 allele specific probe was used. Single cells were positive for both DRB1*11 and DRB1*03 alleles.

The DRB1*11 specific probe was also designed in order to detect the DRB1*11 alleles. This probe has also the ability to detect and distinguish the DRB1*03 alleles. The melting temperature of the DRB1*11 and DRB1*03 alleles were determined as approximately 66.5°C and 55.7°C, respectively. The arrows on the figure showed the melting peaks of the mother, father and the child. The remaining peaks were belong to the blastomeres. The father and the child were positive for both DRB1*03 and DRB1*11 alleles. The mother was positive for the DRB1*11 allele. The figure 5.13 showed the single cells positive for both DRB1*11 and DRB1*03 alleles. The cells inherited both alleles.

Another co-amplifying alleles were HLA-DRB1*15 and DRB1*16. These alleles were amplified with the same sequence specific primers due to the high sequence homology at the primer binding sites. These alleles showed sequence differences in the region between amplification primers. Therefore, the hybridization probes were designed to detect and distinguish these alleles without the need for sequencing analysis. The only situation that made the HLA-DRB1 typing complicated is the co-inheritance of DRB1*15 and DRB1*16 alleles together in one of the parents. As the embryos or children inherited either of these alleles, both of them were amplified and they could not be distinguished by the sequence specific primers. The mother inherited both the DRB1*15 and DRB1*16 alleles, of which the melting peaks were seen as dark blue in the figure 5.14. The child inherited the DRB1*16 allele from his mother. Since the father was negative for both of the alleles, the embryos were inherited either the DRB1*15 or DRB1*16 allele from the mother. The alleles of the parents and the child were determined previously in the preclinical workups prior to PGD cycle. Since DRB1*15 specific hybridization probes were used in this assay, the melting temperature for DRB1*15 and DRB1*16 were detected as approximately 67.5 ^{o}C and 54.2 ^{o}C, respectively.

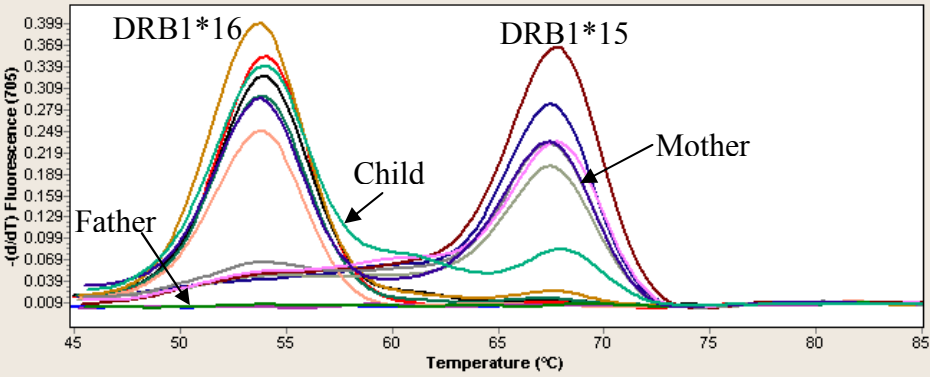

Figure 5.14. Melting curve analysis after real-time PCR for HLA-DRB1*15 and DRB1*16 alleles in single cells. DRB1*15 allele specific probe was used. Single cells were positive either DRB1*15 or DRB1*16 allele.

The co-amplifying alleles were discriminated easily and rapidly by melting curve analysis following PCR with the sequence specific primers. These primers were also used to amplify other HLA-A and HLA-DRB1 alleles in real-time PCR platform as this

platform enabled us to monitor amplification readily. Moreover, it eliminated the post PCR procedures.

The melting peaks for the DRB1*07 and DRB1*14 alleles in single cells were demonstrated in figures 5.15 and 5.16, respectively. Since these alleles were not amplified with any other allele, they were evaluated merely as either positive or negative according to the amplification curves and melting peaks. The melting curve analysis supported the specificity of the amplification. The melting temperature of the DRB1*07 and DRB1*14 alleles were determined as 68.5 ^{o}C in single cells. In figure 5.15, the child and the father were DRB1*07 positive and the mother was negative for this allele. Three blastomeres were found positive for the DRB1*07 allele. In figure 5.16, the child and the mother were DRB1*14 positive and the father was negative. The DRB1*14 positive single cells were pointed out with the arrows. The single cells that were negative for either of the alleles were seen as flat lines at the bottom as they were not amplified.

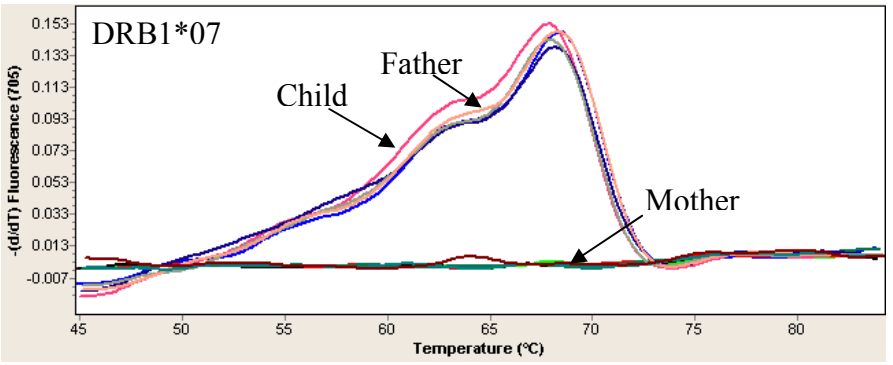

Figure 5.15. Melting curve analysis after real-time PCR for HLA-DRB1*07 allele in single cells. DRB1*07 allele specific probe was used. The melting peaks represented the specific amplification of the DRB1*07 allele in the blastomeres, child and the father.

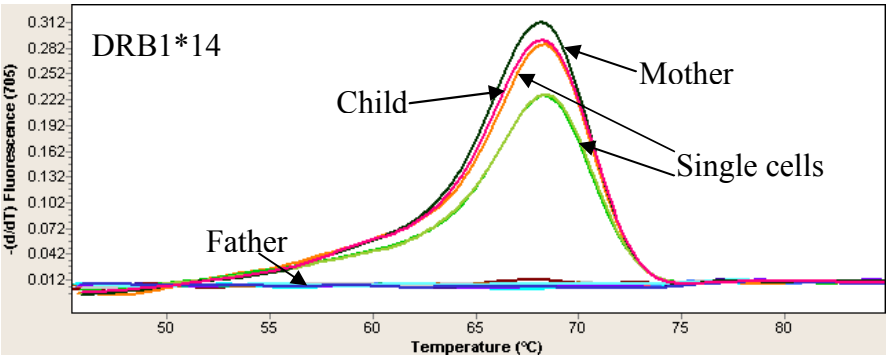

Figure 5.16. Melting curve analysis after real-time PCR for HLA-DRB1*14 allele in single cells. DRB1*14 allele specific probe was used. The melting peaks represented the specific amplification of the DRB1*14 allele in the single cells, child and the mother.

The amplification of the HLA-A alleles with the previously published sequence specific primers were combined with the allele specific hybridization probes. Hybridization probes for each HLA-A allele were designed to bind polymorphic sequences of the alleles. Probe sequences were given in table 3.17. The following figures (Figures 5.17, 5.18 and 5.19) demonstrated the melting peaks for the A*01, A*02 and A*30 alleles in the blastomeres. The samples were amplified for a specific HLA-A allele and a melting peak was obtained after melting curve analysis if the sample was positive.

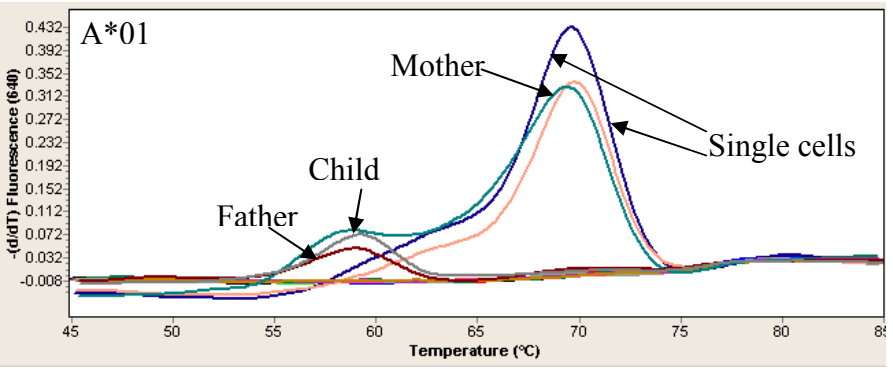

Figure 5.17. Melting curve analysis after real-time PCR for HLA-A*01 allele in single cells. The HLA-A*01 allele specific probe was used. The melting peaks represented the specific amplification of the HLA-A*01 allele. Tm of A*01 allele was 69.5 ^{o}C.

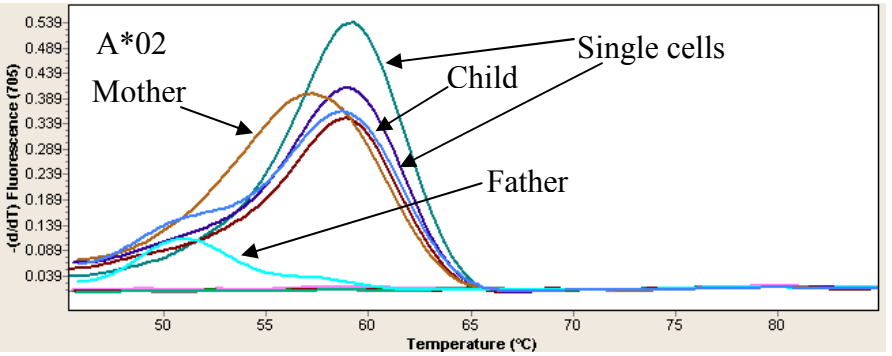

Figure 5.18. Melting curve analysis after real-time PCR for HLA-A*02 allele. The HLA-A*02 allele specific probe was used. The melting peaks represented the specific amplification of the HLA-A*02 allele. Tm of the A*02 allele was 59.8 ^{o}C.

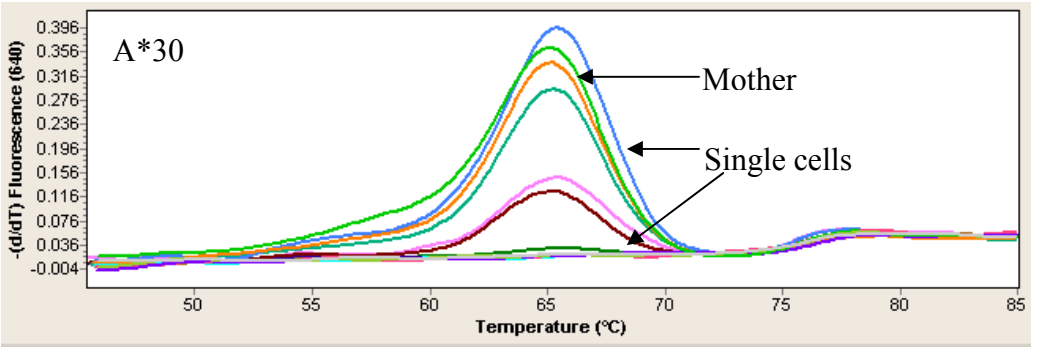

Figure 5.19. Melting curve analysis after real-time PCR for HLA-A*30 allele. The HLA-A*30 allele specific probe was used. The melting peaks represented the specific amplification of the HLA-A*30 allele. Tm of the A*30 allele was 65 ^{o}C.

The real-time PCR and the melting curve analysis of HLA typing was similar to that of sequence based HLA typing in single cells. In both methods, each HLA allele was amplified separately by several successive PCRs. Although real-time PCR based strategy has some advantages over sequence based typing method, both methods failed to determine allele drop out in single cells and the recombination at the HLA locus. Moreover, the patient specific amplification has to be followed in both strategies. Therefore, selection of the HLA compatible embryos could be achieved by eliminating incompatible ones through successive amplifications of targetted HLA-A and DRB1 alleles. The extended HLA locus could not be determined due to analyses of the selected HLA A and DRB1 alleles. Additionally, the amplification of all the HLA alleles of the

HLA A, B, C, DP, DQ and DR genes is not possible in this method in single cells, technically. For this reason, an alternative method which covered whole HLA locus for the selection of the HLA compatible embryos has to be needed for more accurate diagnosis.

5.4. Microsatellites of the Extended HLA Locus Could be Used For Single Cell HLA Typing with More Accuracy and Higher Efficiency

The microsatellites scattered through the extended HLA locus were studied in order to determine the HLA haplotypes in the families. These microsatellites covered approximately 12.2 Mb region in the extended HLA locus. Fourteen microsatellites were studied for each case to determine the informative ones which were selected to be studied in the blastomeres. Prior to clinical PGD cycle, the blood samples were taken from the family members in order to isolate DNA in the preclinical workups. Then, fourteen microsatellites were studied for all the family members to determine the informative microsatellites at the HLA locus. Generally, eight-ten informative microsatellites scattered from the centromeric to the telomeric side of the HLA locus were sufficient to establish accurate HLA haplotypes of the family members. After selection of informative microsatellites at HLA locus, these microsatellites were amplified in single blastomeres after an initial cell lysis step. The microsatellites were amplified directly with the fluorescently labelled primers when the starting material was DNA isolated from the blood sample. Whereas, the same microsatellites were amplified in two steps to obtain a detectable amplification product in the single cells as the starting DNA was only two copies in single cells. All the informative STRs were amplified with outer primers in a single tube with multiplex PCR. Afterwards, two or three microsatellites were amplified from the initial multiplex PCR by using fluorescently labelled nested primers. The microsatellites with different sizes and fluorescent labels were amplified together in order to prevent overlapping of the amplified fragments in the capillary electrophoresis which could lead to misinterpretation. The analysis of microsatellites through the HLA locus provided us to determine allele drop outs and recombinations in this locus in single cells.

Each microsatellite was amplified with different efficiency in single cells (table 5.1). The characteristics of the primers, repetitive freezing and thawing of the primers, the number of primers used and the combinations of different microsatellites in the multiplex

PCR could affect the PCR conditions and the amplification efficiencies. An average of eight - ten microsatellites were amplified in single cells in each multiplex PCR. A total of 510 single cells was analyzed with these markers. The amplification efficiency varied from 79.39% to 97.8% for markers used. The overall efficiency was calculated as 92.35%. The D6S439, LH-1, D6S2792, D6S2811, D6S510 and D6S2972 were the most commonly used markers with an amplification efficiency of at least 93.73%. The D6S2883 and 82-1 markers displayed the lowest amplification efficiencies in single cells. Among the fourteen microsatellites, the D6S2874 and the D6S2810 markers were the least used markers in single cells.

Table 5.1. Amplification efficiencies of microsatellites located in HLA locus.

Marker	***No of Cells Analysed***	***No of Cells with Positive Amplification***	***PCR Efficiency (%)***
D6S291	198	184	92.93
D6S439	345	324	93.91
D6S2874	89	80	89.89
D6S2876	174	168	96.55
D6S2883	246	202	82.11
LH-1	349	334	95.70
82-1	165	131	79.39
D6S2792	319	299	93.73
D6S2810	91	89	97.80
D6S2811	410	401	97.80
D6S265	279	248	88.89
D6S510	368	357	97.01
D6S2972	324	306	94.44
D6S276	259	240	92.66

The allele drop out is basically amplification and detection of only one of the alleles in a heterozygous case. It is one of the major problems in the single cell diagnostics. The allele drop out rate was determined as approximately 8.98% for the HLA locus. The average of ADO occurrence in each PGD cycle was 1.056 with a standard deviation of 1.09.

The successful amplification of different microsatellites in the single cells were presented in the following figures. The figure 5.20 represented amplification of the D6S265 in the blastomeres and in the human genomic DNA. The first and the second rows were belong to two different blastomeres. The maternal and the paternal alleles were seen at the third and the forth rows, respectively. The last row demostrated the alleles of the child. The size of the maternal alleles were determined as 113 bp and 127 bp. The size of the paternal alleles were detected as 117 bp and 119 bp. The child and the blastomere two were inherited 127 bp -sized maternal allele and 119 bp – sized paternal allele. On the other hand, the embryo at the first row inherited other alleles, therefore, it did not share any allele with the second embryo or the child.

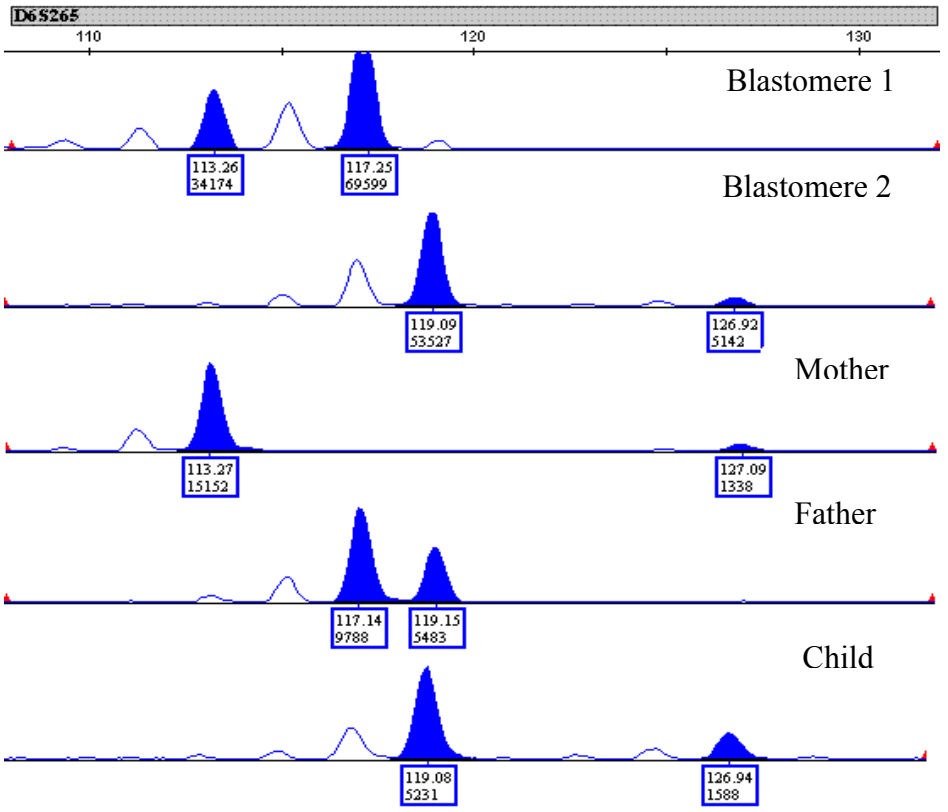

Figure 5.20. Capillary electrophoresis of D6S265 marker in blastomeres and human genomic DNA.

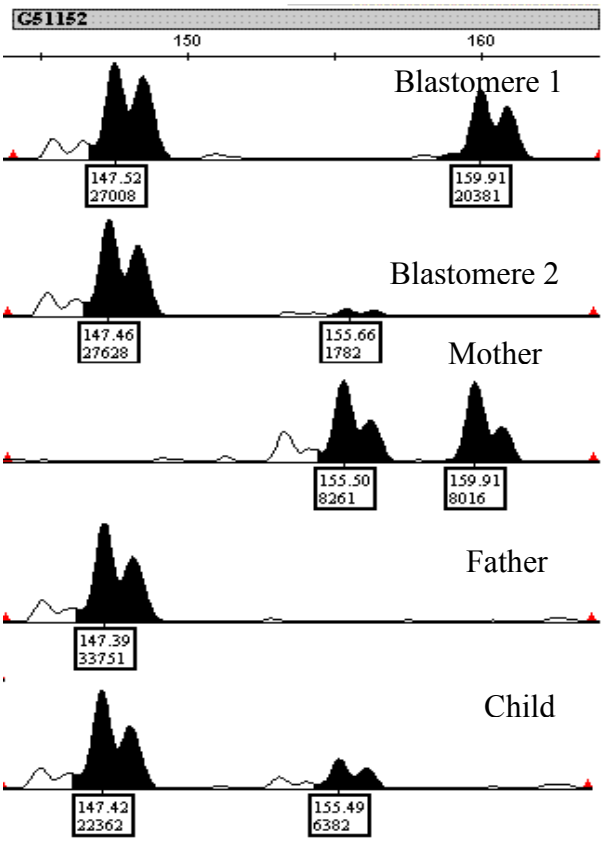

Figure 5.21. Capillary electrophoresis of D6S2876 (G51152) marker in blastomeres and human genomic DNA.

The capillary electrophoresis after the multiplex amplification of the D6S2876 (G51152) marker was presented in figure 5.21. The marker is located centromeric to the HLA-DQB1 gene. As in the case of the D6S2876 marker, the alleles of the sample in the second row (blastomere two) were identical with that of the last row (the child). The paternal allele which was in the forth row was found homozygous for this marker and the size of the allele was 147 bp. All the offsprings were obliged to inherited either of the 147 bp sized paternal allele. The size of the maternal alleles were 155 bp and 159 bp. The child and the blastomere two were inherited the maternal allele with 155 bp long. Since the paternal alleles were found in homozygous state, they were considered as uninformative.

The D6S2811 (HLABC-CA) which was the most frequently amplified marker in the blastomeres was presented in figure 5.22. This marker is located between the HLA-B and the HLA-C genes. The sizes of the maternal alleles were 139 bp and 141 bp. The size of the paternal alleles were 146 and 148 bp. The parents did not share any common allele

size, therefore, the marker was very informative. As seen in the previous markers, the child and the blastomere two inherited the same alleles from the parents. The allele sizes were detected as 139 bp and 148 bp. The blastomere one was inherited the maternal and paternal alleles with sizes of 141 bp and 146 bp, respectively.

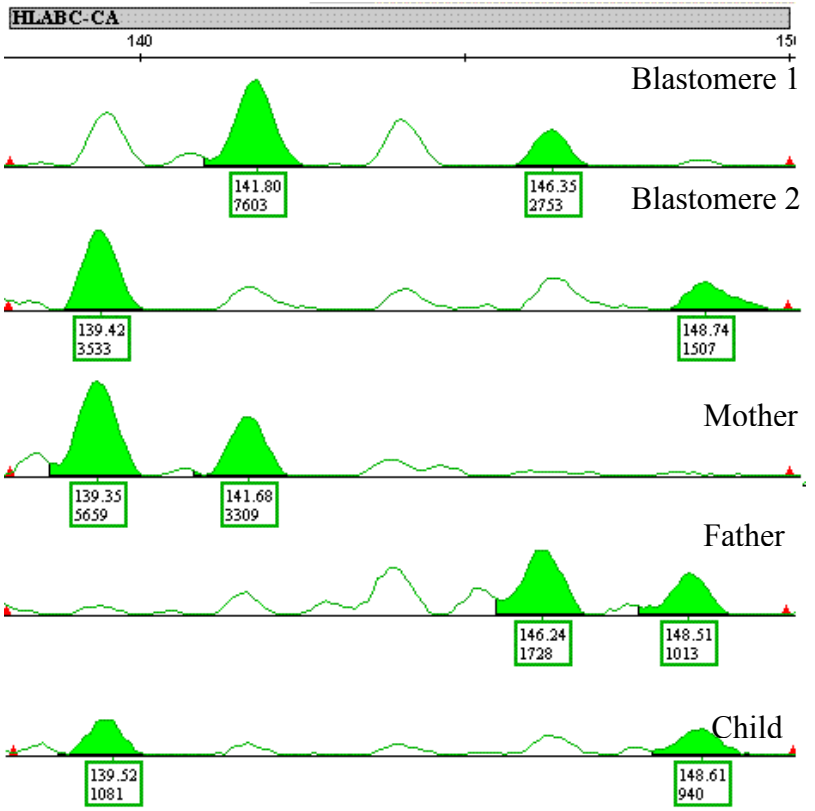

Figure 5.22. Capillary electrophoresis of D6S2811 (HLABC-CA) marker in blastomeres and human genomic DNA.

The D6S2972 (MOG-CA) which is located at the telomeric side of the HLA-A gene was presented in figure 5.23. The sizes of the maternal alleles were 213 bp and 228 bp. The size of the paternal alleles were 215 and 221 bp. As in the case of D6S2972 marker, the parents did not share any common allele size. The child and the blastomere two inherited the same alleles from the parents. The allele sizes were detected as 213 bp and 221 bp. The blastomere one was inherited the maternal and paternal alleles with the sizes of 228 bp and 215 bp, respectively.

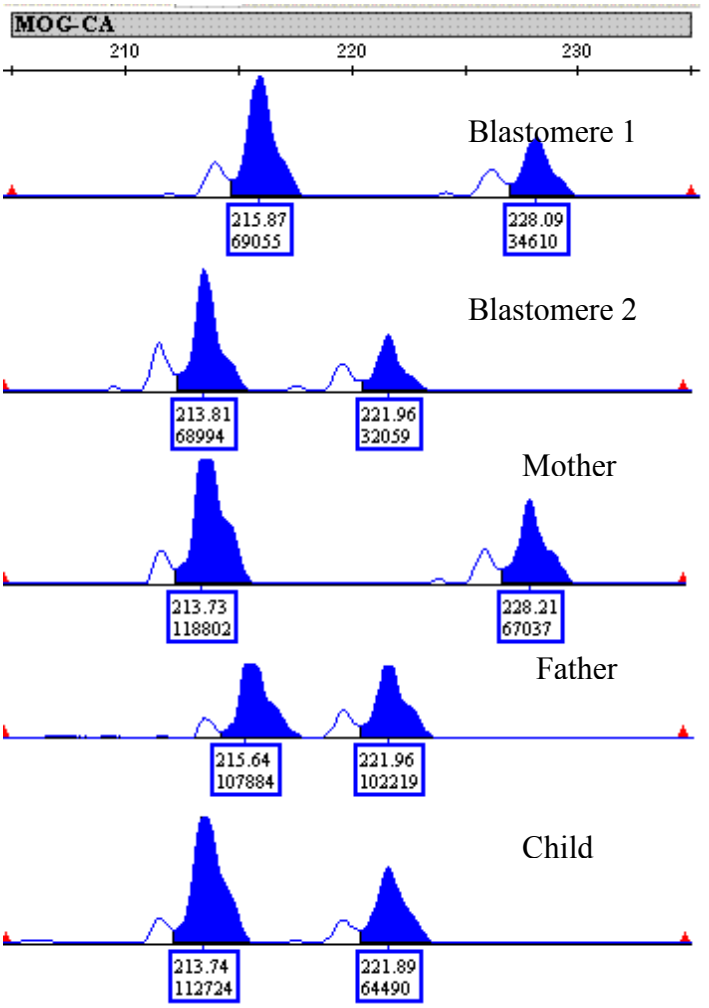

Figure 5.23. Capillary electrophoresis of D6S2972 (MOG-CA) marker in blastomeres and human genomic DNA.

The figure 5.24 represented the amplification of the D6S2792 (TNFa) marker in the blastomeres and in the human genomic DNA. The microsatellite is located between the HLA-DRA and the HLA-B gene in the class III region. The first and the second rows were belong to two different blastomeres, as previously presented. The size of the maternal allele was determined as 104 bp. Since the mother was found homozygous for this marker, all the offsprings were inherited 104 bp sized allele. Therefore, maternal alleles were uninformative. The size of the paternal alleles were detected as 97 bp and 106 bp. The child and the second blastomere were inherited the paternal allele with a size of 106 bp. The first blastomere was inherited the other allele of the father.

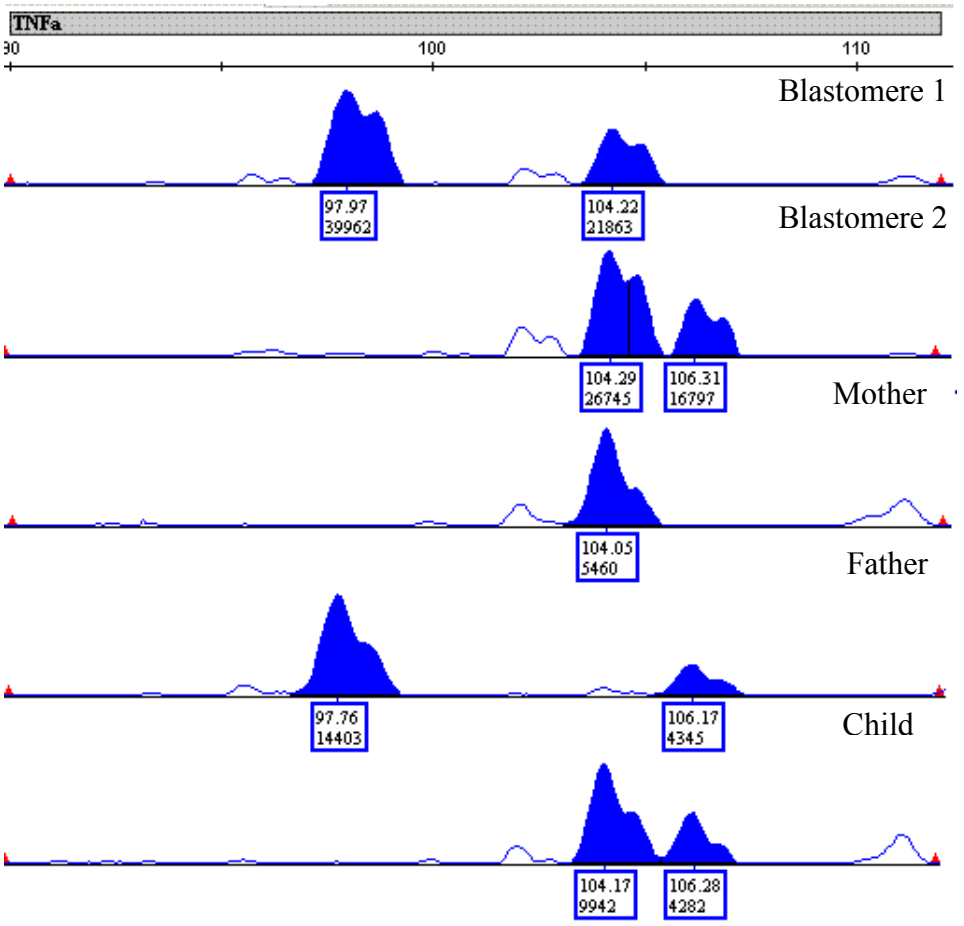

Figure 5.24. Capillary electrophoresis of D6S2792 (TNFa) marker in blastomeres and human genomic DNA.

As shown in the previous figures, each microsatellite was analyzed individually to determine each segregating allele and the HLA haplotype of each cell. The pedigree given in figure 5.25 represented the data derived from one of the preimplantation genetic diagnosis cycles. Eleven embryos were biopsied in the cycle in order to be analyzed for the sickle cell mutation and the HLA typing. Five out of eleven blastomeres were showed in the pedigree. Ten informative microsatellites located at the HLA locus were studied for each blastomere. The haplotype analysis for the extended HLA locus has been presented here. The maternal haplotypes were represented as “A” and “B”. Similarly, the paternal haplotypes were named as “C” and “D”. Therefore, the expected haplotype combinations in the offsprings were “AC”, “BC”, “AD” and “BD”. The child’s genotype was assumed as “AC” everytime, and therefore, “AC” genotype in the embryos were regarded as HLA-compatible with the child. The HLA haplotype of blastomere 203 was determined identical

with that of 201. The allele drop out of the D6S2874 and D6S291 was detected in the maternal alleles. The allele drop out of 82-1 and D6S276 was detected in the paternal alleles. The allele drop out of the whole maternal allele was detected in 206 since none of the microsatellites were amplified and detected for the maternal allele. Allele drop out occurs randomly in single cells.

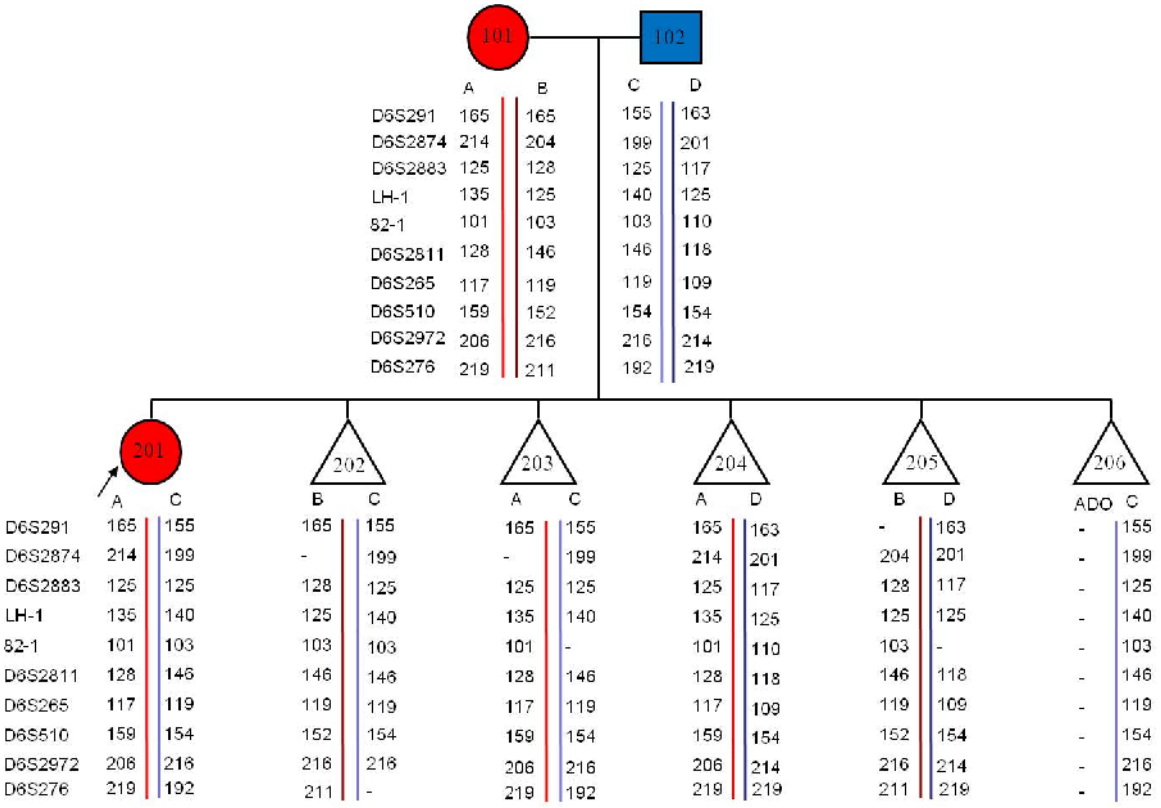

Figure 5.25. Haplotype analysis for extended HLA locus in blastomeres. Embryos were pepicted with triangles. Maternal alleles were demostrated with red and brown lines. Paternal alles were demonstrated with blue and dark blue lines.

The electropherograms showing the allele drop out of the D6S439 and LH-1 for different cases were given in figures 5.26 and 5.27, respectively. As seen in the the third and the forth rows, the maternal and the paternal alleles were detected as heterozygous with different allele sizes. Thus, each offspring was expected to be heterozgous for D6S439 unless an expansion or deletion has not been occured in the repeat region. The heterozygous case was shown in the second row (blastomere four). However, single allele with 124 bp size was detected in the first row (blastomere three), suggesting that only the

maternal allele was amplified and the paternal allele was dropped out or one of the paternal alleles was subjected to expansion which is less likely when considered the amplification failure of other markers for the paternal haplotype.

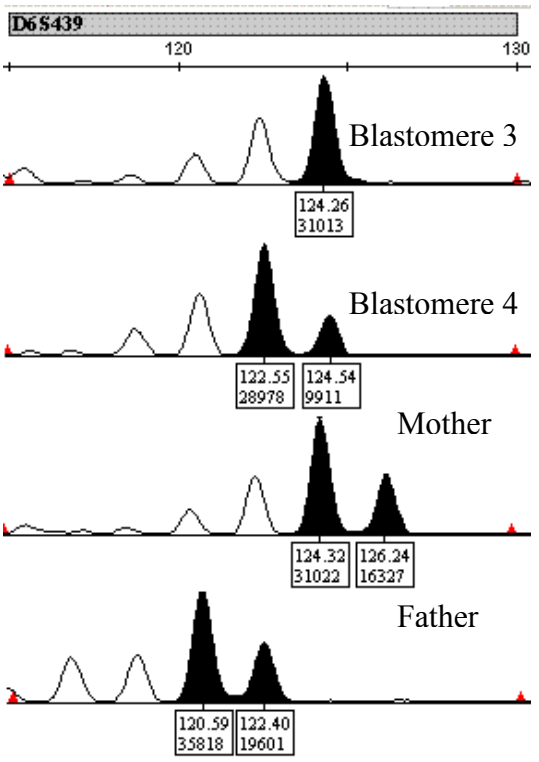

Figure 5.26. Allele drop out of D6S439 marker in a single cell. Only maternal allele was detected in blastomere three. None of the paternal alleles was detected.

The allele drop out in LH-1 marker was seen is the first row (blastomere five). The third and the forth rows demonstrated the maternal and the paternal alleles, respectively. Only the amplification of the maternal allele was detected in blastomere five. However, none of the paternal alleles was detected in this sample.

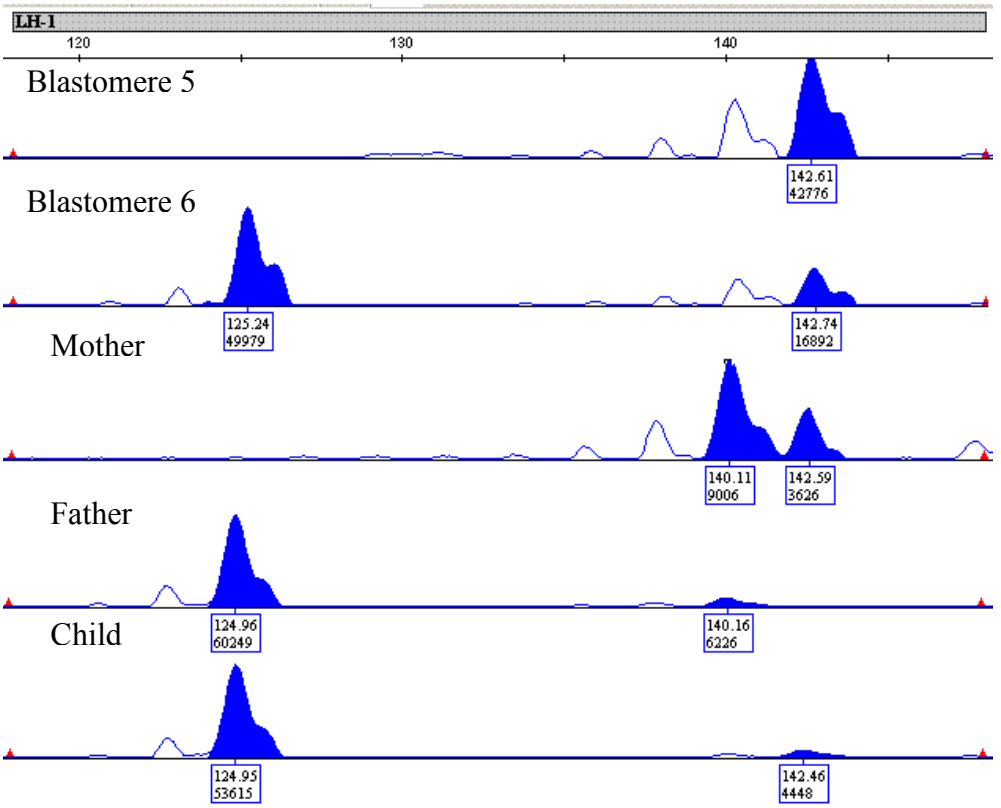

Figure 5.27. Allele drop out of LH-1 marker in a single cell. Only the maternal allele was detected in blastomere five. None of the paternal alleles was detected.

Use of microsatellites scattered through the extended MHC locus provided to determine the whole HLA alleles in the blastomeres. By using at least eight markers, typing of HLA-A, B, C, DP, DQ and DR genes is possible in the blastomeres. Since transplantation biology requires full HLA compatibility in at least ten alleles, this strategy allows obtaining information from the alleles of six genes and the non-HLA genes indirectly. This method does not require successive PCR cycles. Therefore, selection of HLA compatible embryos could be accomplished very rapidly. Since the capillary electrophoresis has a high sensitivity to resolve one base pair size differences, an accurate HLA haplotyping can be accomplished by running fluorescently labelled HLA microsatellites. Use of microsatellites enables to determine the recombinations at the extended HLA locus. Therefore, more accurate HLA typing allows selection of the HLA compatible embryos. Since a set of informative markers was used in each case, the single cell testing did not require a patient specific strategy anymore, resulting in a diagnostic standardisation in the single cells. Another advantage is detection of the allele drop out in

the single cells. Since at least eight microsatellites were used for each cell, the allele drop out and the origin of the failed allele could be determined. Overall, due to the increased accuracy of the HLA typing, this strategy enables transfer of more embryos for implantation by eliminating the ambiguity resulted from the sequence specific typing of the HLA alleles in the single cells.

5.5. Detection of Various Beta-Globin Mutations was Achieved in Single Cells

Twelve beta-globin mutations were tested in the single cells combined with the HLA typing. These mutations were found in different combinations which were listed in table 5.2 with the corresponding case numbers. The most common genotype was homozygous IVS I:110 mutation which was found in seven beta-thalassemia cases. Therefore, it was the most frequently studied beta-globin mutation in the single cells.

Table 5.2. Beta-globin genotypes that were analyzed in single cells and number of cases encountered in affected children.

Mutations	*Number of Cases*
IVS I:110 / IVS I:110	7
Codon 6 / Codon 6	4
IVS I:6 / IVS I:110	3
IVS II:1 / Codon 39	3
Codon 8/9 / Codon 8/9	2
IVS I:1 / Codon 44(-C)	2
IVS I:110 / Codon 5(-CT)	2
IVS I:6 / IVS II:745	2
IVS II:1 / IVS II:1	2
IVS II:745 / IVS II:745	2
IVS I:110 / IVS II:1	1
IVS I:1 / Codon 8(-AA)	1
IVS I:1 / IVS I:1	1
IVS I:1 / IVS I:110	1
IVS I:110 / Codon 44(-C)	1
IVS I:110 / IVS II:745	1
IVS I:5 / Codon 8(-AA)	1
IVS I:6 / Codon 39	1
IVS I:6 / IVS I:6	1
IVS II:1 / Codon 8/9	1

As presented in table 5.2, different combinations of several beta-globin mutations have been studied in the single cells. Therefore, different amplification and detection strategies were develeoped for the mutation analysis in the single cells. Except IVS II:745 (C>G) mutation, all the other beta globin mutations could be amplified in the single amplicon. The beta-globin gene of cases which were carrying IVS II:745 (C>G) mutation and an additional HBB mutation were amplified as two separate amplicons simultaneously. After the initial amplification of the region containing the beta-globin mutations in the multiplex PCR with the HLA-related primers, the region was amplified with either the nested primers in order to detect the mutations by sequencing analysis or the real-time PCR primers and the hybridization probes in order to determine the genotype of the single cells by melting curve analysis.

For the sequencing analysis, a nested PCR was carried out from the initial multiplex PCR in the single cells in order to obtain sufficient amount of DNA for sequencing analysis. The primers given in table 3.7 were used to amplify the beta-globin gene in the nested PCR. A 200 bp DNA fragment was amplified to detect IVS II:745 (C>G) mutation by sequencing analysis. The bands of this fragment on the agarose gel were presented in the figure 5.28.

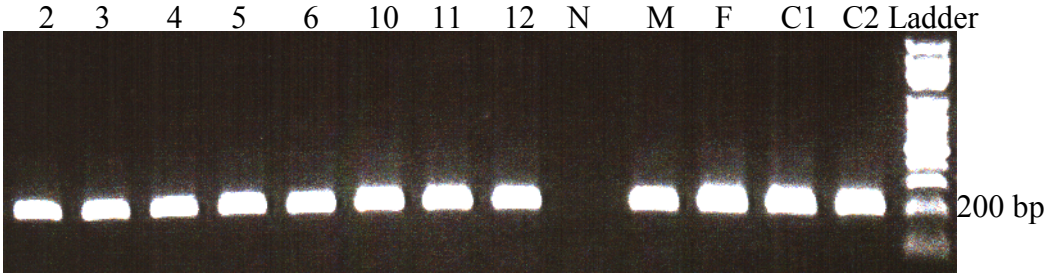

Figure 5.28. Amplification of region covering IVS II:745 mutation site in the beta-globin gene of blastomeres. The blastomeres were enumarated. N, M, F, C1 and C2 denote for negative control, mother, father, child one and child two, respectively.

Most of the mutations of the beta globin gene were clustered in the first exon and the first intron. Therefore, two alternative primers comprising this region were used in the single cells. The figure 5.29 and the figure 5.30 demonstrated the amplification bands for region covering from exon one to intron two of the beta globin gene. Both primer pairs

amplified the region very efficiently as shown in the agarose gel images below. The blastomeres were enumarated. N, M, F, C1 and C2 denoted for negative control, mother, father, child one, and child two, respectively. All the amplicons were sequenced in order to determine the mutation status of the blastomeres.

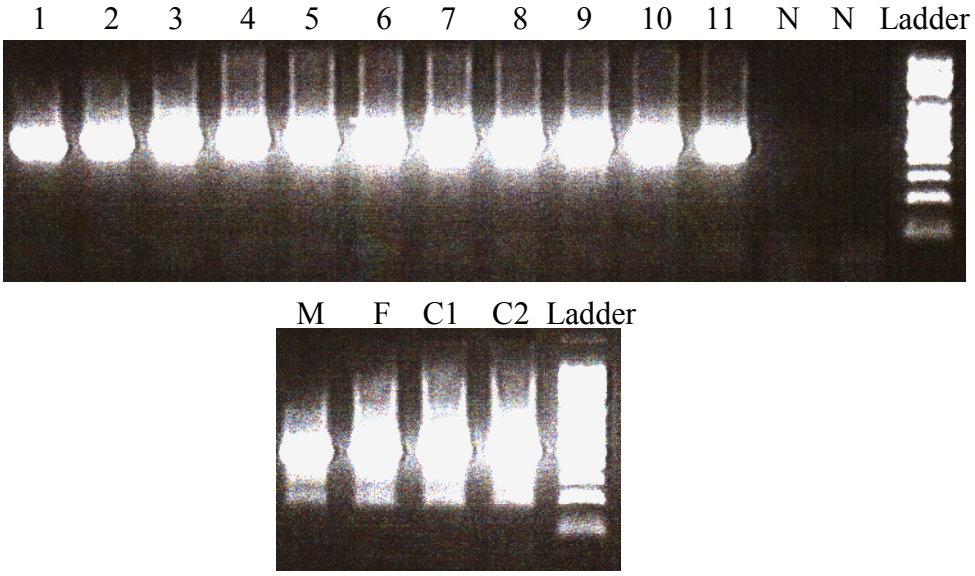

Figure 5.29. Amplification of region (550 bp) covering from exon one to intron two of beta-globin gene in blastomeres.

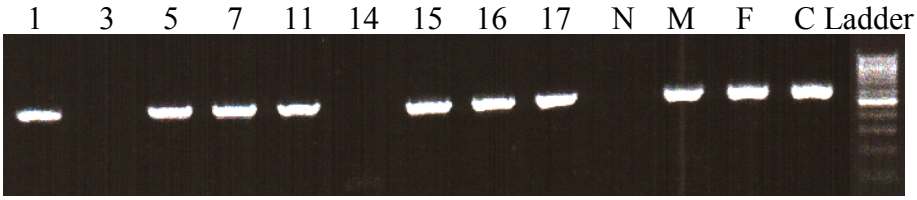

Figure 5.30. Amplification of region covering (600 bp) from exon one to intron two of beta-globin gene in blastomeres with alternative primer pairs.

Sequencing is the gold standard method to determine the sequence variations. Sequencing was used to determine the beta-globin mutations in single cells. The success of sequencing was 100% for all the amplified fragments from blastomeres. The following electropherograms exhibited various beta-thalassemia mutations which were detected in different blastomeres.

The starting DNA in single cell studies is only two copies. Two copies of DNA were amplified in two steps to obtain million copies of the DNA fragments. One of the copies could be lost at the beginning of the PCR and resulted in the allele drop out or favored and resulted in the selective amplification of one of the alleles. These problems could be observed at the mutation and the polymorphic sites. The figure 5.31. demonstrated the IVS I:1 (G>A) mutation in which the mutation (A) was detected much more stronger than the wild type (G) allele.

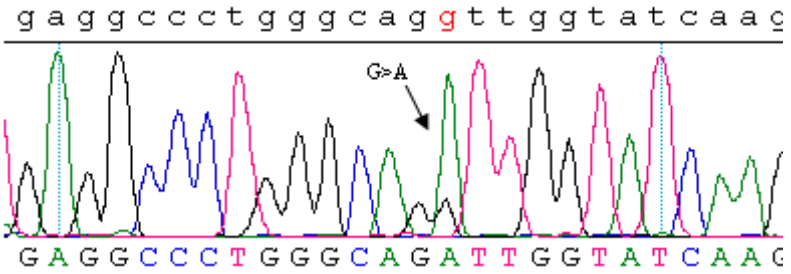

Figure 5.31. Electropherogram demonstrating heterozygous IVS I:1 (G>A) mutation in a single cell. The upper sequence is the reference sequence of the HBB gene.

Sequencing was done in both directions to obtain a consensus result. Because the mutation or the wild type peaks were not prominent sometimes due to the chemistry, composition of the sequence and the run parameters. Therefore, sequencing in both directions clarifies the ambiguous results in single cells. The IVS I:5 (G>T) mutation was seen in both directions in figure 5.32. At the top electropherogram, the mutant T was very low in height and at the bottom, both the appearance of the mutant T and the wild type G were not similar to the neighbouring nucleotides due to the chemistry or run conditions. Therefore, the analysis of the sequences in both directions is absolutely necessary in single cell genetic testing.

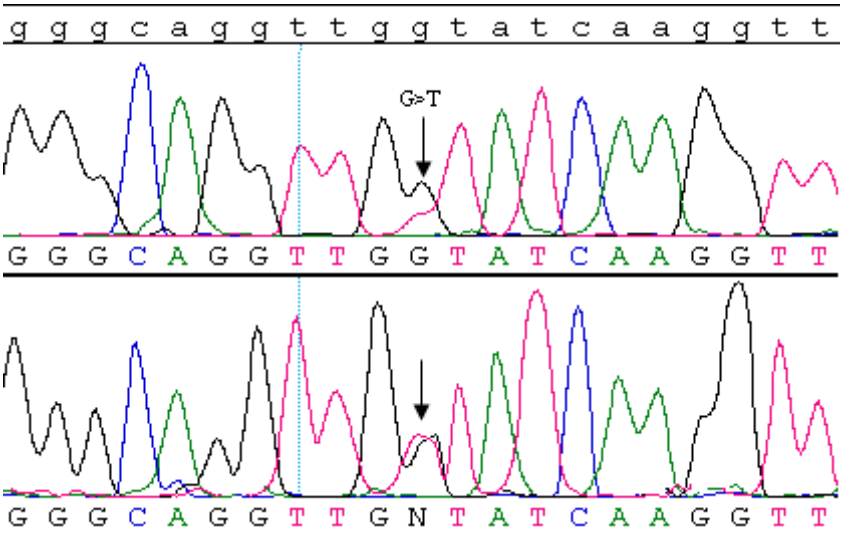

Figure 5.32. Electropherogram demonstrating heterozygous IVS I:5 (G>T) mutation in both directions in a single cell.

The following electropherograms were representing heterozygous IVS I:6 (T>C), IVS I:110 (G>A), IVS II:1 (G>A) and codon 5(-CT) mutations in single cells.

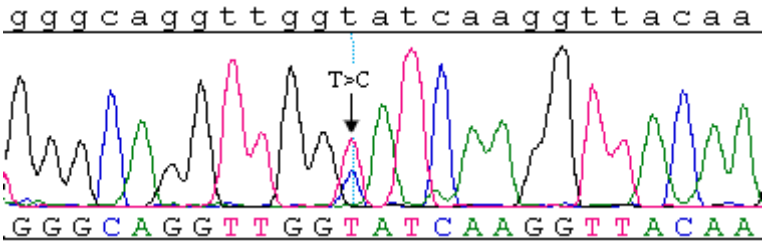

Figure 5.33. Electropherogram demonstrating heterozygous IVS I:6 (T>C) mutation in a single cell.

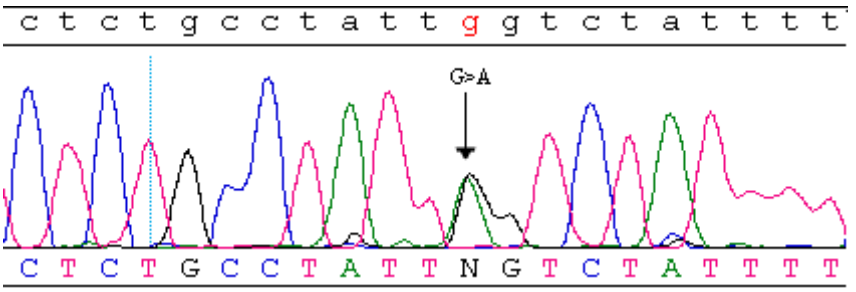

Figure 5.34. Electropherogram demonstrating heterozygous IVS I:110 (G>A) mutation in a single cell.

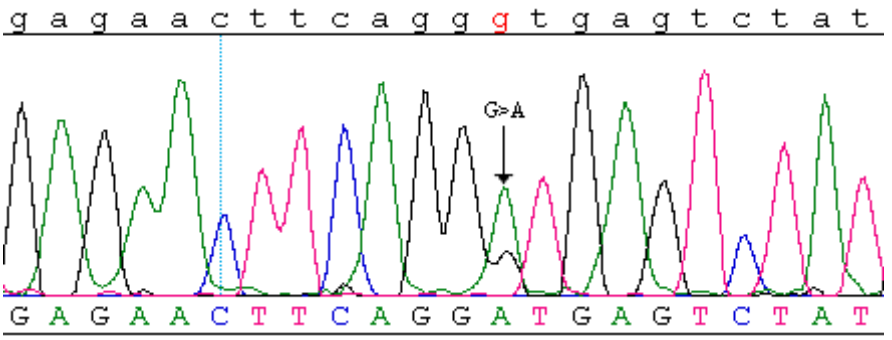

Figure 5.35. Electropherogram demonstrating heterozygous IVS II:1 (G>A) mutation in a single cell.

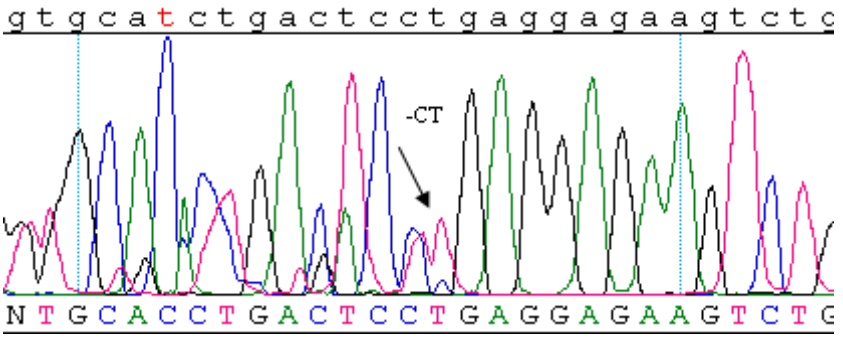

Figure 5.36. Electropherogram demonstrating heterozygous codon 5 (-CT) mutation in a single cell. The electropherogram was obtained from sequencing with the reverse primer.

The following electropherogram shows the homozygous IVS II:745 (C>G) mutation in a single cell.

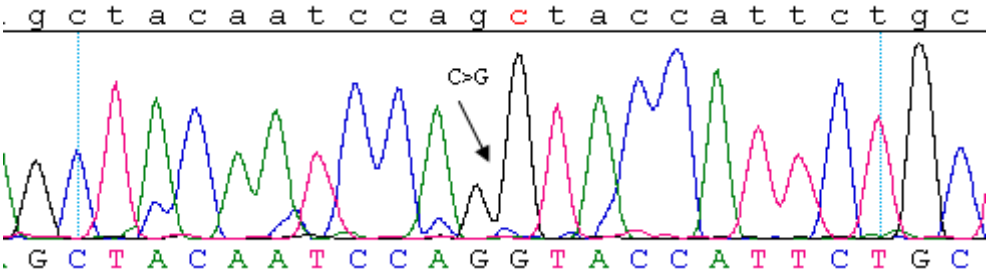

Figure 5.37. Electropherogram demonstrating homozygous IVS II:745 (C>G) mutation in a single cell.

The allele drop out is one of the major problems in single cell genetic testing which leads to misdiagnosis of the mutations. The allele drop out can be observed directly in sequencing of cases where the mutations were expected to be found in heterozygous or compound heterozygous state. The finding of a mutation in homozygous state as presented in figure 5.38 and 5.39 supports the allele drop out in the cell.

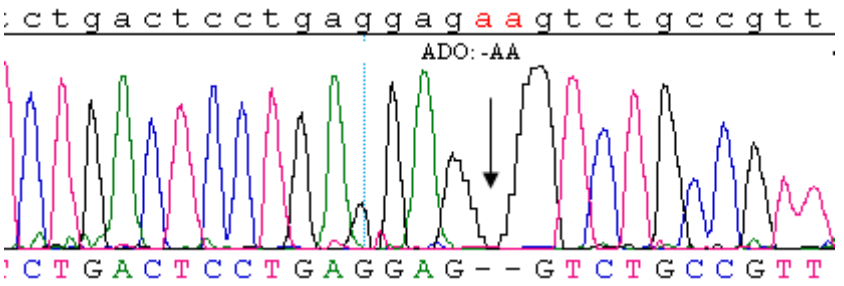

Figure 5.38. Electropherogram representing the allele drop out in a single cell. Homozygous codon 8(-AA) mutation was detected. The affected child was compound heterozygous for codon 8(-AA) and IVS I:1(G>A).

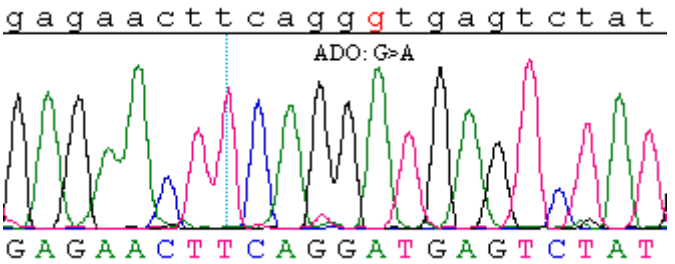

Figure 5.39. Electropherogram representing the allele drop out in a single cell. Homozygous IVS II:1(G>A) mutation was detected. The affected child was compound heterozygous for IVS I:110 (G>A) and IVS II:1(G>A).

Sequencing was performed successfully in single cells for detection of various beta-globin mutations. As this method requires various laboratory work and time, real-time PCR and melting curve analysis which is a rapid, simple and sensitive method was applied to single cells to detect the mutations in the beta-globin gene.

The melting curve peaks for the codon 39 (C>T) mutation were presented in figure 5.40. The father was carrier of IVS II:1 (G>A) mutation. Therefore, his DNA was detected as wild type for codon 39 (C>T) mutation in the melting curve analysis. The mother was heterozygous for codon 39 (C>T) mutation. The child was compound heterozygous for codon 39 (C>T) and IVS II:1 (G>A) mutations. The melting curve analysis for codon 39 (C>T) mutation demonstrated the child and the mother as heterozygoous for this mutation. The single cell represented with a red line was found heterozygous. The other single cells were detected as homozygous wild type at the site of codon 39 (C>T) mutation. The melting temperature for the wild type and the mutant allele were found as 71.5 ^{o}C and 66 ^{o}C, respectively.

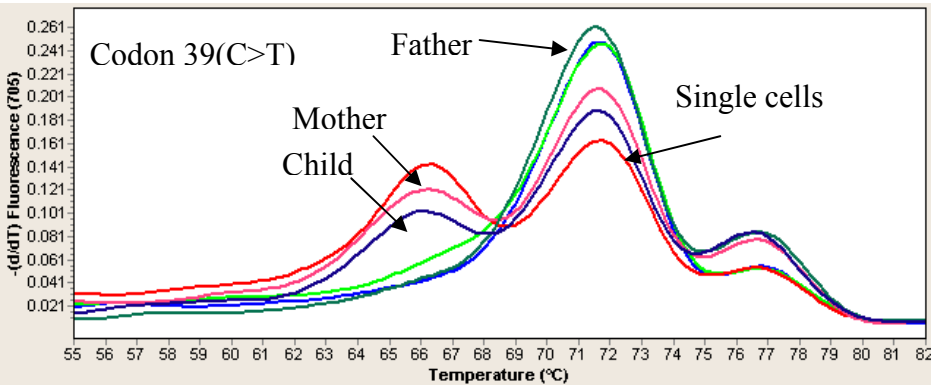

Figure 5.40. Melting peaks for codon 39 (C>T) mutation in single cells. The father, mother and child were used as controls. The father was wild type. The mother and the child were heterozygous for the codon 39 (C>T) mutation.

The codon 8/9 (+G) mutation was studied in single cells, father, mother and the child. As seen in the figure 5.41, the mother and the child were heterozygous and the father was homozygous wild type for the codon 8/9 (+G) mutation. According to this situation, the single cells were expected to be either heterozygous or homozygous wild type for this mutation. Whereas, one of the single cells which was represented by a dark green line was detected as homozygous mutant for the mutation. This could be interpretted by the allele drop out of the other allele and detection of only the mutant allele. The melting temperature of the wild type and the mutant allele were found as 65.5 ^{o}C and 57 ^{o}C, respectively.

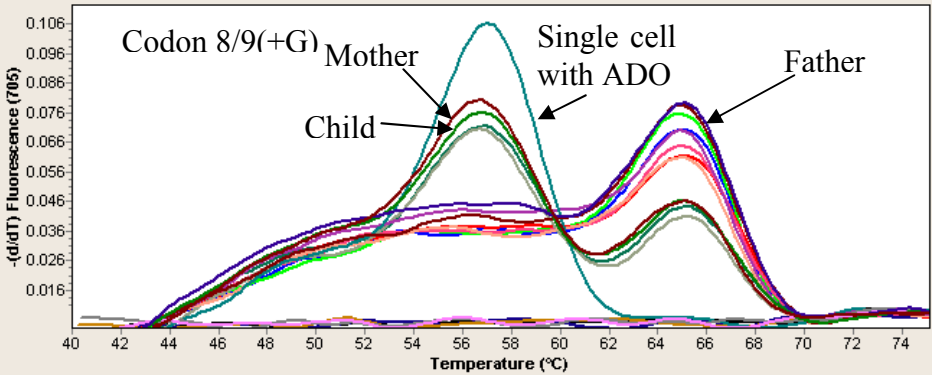

Figure 5.41. Melting peaks for codon 8/9 (+G) mutation in single cells. The father was wild type. The mother and the child were heterozygous. Allele drop out was observed in one of the blastomeres (represented by a dark green line).

Various beta-globin mutations were studied with real time PCR in single cells. The following figures were demonstrating the melting peaks of IVS I:1 (G>A), IVS I:5 (G>T), IVS I:110 (G>A) and IVS II:1 (G>A) mutations in single cells.

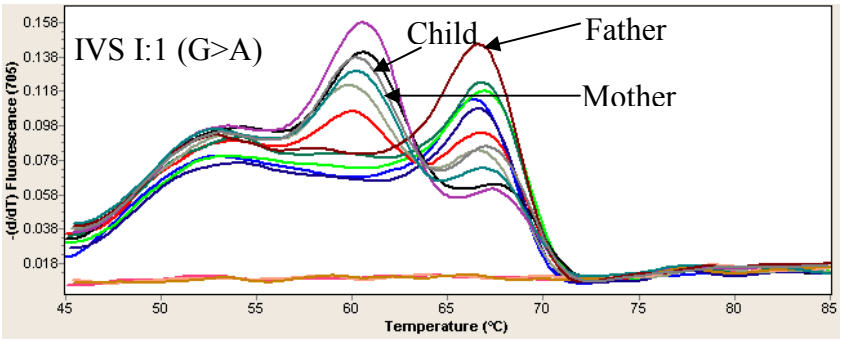

Figure 5.42. Melting peaks for IVS I:1 (G>A) mutation in single cells. The father was wild type (brown line). The mother and the child were heterozygous. The melting temperature of the wild type and the mutant allele were 68 ^{o}C and 60 ^{o}C, respectively.

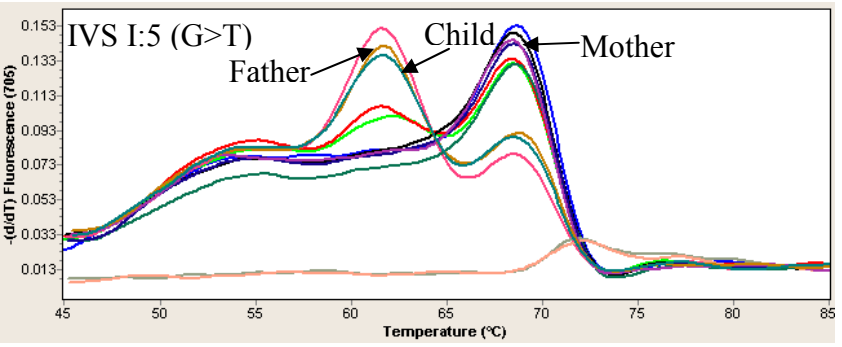

Figure 5.43. Melting peaks for IVS I:5 (G>T) mutation in single cells. Mother was wild type (violet line). The father and the child were heterozygous for IVS I:5 (G>T) mutation. Melting temperature of wild type and mutant allele were 69 ^{o}C and 62.5 ^{o}C, respectively.

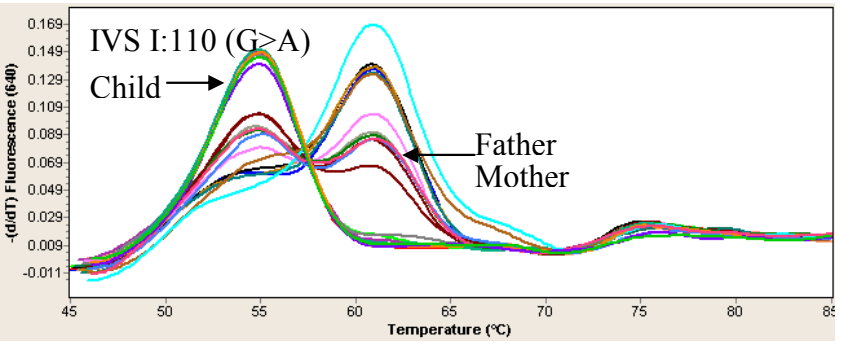

Figure 5.44. Melting peaks for IVS I:110 (G>A) mutation in single cells. The mother and the father were heterozygous. The child was homozygous. Melting temperature of wild type and mutant allele were 61.5 ^{o}C and 55 ^{o}C, respectively.

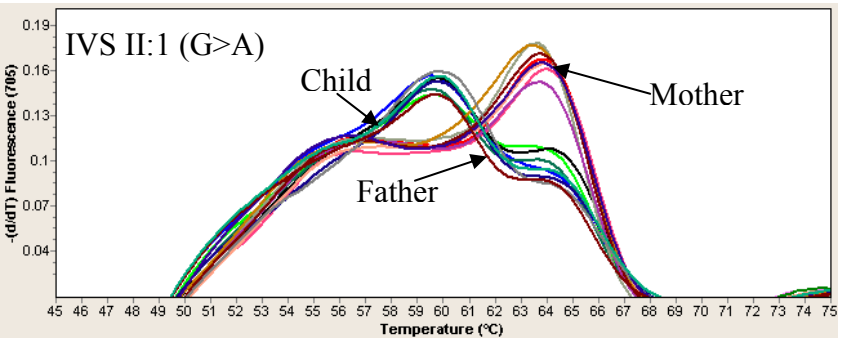

Figure 5.45. Melting peaks for IVS II:1 (G>A) mutation in single cells. The mother was wild type (dark blue line). The father and the child were heterozygous for IVS II:1 (G>A) mutation. Tm of wild type and mutant allele were 64.5 ^{o}C and 59.5 ^{o}C, respectively.

The sickle cell mutation (codon 6 (A>T)) was studied by the real-time PCR and the melting curve analysis (figure 5.46). The mother and the father were carriers of the sickle cell mutation and the child was homozygous mutant. The single blastomeres biopsied from the embryos were analyzed for the sickle cell mutation. One of the embryos (black line) was found homozygous for the codon 6 (A>T) mutation. The others were found either homozygous or heterozgous wild type. The peaks for the mother, father and the child were pointed out with the arrows. The melting temperature of the wild type allele was found as 65.5 ^{o}C. The melting temperature of the mutant allele was detected as 60 ^{o}C.

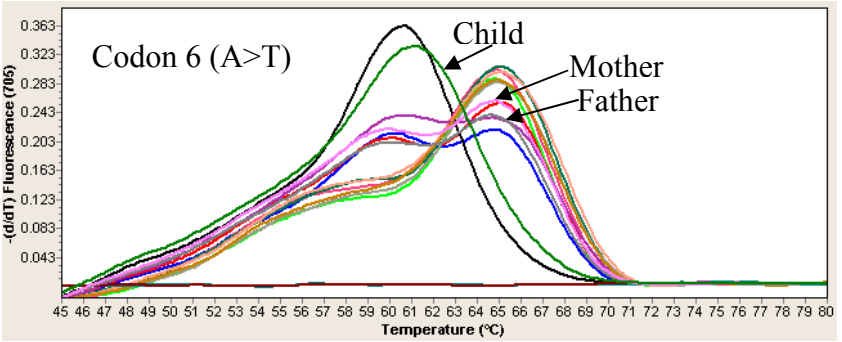

Figure 5.46. Melting peaks for codon 6 (A>T) mutation in single cells. The mother and the father were heterozygous. The child was homozygous for the codon 6 (A>T) mutation. Tm of the wild type and the mutant allele were 65.5 ^{o}C and 60 ^{o}C, respectively.

The amplification of a fragment of the beta-globin gene, in combination with the HLA genes or the microsatellites, was achieved by either the conventional PCR or the real-time PCR in single cells. As a detection method, sequencing or melting curve analysis were two alternative methods to detect the mutations in single cells. Sequencing analysis was generally prefered for the rare beta globin mutations in single cells since it does not require mutation specific probes. The probes were designed for the most common ten mutations. Therefore, the real-time PCR and the melting curve analysis was used for detection of those mutations.

5.6. Allele Drop Out was Controlled by Amplification of the Linked Microsatellites

Mutation detection in the beta-globin gene was achieved either by the real-time PCR or the sequencing analysis. The inherited mutations could be found in compound heterozgous or homozygous state in the family. In the single cells, the starting DNA is found in two copies only and the allele drop out is one of the major pitfalls of the single cell diagnostics. In the case of homozygous inheritance of the mutations, use of the linked microsatellites is needed in order to prevent misdiagnosis in the single cells. Therefore, six linked microsatellites for the beta globin gene were used in order to exclude the allele drop out in the beta-globin gene. The microsatellites were studied initially in the parents and the children in order to identify the informative ones. Generally, two or three of the informative microsatellites were used in the single cells. The microsatellites were amplified in the nested or the heminested strategy as described in the section 4.6.

The figure 5.47 displayed the capillary electrophoresis the results of the amplified D11S1331 marker in the single cells and in the human genomic DNA. The microsatellite was amplified initially with the outer primers in the single cells. Afterwards it was further amplified with the fluorescently labelled nested primers and subjected to the capillary electrophoresis. In the figure 5.47, the mother and the father were heterozygous for D11S1331. Actually, they had common allele with a size of 144 bp and this allele was found in homozygous state in the affected child. Three blastomeres were presented here. All of them were found in heterozygous state, indicating that the allele drop out of the beta globin gene was excluded in these blastomeres.

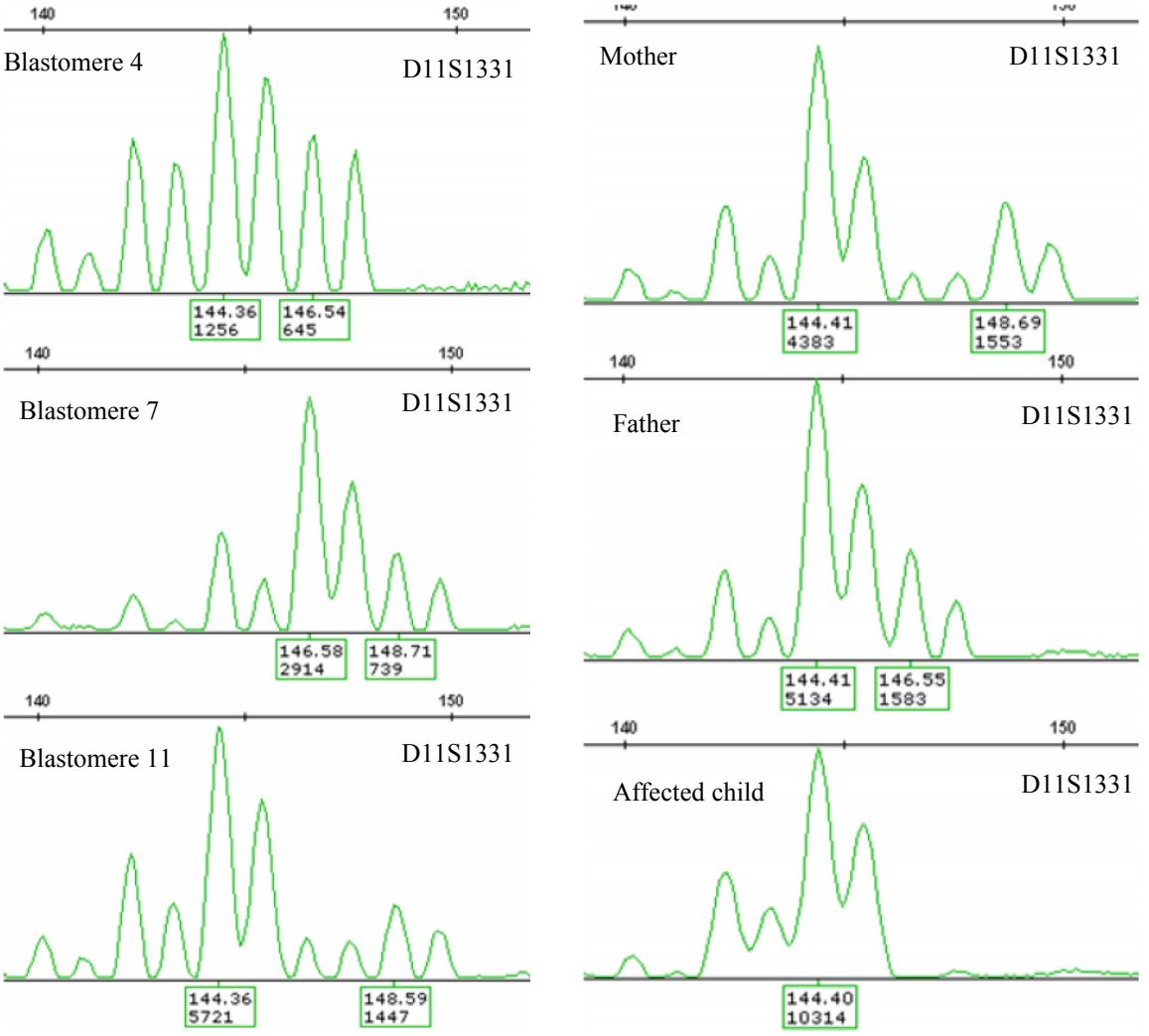

Figure 5.47. Presentation of D11S1331 marker in single cells. The three blastomeres represented here were found in heterozygous state. Allele drop out was excluded.

The D11S1997 marker was found as heterozygus for the parents and the affected child, as displayed in figure 5.48. The size of the maternal alleles were 140 bp and 149 bp. The size of the paternal alleles were 136 bp and 140 bp. Parents had one common allele size. The affected child inherited 140 bp sized paternal allele and 149 bp sized maternal allele. The blastomere four and the blastomere seven were found in heterozygous state. However, the blastomere eleven had only one allele. Since the allele size was 140 bp which was the size of common allele of parents, ADO could not be excluded or included. The microsatellite was regared as uninformative for this sample. Other microsatellites should be evaluated in order to obtain a conclusive result.

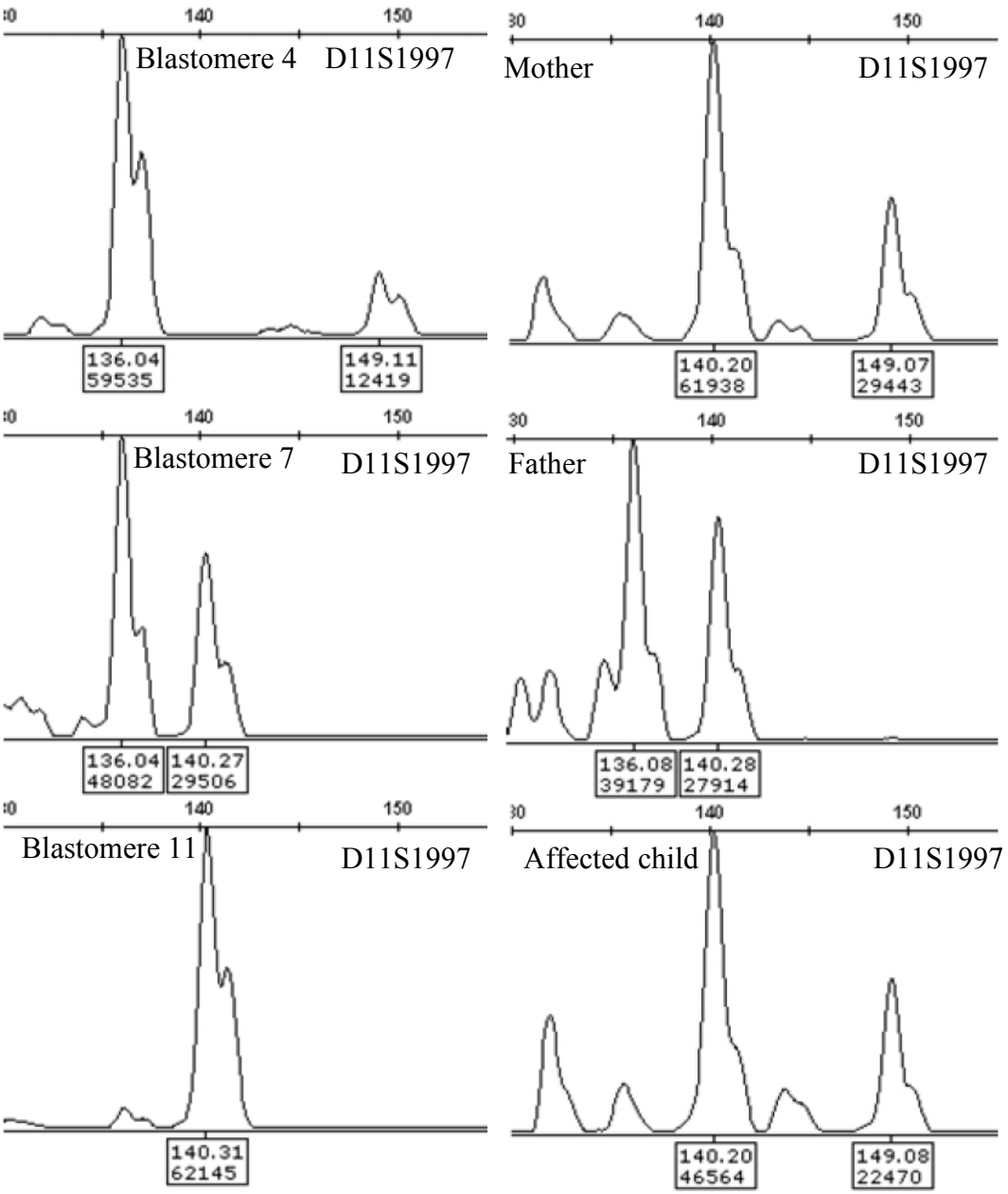

Figure 5.48. Capillary electrophoresis of D11S1997 marker in single cells after fluorescent multiplex PCR.

The figure 5.49 demonstrated alleles of D11S4149 marker in the single cells, parents and the affected child. The mother was homozygous for this allele. So all the offsprings were obliged to carry the allele with a size of 158 bp. The size of the paternal alleles were 144 bp and 156 bp. The father was found heterozygous and did not carry common allele with the mother. Therefore, all the offsprings were expected to be in heterozygous state

which was the situation seen in figure 5.49. As a result, the allele drop out of the beta globin locus has been excluded for all of the blastomeres.

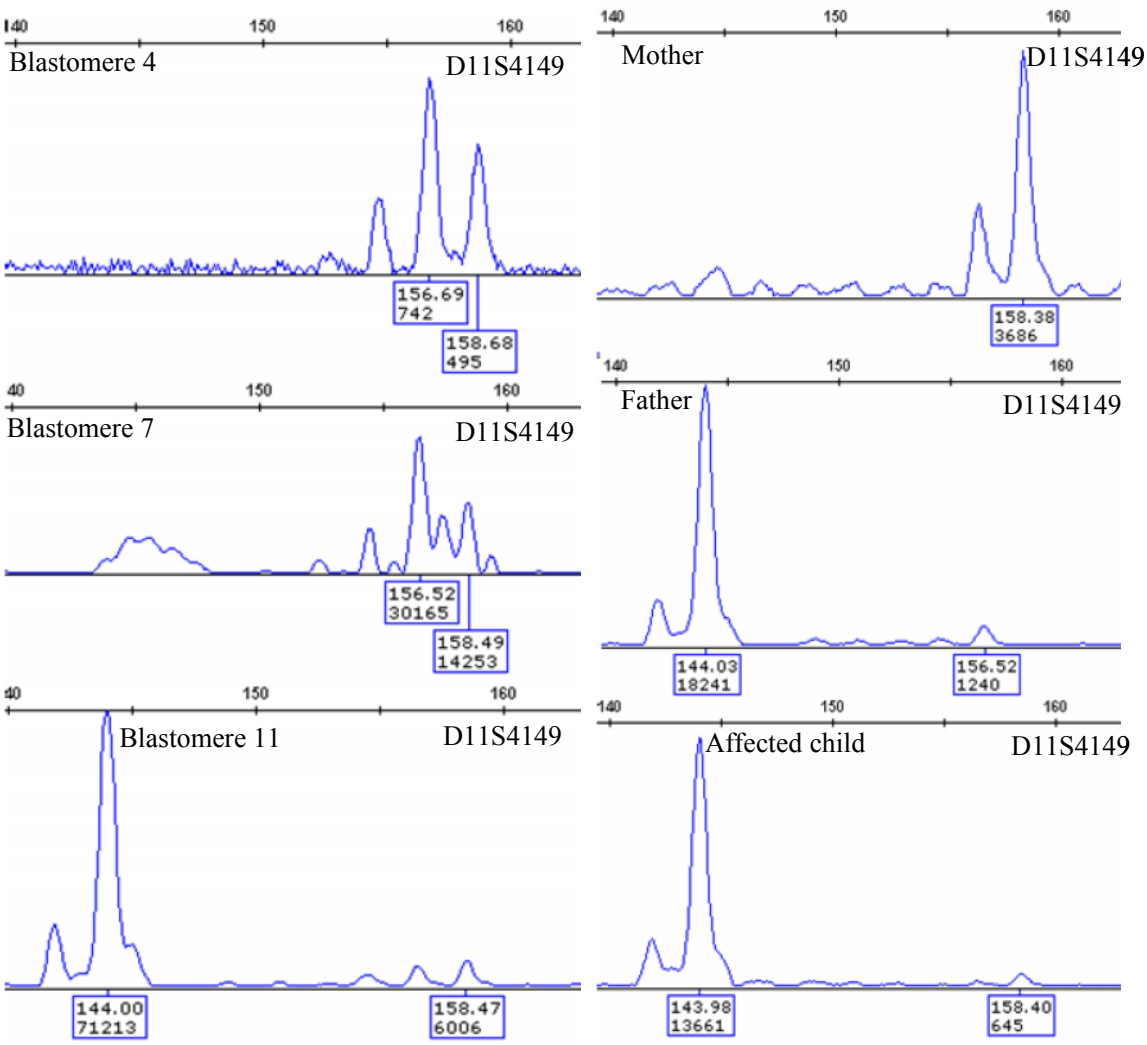

Figure 5.49. Capillary electrophoresis of D11S4149 marker in single cells after fluorescent multiplex PCR. The affected child and the three blastomeres represented here were found in heterozygous state. For this reason, allele drop out was excluded in blastomeres.

The allele drop out rate was determined as approximately 11% for the beta-globin gene in this study. The average of ADO occurrence in each PGD cycle was 1.27 with a standard deviation of 1.36.

5.7. Preimplantation Genetic Diagnosis Enabled Selection of Healthy and HLA Compatible Embryos

A total of forty-two couples were treated in this study. Among them, thirty-nine couples were undertaken the PGD cycle for the HLA typing in combination with monogenic disorders (beta-thalassemia or sickle cell disease). The remaining three couples were treated for only selection of the HLA compatible embryos. The average maternal age was 30.6 years for all the patients (table 5.3). The youngest and the oldest patients were twenty and forty-six years old, respectively.

An *in vitro* fertilization cycle includes, basically, stimulation of ovaries by intense hormone theraphy, retrieval of oocytes, fertilization of them with sperms in the laboratory and transfer of high quality and healthy embryos back to the patient for implantation. A total of 107 *in vitro* fertilization cycles were performed (table 5.3). Among them, 101 *in vitro* fertilization cycles were performed for selection of the HLA compatible and the beta-globin mutation free embryos. The other six cycles were carried out for only the HLA compatibility. The average number of the cycles performed per couple was two, suggesting that two or three *in vitro* fertilization cycles were performed for a couple. The average number of oocytes retrieved per cycle was 19.8. Approximately 12.5 oocytes were fertilized per *in vitro* fertilization cycle. A total of 1180 embryos were analyzed in total of 107 *in vitro* fertilization cycles. In each cycle, eleven embryos were analyzed in average. The number of analyzed embryos were 1103 and 77 for the beta-thalassemia/sickle cell plus HLA PGD and HLA-only PGD, respectively. After the genetic analysis, 122 embryos for beta-thalassemia/sickle cell plus HLA PGD and ten embryos HLA-only PGD were found genetically suitable for transfer. Among them, a total of 94 embryos were transferred back to the patients in 62 transfer cycles, suggesting that 58% of the cycles were completed with an embryo transfer. The ratio of cycles with transfer was reached to approximately 67% for HLA only PGDs. The average number of embryos transferred per cycle was 1.47, indicating that one or two embryos were transferred in each transfer cycle. Two embryos on average were transferred in the HLA only PGDs.

Implantation is simply the adherence of the embryo to the wall of the uterus. Since the number of embryos implanted was nineteen, the implantation rate was 20.2% (table

5.3). Among them, sixteen embryos were implanted in the PGD cycles of beta-thalassemia/sickle cell plus HLA.

A clinical pregnancy is a pregnancy which is confirmed by both high levels of human chorionic gonadotropins (hCG) and ultrasound examination of fetal heartbeats. The number of clinical pregnancies per cycle and per transfer were estimated approximately 12.2% and 21%, respectively (table 5.3). The number of clinical pregnancies per cycle and per transfer, on the other side, were reached to 33.3% and 50% for the HLA-only PGDs due to the limited number of patients in this group. As a result, sixteen pregnancies were obtained. Thirteen of them were clinical pregnancy. Beta-thalassemia/sickle cell plus HLA PGDs were resulted with eleven clinical pregnancies.

A biochemical pregnancy is only confirmed by elevated levels of human chorionic gonadotropins (hCG) and it ends in miscarriage prior to ultrasound examination. Ectopic pregnancy, on the other hand, is a complication of pregnancy in which the embryo implants outside the uterus. Two of the pregnancies were biochemical and one of them was ectopic (table 5.3). One of the pregnancies was terminated due to miscarriage. One of clinical pregnancies was terminated due to USG findings of finnish nephrosis in the fetuses. A twin pregnancy is ongoing. Therefore, ten pregnancies went to termination. Ten healthy and HLA compatible babies were born after 101 cycles of beta-thlassemia/sickle cell plus HLA PGD. Additionally, two babies were born after the HLA-only PGDs. The live birth rate was determined as 9.3% for all PGD cycles. Since 94.4% of all PGDs were applied for HLA selection with beta-globin analysis, the live birth rate was 8% for this group. On the other hand, live birth rate was 33% for the HLA-only PGDs since only six cycles were performed in this study. Whole parameters and the data were summarized in table 5.3.

Table 5.3. Clinical data for preimplantation genetic diagnosis.

Parameters	***Total PGDs***	***B-thal / SC + HLA***	***Only HLA***
No of couples treated	42	39	3
Maternal age	30.6	30.7	29
Number of cycles performed	107	101	6
Number of cycles performed per couple	2.5	2.5	2

Table 5.3. Clinical data for preimplantation genetic diagnosis (continued).

Parameters	***Total PGDs***	***B-thal / SC + HLA***	***Only HLA***
Number of oocytes retrieved per cycle	19.8	19.8	19.8
Number of oocytes fertilized per cycle	12.5	12.4	14.5
Number of embryos analyzed	1180	1103	77
Number of embryos analyzed per cycle	11	10.9	12.8
Number of cycles with transfer (%)	62 (58%)	58 (57%)	4 (66.6%)
Number of embryos suitable for transfer	132	122	10
Number of embryos transferred	94	86	8
Transferred embryos per cycles with transfer	1.47	1.48	2
Number of embryos implanted	19	16	3
Implantation rate	20.2%	18.6%	37.5%
Number of pregnancies	16	13	3
Number of clinical pregnancies	13	11	2
Clinical pregnancies per cycle	12.2%	10.9%	33.3%
Clinical pregnancies per transfer	21%	19%	50%
Number of miscarriages	2	2	0
Number of biochemical	2	1	1
Number of ectopic pregnancies	1	1	0
Number of pregnancies ongoing	1 (twin)	1 (twin)	0
Number of pregnancies went to term	10	8	2
Number of babies born	12	10	2
Live birth rate per cycle	9.3%	8%	33%

All the PGD cycles were performed for selection of the HLA matched embryos. A total of 101 cycles (94.4%) were applied for the selection of the HLA matched and sickle cell or beta-thalassemia free embryos (Table 5.4). Among 1180 analyzed embryos, 183 embryos were found uninformative after the genetic analysis due to the allele drop out, the amplification failure and contamination. Therefore, average number of uninformative cells per cycle was 1.7 for all PGD cycles. Since the number of cells were very limited for HLA-only PGDs, it reached to 2.3 cells. As a result, 997 cells were genetically analyzed and found informative for all the PGD cycles.

Table 5.4. Genetic analysis data of preimplantation genetic diagnosis cycles

Parameters	***Total PGDs***	***B-thal / SC + HLA***	***Only HLA***
Number of cycles performed	107	101	6
Number of embryos analyzed	1180	1103	77
Number of uninformative cells	183	169	14
Number of uninformative cells per cycle	1.7	1.7	2.3
Number of cells with informative PCR signal	997	934	63
Total number of HLA compatible embryos resulted	201	190	11
Number of HLA compatible embryos per cycle	1.9	1.9	1.8
Ratio of HLA compatible embryos to embryos with informative PCR signal	20.1%	20.3%	17.5%
Total number of HLA compatible and unaffected embryos	NA*	122	NA
Number of HLA compatible and unaffected embryos per cycle	NA	1.2	NA
Ratio of HLA compatible and unaffected embryos to embryos with informative PCR signal	NA	13%	NA

**NA: Not applicable.*

Complete HLA haplotype could be constituted by the microsatellite analysis at the extended HLA locus. Since the SSP typing of the single cells determine only targetted alleles and the embryos with no amplification for the targetted allele were eliminated, complete HLA haplotype could not be determined. Therefore, the data were not available for complete HLA haplotype. On the other hand, selection of 201 HLA compatible embryos was achieved by both methods. Under normal conditions, the probability of finding of an HLA compatible embryo is 25%. In our study, the ratio was found as 20% among the embryos with informative result. In average, 1.9 embryos were found HLA compatible in each PGD cycle. A total of 122 embryos were evaluated as the HLA compatible and unaffected by sickle cell disease or beta-thalassemia. The average number

of HLA compatible and unaffected embryos per cycle was 1.2. When the number of embryos with informative PCR signal was considered, 13% of the embryos were found healthy and HLA compatible. The probability of finding an healthy and HLA matched embryo is 3/16 or 18.75%.

5.8. HLA Haplotyping Revealed Recombination Frequencies and Unique Patterns at MHC Locus in Single Cells

Fourteen microsatellites through the extended human MHC locus encompass approximately 12.2 Mb region. The location and the coordinates of microsatellites were shown in the figure 5.50. All the microsatellites have been analysed by capillary electrophoresis of PCR amplified fluorescent fragments from the PGD candidate couples and their children to determine the heterozygous alleles. After selection of informative microsatellites covering the whole MHC, the single cells from the embryos of the family have been analyzed for the recombinations beside the analysis for the HLA compatibility. The target region comprises the class I (HLA-A, B and C), class II (HLA-DRB1, DQA1, DQB1, DPA1 and DPB1) and the class III (non HLA genes) genes. The target region extends 3.2 Mb centromeric from the HLA-DPB1 gene in the class II and 5.7 Mb telomeric from the HLA-A gene in the class I. The region between the microsatellites D6S2972 and D6S2810 (1.7 Mb) has been regarded as the class I region, between D6S439 and D6S2883 (0.67 Mb) as the class II, and between D6S2810 and LH-1 (0.99 Mb) as the class III.

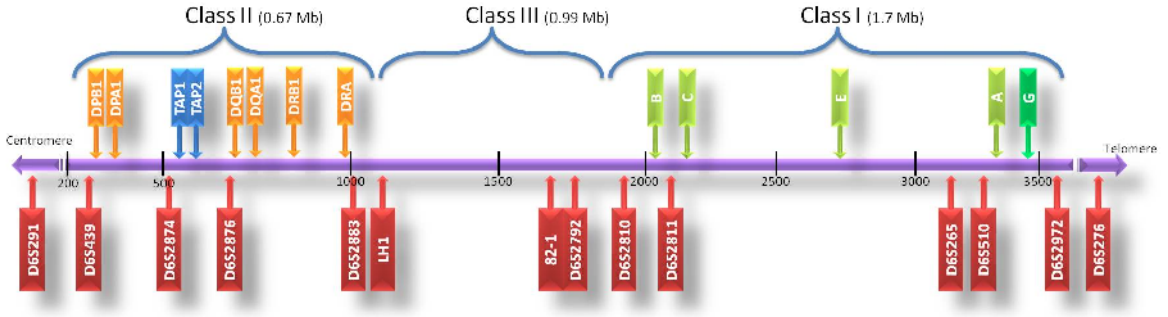

Figure 5.50. Location of microsatellites used in this study and HLA genes at HLA locus. The class I and the class II genes were shown with green and orange arrows, respectively. The microsatellites were demonstrated with red arrows.

Study of at least nine informative microsatellites in this region enabled to estimate the meiotic recombination rate at the extended HLA locus in the PGD embryos. For this purpose, a total of 366 cells from twenty six couples were taken into this study. These embryos were obtained from forty-eight *in vitro* fertilization cycles. The microsatellite typing results of a couple and their six embryos were shown in the figure 5.51 as representative of twenty-six couples and 366 embryos studied. The paternal allele of embryo 206 has been different from the original paternal alleles in that the region between LH-1 and D6S439 has been exchanged with the same region of the other paternal allele. The allele has been recombined a region between LH-1 and 82-1 microsatellites which are located within the class III region. Therefore, the whole class II genes have been expected to be recombined. Any other recombination of the target region has not been observed in the other represented embryos of this couple.

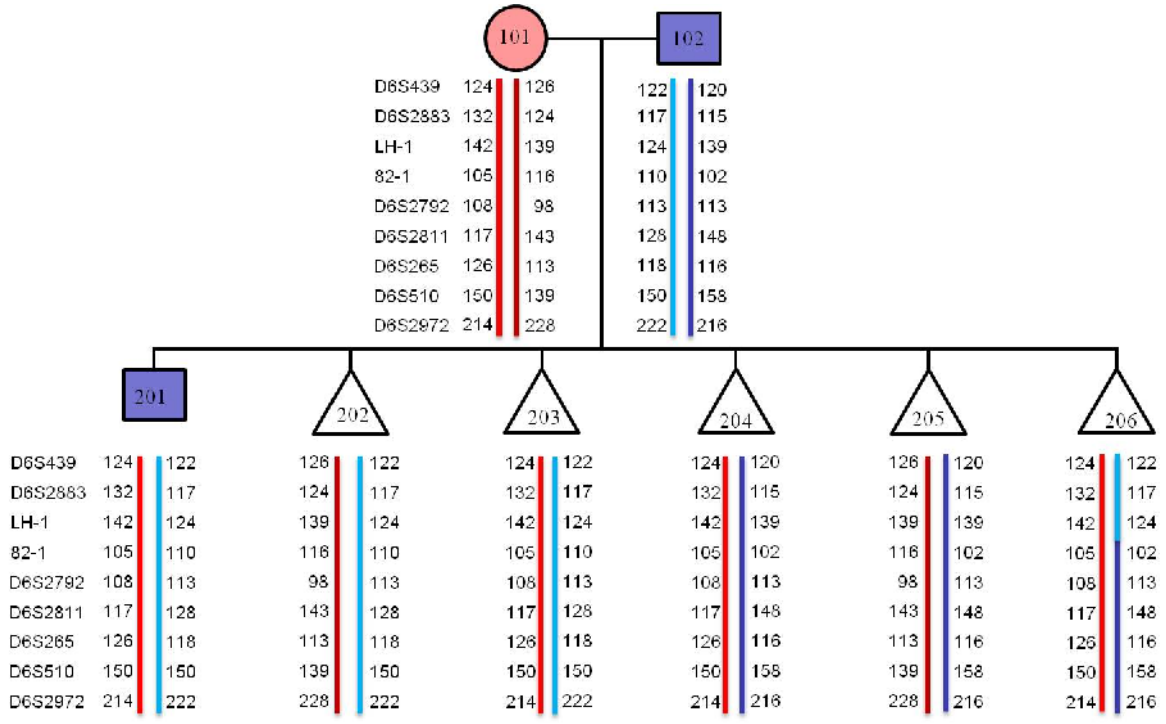

Figure 5.51. Haplotype analysis of extended MHC. A recombination at one of the paternal alleles was detected. Embryos were depicted with triangle. Maternal alleles were shown with red and brown lines. Paternal alleles were demonstrated with blue and dark blue lines.

Total of 39 recombinant MHC alleles have been observed in 366 embryos. Average recombinant allele frequency in the whole extended MHC region of 12.2 Mb was 5.33%

with the recombination rate of 0.44 cM/Mb (table 5.5). The recombinant allele frequencies were on average 1.23% (0.17 cM/Mb) for the extended class I, 3.69% (0.92 cM/Mb) for the extended class II and 0.41% (0.41 cM/Mb) for the class III.

Table 5.5. Total number of chromosomes with recombination and corresponding recombination rates.

		Total	*Ratio (%)*	*Region size (Mb)*	*cM/Mb*
TOTAL	Recombinations at HLA	39	5.33	12.20	0.44
	Extended Class I region	9	1.23	7.30	0.17
	Extended Class II region	27	3.69	4.00	0.92
	Class III region	3	0.41	0.99	0.41
MATERNAL	Recombinations at HLA	31	8.47	12.20	0.69
	Extended Class I region	9	2.46	7.30	0.34
	Extended Class II region	21	5.74	4.00	1.44
	Class III region	1	0.27	0.99	0.27
PATERNAL	Recombinations at HLA	8	2.19	12.20	0.18
	Extended Class I region	0	0.00	7.30	0
	Extended Class II region	6	1.64	4.00	0.41
	Class III region	2	0.55	0.99	0.55

The recombinations were detected in thirty-one maternal chromosomes and in eight paternal chromosomes (table 5.5). The recombination rate for the chromosomes with maternal origin was 8.47% (0.69 cM/Mb). On the other hand, the recombination rate was reduced to 2.19% (0.18 cM/Mb) for the paternal chromosomes, suggesting that 3.87 fold difference was observed in the recombination rate between the maternal and the paternal chromosomes. All the paternal recombination events were detected at the extended class II region. Among them, two recombinations were extended to the class III region since the recombination has been detected at the LH-1 marker. As a result, the recombination rate of the paternal chromosomes at the class II and the class III regions were 1.64% (0.41 cM/Mb) and 0.55% (0.55 cM/Mb), respectively. Nine maternal recombinations were detected at the extended class I region which extends 7.3 Mb. The maternal recombination rate for this region was calculated as 2.46% (0.34 cM/Mb). Twenty-one maternal recombinations were detected at the extended class II region. Moreover, one of these

recombinations were extended to the LH-1 marker which is located at the class III region. The recombination rate was, therefore, estimated as 5.74% (1.44 cM/Mb) for four megabases for the extended class II region. The ratio was 0.27% (0.27 cM/Mb) for 0.99 Mb region covering the class III region in the maternal chromosomes.

Significant variations in the recombination rates have been observed inside 12.2 Mb extended MHC region among the individuals (table 5.6). No recombination in any allele was observed in total of ninety embryos from nine couples. The disparity was found fourteen fold with the recombination rates ranging on average 0.19 – 2.73 cM/Mb among the families (cases A and C) when 276 embryos were analysed from seventeen couples. The variations in individual recombination rates were also observed within the alleles from the same gender. There were no recombinations at the maternal alleles of 142 embryos from twelve couples and the paternal alleles of 252 embryos from nineteen couples. The range of variation between couples was as high as fourteen fold (0.39 – 5.46 cM/Mb) for the maternal alleles (cases C and N) and 2.6 fold (0.37 – 0.96 cM/Mb) for the paternal alleles (cases A and D).

Table 5.6. Number of recombinations and recombination frequencies detected in maternal and paternal alleles of each cases.

	Total				***Maternal***			***Paternal***		
Cases	***No of Embryos***	***No of Recombinant Alleles***	***Recombination Rate (%)***	***cM/Mb***	***Number of Recombinant Alleles***	***Recombination Rate (%)***	***cM/Mb***	***Number of Recombinant Alleles***	***Recombination Rate***	***cM/Mb***
A	22	1	2.27	0.19	0	0.00	0.00	1	4.55	0.37
B	14	2	7.14	0.59	1	7.14	0.59	1	7.14	0.59
C	3	2	33.33	2.73	2	66.67	5.46	0	0.00	0.00
D	17	2	5.88	0.48	0	0.00	0.00	2	11.76	0.96
E	13	1	3.85	0.32	0	0.00	0.00	1	7.69	0.63
F	14	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
G	19	1	2.63	0.22	1	5.26	0.43	0	0.00	0.00

Table 5.6. Number of the recombinations and recombination frequencies detected in maternal and paternal alleles of each cases (continued).

	Total				*Maternal*			*Paternal*		
Cases	***No of Embryos***	***No of Recombinant Alleles***	***Recombination Rate (%)***	***cM/Mb***	***Number of Recombinant Alleles***	***Recombination Rate (%)***	***cM/Mb***	***Number of Recombinant Alleles***	***Recombination Rate***	***cM/Mb***
H	18	2	5.56	0.46	2	11.11	0.91	0	0.00	0.00
I	6	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
J	11	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
K	12	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
L	3	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
M	15	3	10.00	0.82	3	20.00	1.64	0	0.00	0.00
N	21	2	4.76	0.39	1	4.76	0.39	1	4.76	0.39
O	11	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
P	17	2	5.88	0.48	2	11.76	0.96	0	0.00	0.00
Q	11	4	18.18	1.49	4	36.36	2.98	0	0.00	0.00
R	16	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
S	22	2	4.55	0.37	2	9.09	0.75	0	0.00	0.00
T	9	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
U	50	7	7.00	0.57	7	14.00	1.15	0	0.00	0.00
V	4	2	25.00	2.05	2	50.00	4.10	0	0.00	0.00
W	16	2	6.25	0.51	1	6.25	0.51	1	6.25	0.51
X	3	1	16.67	1.37	1	33.33	2.73	0	0.00	0.00
Y	8	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
Z	11	3	13.64	1.12	2	18.18	1.49	1	9.09	0.75
Total	**366**	**39**	**5.33**	**0.44**	**31**	**8.47**	**0.69**	**8**	**2.19**	**0.18**

The haplotype analysis has allowed to define the regions of the recombination at the extended MHC locus (figure 5.52). The recombination breakpoints have been resolved to regions of 0.670 – 5.7 Mb for 39 recombinant alleles. Three of the nine recombinations within the extended class I region were involving a region of the class I HLA genes whereas the other six (cases H, Q, U, X, Z) were far telomeric regions (5.5 Mb) that have not hit the HLA class I exons. The breakpoints in twenty of the twenty seven recombinations at the extended class II region have been mapped to a region between the DRB1 and the DPB1 exons covering the TAP1 and the TAP2 genes whereas the other seven breakpoints (cases D, Q, W) have been mapped far centromeric (3.2 Mb) to the

extended class II region, beyond the DPB1. The breakpoints of only three recombinations (cases E, M, N) have been mapped to the class III region. The low amount of DNA from the single blastomere and the limited number of the informative microsatellites restricted the finest microsatellite mapping of the recombination breakpoint. However, the recombination hotspot has been previously mapped to the second intron of the TAP2 gene within the MHC II region (Cullen *et al.,* 1995). Our observation is inline with the previous finding since 74% of the recombinations at class II region has covered the TAP2 region.

The microsatellites which were located at the centromeric or the telomeric side of the HLA locus showed the highest recombination frequency. The region flanking between D6S439 and D6S2972 encompasses approximately 3.4 Mb region of the HLA locus. In cases, where D6S439 was uninformative, D6S291 marker was used to determine the recombinations. The recombinations at D6S291, D6S439 and LH-1 were observed in one maternal (case M) and two paternal originated chromosomes (cases E and N). The recombinations at D6S2876 and D6S2883 were encountered at only paternal chromosomes. The microsatellites with recombinations observed at only maternal chromosomes were D6S510, D6S276 and D6S2972 (Figure 5.52).

Among the thirty-one maternal recombinant chromosomes, seventeen (55%) of them have been mapped to the region between D6S439 and LH-1 markers, which extends approximately 0.97 Mb and covers the TAP2 gene. Four recombinant chromosomes of the maternal origin (13%) were mapped to 3.2 Mb region between the D6S439 and D6S291 which is at the centromeric side of the class II region. One recombination was localized to a region of the class I between D6S2811 and D6S510 microsatellites (approximately 1.5 Mb). Six of the maternal recombinations (20%) were mapped to a region between D6S2972 and D6S276 which extends approximately 5.5 Mb at the telomeric side of the class I region. Finally, one maternal recombination was localized to the class III region.

Five recombinant chromosomes of the paternal origin (63%) were mapped to 3.9 Mb region between LH-1 and D6S291 microsatellites which covers the TAP2 recombination hotspot region. It is, therefore, expected that the recombinations have been occurred at the TAP2 hotspots and the alleles of the DPA1 and the DPB1 genes have been affected. One of the recombinations was localized to 3.2 Mb region between D6S439 and D6S291 which

is at the centromeric side of the class II region. Additionally, two recombinations were extended to the class III region. No paternal recombination was observed at the extended class I region.

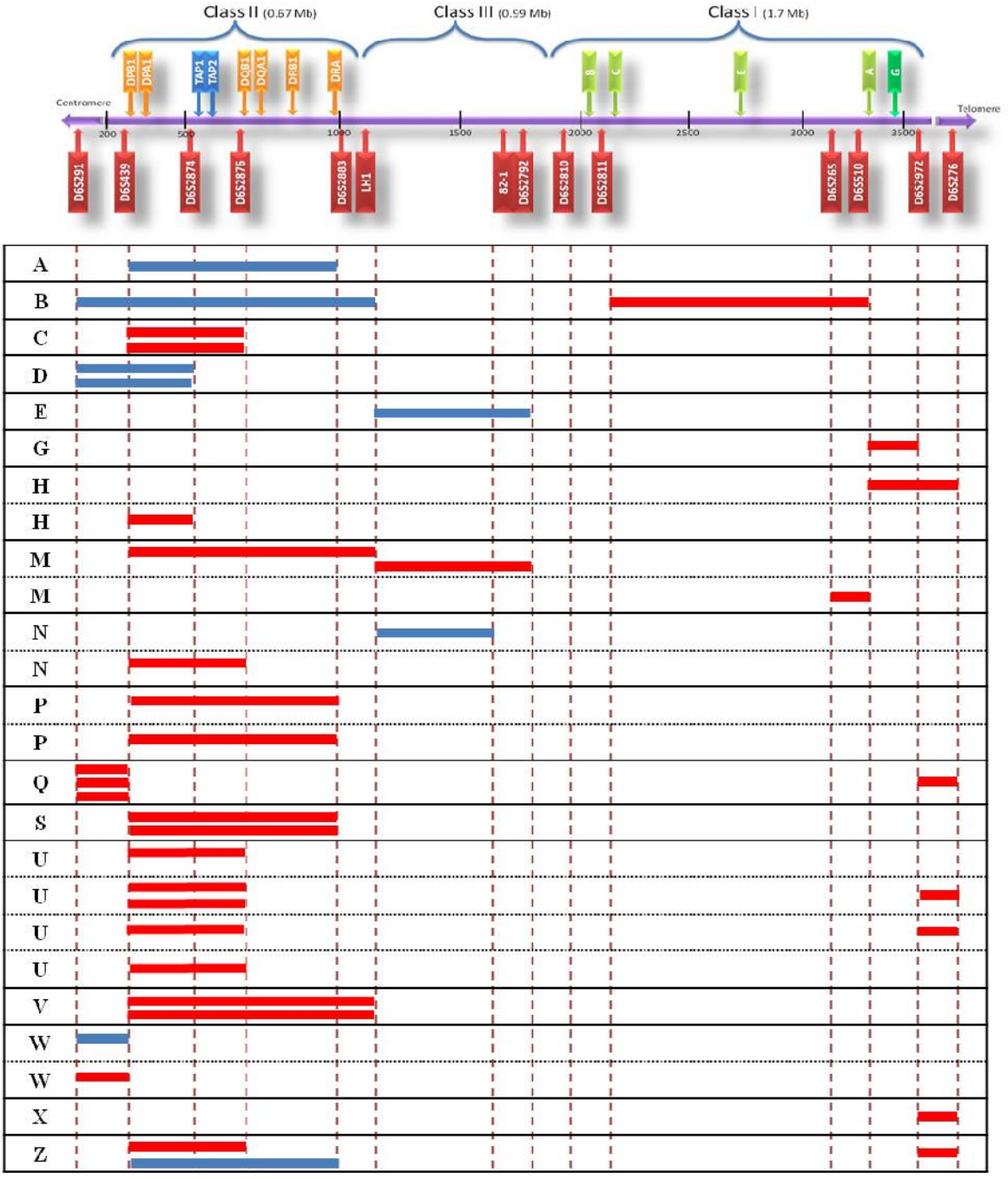

Figure 5.52. Location of each recombination has been mapped on extended MHC for each embryo. Red and blue bars represent chromosomes of maternal and paternal origin, respectively. The couples were identified with capital letters at the beginning of each row. Different cycles of the couples were separated from each other with horizontal dotted line.

The HLA class I (green arrows) and class II genes (orange arrows) and the microsatellites (dark red arrows) were shown in the HLA map.

6. DISCUSSION

6.1. Evaluation of Amplification in Single Cells

The analysis of single cells require use of distinct molecular biology methods to reach a conclusive genetic test result. For this purpose, different molecular biology methods have been adapted for single cell use in this study. This study covered the use of different polymerase chain reactions and the detection methods in single cells. As a diagnostic model, analysis of the beta-globin mutations and the HLA typing in single cells have been performed.

We targetted to identify the single nucleotide variations such as point mutations and small insertions/deletions, therefore, this study focused on the establishment of amplification techniques in single cells. Other genetic variations require use of different molecular biology techniques in single cells. Gross chromosomal changes such as translocations and aneuploidy screening have been analysed by fluorescence *in situ* hybridization technique (Harper and Wells, 1999). Comparative genomic hybridization has been used as an alternative of FISH in order to detect chromosomal abnormalities in single cells. However, its use has been limited in single cells compared to that of FISH (Voullaire *et al.,* 1999).

In this study, multiplex PCR has been established for the simultaneous amplification of different gene regions in the single cells. After the complete lysis of the cells with proteinase K / SDS lysis buffer, at least four genomic regions were amplified from the single cell DNA. The regions included a partial fragment of beta-globin gene and relevant regions of the HLA genes and/or informative microsatellites located at these regions. Use of nested and hemi-nested amplification strategies enabled detection of the HLA alleles and further amplification of the target region of the beta-globin gene. Fluorescent multiplex nested PCR strategy has been applied to amplify the microsatellite markers. Moreover, in PGD of single gene disorders, use of linked microsatellites allows identification of the genotype in the cases that the disease causing mutation has not been determined. Therefore, the method combined with the fluorescent multiplex PCR and the

capillary electrophoresis can be easily applied in order to analyse various types of mutations indirectly without any futher optimization (De Vos *et al.,* 1998, Apessos *et al.,* 2001, Piyamongkol *et al.,* 2001).

The amplification in the single cells has some obstacles which are not encountered in the amplification of the conventional DNA samples. The problems arise from the copy number of the DNA as it is found only two copies in the single cells. As the number of target regions to be amplifed increases, the amplification efficiency may decrease due to the increasing concentration of the primers, thermodynamics of the reaction and the competition of the primers to bind the target. Therefore, the PCR conditions at each step are extremely sensitive to any alteration in the single cells. The amplification failure, allele drop out, contamination and the preferential amplification are the major drawbacks of the multiplex PCR in the single cells. The preferential amplification is generally termed for inefficient amplification of one of the alleles. Usually shorter alleles are amplified efficiently in the case of amplification of heterozygous microsatellites. On the other hand, the amplification failure is generally caused by loss of the cell during the transport of cell into the PCR tube in IVF laboratory and incomplete cell and nucleus lysis. Two regions of the beta globin gene have been previously amplified with an amplification efficiency of 79% in the single cells (Hussey, *et al.,* 2002). In this study, as the total amplification failure was 14%, general amplification efficiency was determined as 86%. A general amplification efficiency of 84.4% in single gene disorders has been previously reported (Gutiérrez-Mateo *et al.,* 2009). At this point, whole genome amplification is considered as a promising application in the recent years. Although the allele drop out and the preferential amplification have been reported as drawbacks of the whole genome amplification (WGA), multiple displacement amplification showed the best amplification results in single cells compared to other whole genome amplification methods which are known as primer extension preamplification and degenerate oligonucleotide primed PCR (Handyside *et al.,* 2004, Hellani *et al.,* 2005, Spits *et al.,* 2006, Renwick *et al.,* 2006). The WGA allows study of more than twenty regions (Handyside *et al.,* 2004, Hellani *et al.,* 2005). Therefore, it is suitable in single cells to couple PGD with aneuploidy screening or amplify more than fifteen informative linked microsatellites.

The allele drop out is a significant concern of the single cell PCR. In order to prevent the misdiagnosis, mutation analysis has been coupled with linked microsatellites as an internal control for ADO in the single cells (Rechitsky *et al.,* 1998, Fiorentino *et al.,* 2006). An average ADO rate of 7.5% has been reported previously (Fiorentino *et al.,* 2006). Reported ADO rates showed variations from one laboratory to another. In this study, ADO rate was reported as 8.98% for HLA locus and 11% for beta-globin gene. These results are concordant with the literature. However, a slightly higher ADO rate was observed for the beta-globin region compared to the HLA locus. The length of the beta-globin region to be amplified, characteristics of the primers and beta-globin sequence may influence the ADO rate. In autosomal recessive conditions, the single cells with a genotype of homozygous wild type were considered to be unaffected although mutant allele could not be detected due to the allele drop out. The major risk was encountered in the autosomal dominant conditions. Since the detection of homozygous wild type genotype did not exclude the allele drop out of the mutant allele by the mutation analysis, use of informative linked microsatellites was of utmost importance to prevent the misdiagnosis. In this study, since beta-globin mutations were inherited in autosomal recessive manner, use of informative linked microsatellites were decided according to the number and the amount of the primers used in the initial multiplex PCR. The presence of inherited mutations in compound heterozygous or homozygous condition in the offsprings affected the decision on use of linked microsatellites.

Additionally, haplotype analysis allowed detection of the contamination in the single cells. The fingerprint of the contaminant does not match with the alleles of the parents. The contamination rates in the single cells have been reported between 1.8% to 8.1% for eight laboratories (Gutiérrez-Mateo *et al.,* 2009). On the other hand, presence of an additional parental allele could be interpretted as trisomy of the chromosome on which microsatellite is located. As the maternal age increased, the occurence of aneuploidy increases proportionally (Hassold and Jacobs, 1984). Trisomy of chromosome six has been reported between 1.5% to 2% (Van de Velde, *et al.,* 2009). Detection of only one set allele which belongs to one of the parents could be evaluated as monosomy or uniparental disomy. The monosomy of chromosome six and uniparental disomy have been reported between 3.7% to 10.3% and 0.3% to 0.5%, respectively (Van de Velde, *et al.,* 2009). This data have not been evaluated for the rate of trisomy, monosomy and uniparental disomy. Since they are

not clinically significant, all the embryos which were affected either of the aneuploidy were eliminated.

6.2. Development of Conventional HLA Haplotyping Techniques for Single Cells

The need of HLA typing in the single cells arises from the need of an HLA compatible donor for an affected child waiting for the stem cell transplantation. HLA typing in the single cells has been first reported in 2001 by Yury Verlinsky for the preimplantation genetic diagnosis of Fanconi anemia (Verlinsky *et al.,* 2001). In that study, preimplantation genetic testing for IVS4+4A>T mutation has been performed in order to select the unaffected embryos. Twenty four embryos have been found unaffected and tested for the HLA-A (A*02 and A*26) and the HLA-B (B*35 and B*44) genes in order to determine the HLA compatible embryos. Only the class I genes (HLA-A and HLA-B) have been amplified and the class II genes have not been tested.

In this study, alternative strategies were used in the single cells to determine the HLA compatibility. The first method was based on amplification of the HLA alleles in the single cells by using the sequence specific primers and the selection of HLA compatible cells due to the amplification status of the HLA alleles. This method provided basically a low resolution HLA typing. This strategy is based on amplification of the alleles of the HLA-A and the HLA-DRB1 genes which are expected to be inherited from the parents. The strategy is successful to amplify each the HLA-A and the DRB1 alleles. The HLA typing by using the sequence specific primers has an advantage of direct determination of the HLA alleles in the single cells. The most polymorphic exons were amplified with the consensus primers initially in the single cells. It requires the post PCR processes which raise the risk of contamination after the PCR. The amplification of the HLA alleles with the sequence specific primers provided information from only two genes with eight alleles simultaneously. The determination of the HLA alleles requires different PCR protocols and optimizations for each allele which, in turn, may require too much laboratory work and time. Moreover, as each family has distinct combinations of the HLA alleles, family specific amplification strategies have to be developed.

An obstacle of this strategy was emerged when the co-amplifying alleles were inherited together. The amplification of alleles with high sequence homology could be resulted in misdiagnosis or misinterpretation. Therefore, the co-amplification of the alleles with high sequence homology directed the study to sequence the amplified products to resolve the ambiguity. Sequencing of the HLA alleles provided a high resolution HLA typing.

Since the HLA locus extends 3.3 Mb region, it is vulnerable to the recombinations. As the HLA-DRB1 and the HLA-A genes are located at the centromeric and telomeric side of the HLA locus, respectively, the allele typing of both genes provides partial typing of the HLA locus without excluding the recombination in or around the HLA locus. This method failed to detect the recombinations, since only targetted HLA alleles were amplified and the status of the other HLA genes could not be evaluated. Complete the HLA locus could not be analyzed for all HLA alleles. The analysis of the HLA-DRB1 and the HLA-A alleles were inefficient to determine the recombinations at the HLA locus. The results may lead to misdiagnosis or incomplete HLA typing.

Moreover, patient specific amplification has to be followed in both strategies. Therefore, selection of the HLA compatible embryos could be achieved by eliminating the incompatible ones through successive amplifications of targetted HLA-A and DRB1 alleles. The extended HLA locus could not be determined due to the analysis of the selected HLA A and DRB1 alleles. Additionally, the amplification of all HLA alleles of the HLA A, B, C, DP, DQ and the DR genes is not possible in this method in the single cells, technically. For this reason, an alternative method which covers whole HLA locus for selection of the HLA compatible embryos has to be needed for more accurate diagnosis.

6.3. Determination of Heat Dissociation For Rapid Resolution of Single Nucleotide Polymorphisms Allowed Efficient and High Range Analysis

The strategies to analyze the beta-globin mutations genetically in the single cells have been developing for approximately twenty years in order to establish a rapid, accurate

and reliable method. In this study, direct sequencing and real-time PCR methods were established succesfully in combination with the HLA match in the single cells.

In this study, 1103 single cells biopsied from the embryos were analyzed for the beta-globin mutations coupled with the HLA-compatibility testing in order to prevent from either the sickle cell disease or the beta-thalassemia and to select the HLA compatible embryos. Since the inherited beta-globin mutations in the family and the affected child were detected by sequencing analysis prior to analysis in the single cells, targetted mutation analysis was preferred. The beta-globin gene which contained the mutations was amplified in two steps. An initial multiplex PCR and a following nested PCR were applied in order to get a successful amplification. The mutation detection was achieved by either sequencing or melting curve analysis for the beta-globin mutations in the single cells. Direct sequencing of the single cell PCR products to detect the beta-globin gene was achieved long after the initial attempts of PGD for the beta-globin mutations. Sequencing of all the amplified beta-globin fragments were achieved although the amplification efficiency was reported as 79% (Hussey, *et al.,* 2002). All the amplified fragments of the beta-globin gene were sequenced with a 100% success in this study. Although direct sequencing is a very reliable method, it requires laborious work and time to get the final results. Therefore, real-time PCR was optimized to detect the beta-globin mutations in the single cells in this study. Technically, real-time PCR coupled with melting curve analysis is a more suitable technique for the single cell diagnostics since it is single step, easy and rapid for the mutation detection.

Ten different beta-globin mutations have been detected by heat dissociation of the DNA probe from the amplified fragment of the beta-globin gene. Moreover, this method has been applied to the identification of the HLA alleles by using hybridization probes in the single cells for the first time. Highly polymorphic sequence of the HLA genes allowed to develop a new, rapid and alternative method.

Development of the embryos at the laboratory conditions are time-restricted and they have to be transferred at the very latest day five of fertilization. Therefore, a more rapid amplification and genotyping strategy has to be developed before the embryos stop development *in vitro* conditions. The real-time PCR and the melting curve analysis based

HLA analysis is similar to that of the sequence based HLA typing in the single cells. The technique enabled direct detection of the amplified alleles without any need of gel electrophoresis and sequencing analysis for ambigous allele combinations. Since the HLA typing is fundamental prior to stem cell and organ transplantation, the real-time PCR for HLA typing provides a rapid and reliable determination of the HLA alleles in the donor candidates and the recipient. Rapid typing of the HLA-A, B and DRB1 alleles were established by using the sequence specific primers in combination with either the hybridization or the TaqMan probes in the cell lines and the clinical samples (Slateva *et al.,* 1998, Slateva, *et al.,* 2001, Casamitjana *et al.,* 2005, Faner *et al.,* 2006). A novel strategy which uses the allele specific hybridization probes in combination with previously published sequence specific primers for the HLA-A and the HLA-DRB1 alleles in the single cells has been developed. This strategy provided a highthroughput and high resolution allele match between the embryos and the affected child since it has the ability to discriminate the co-amplifying alleles. Actually, co-amplifying alleles have to be sequenced when they are amplified with PCR-SSP method.

Although the real-time PCR based strategy has some advantages over sequence specific amplification method, both methods fail to determine the allele drop out in the single cells and the recombinations at the HLA locus which could be resulted in misdiagnosis. Since the strategy is based on positive amplification of the allele, it has inability to differentiate the negative allele and the allele drop out. Therefore, the allele drop out could not be assessed by this method.

6.4. Microsatellites of the Extended HLA Locus Could Provide Diagnostic Level Standardisation of Single Cell HLA Typing with More Accuracy and Higher Efficiency

In this study, short tandem repeats were used for two purposes. The first one is linkage analysis for the beta globin mutations in order to exclude the allele drop out in the single cells. Finding of heterozygous allele sizes excludes the allele drop out. The second aim is to determine the HLA haplotype to find out the HLA compatible embryos. The method is based on indirect typing of the HLA alleles in single cells by using the microsatellites throughout the HLA locus. Use of informative microsatellites scattered

through the HLA locus enables to determine the whole HLA haplotype, therefore, provide to ascertain the HLA compatible embryos. Due to restrictions of the allele specific amplification strategy, use of informative microsatellites of the extended HLA locus preferred instead of amplifying each HLA allele. In this study, approximately eight-ten informative microsatellite were used.

Four microsatellites were initially used in order to determine the HLA haplotypes in six clinical PGD cycles for two families (Van de Velde *et al.,* 2004). Seven markers have been studied initially in the family in order to select the informative four markers to be further used in the single cells. One marker was found in the telomeric side, three markers were located within the class I region. The others were close to the class II region and centromeric side of the HLA locus. Shortly after, minisequencing-based genotyping of the HLA regions A, B, C and the DRB1 in the single cells have been combined with analysis of different microsatellites scattered through the HLA locus (Fiorentino *et al.,* 2004). Approximately thirty additional markers were reported for the use of HLA haplotyping in the single cells (Fiorentino *et al.,* 2004, Verlinsky *et al.,* 2004). Since the availability of tens of informative microsatellites throughout the HLA locus, six to fifteen informative markers were optimized to use in the single cells in order to ascertain complete HLA haplotype and reduce the misdiagnosis due to the recombination, allele drop out and the preferential amplification. In average, use of informative eight-twelve microsatellites throughout the HLA locus provide fingerprints of the HLA alleles since the maternal and the paternal chromosome six were identified prominently in the single cells. This strategy provides not only testing of the HLA A, B, C, DP, DQ, and DR genes for stem cell transplantation but also evaluation of non-HLA genes which are related with the immunity and other genetic conditions. Determination of the compatibility of the extended HLA haplotype in the single cells will lead to a better stem cell source and resulted in more successful stem cell transplantation due to the high compatibility between the donor and the recipient.

The capillary electrophoresis has a high sensitivity and ability to resolve at least one base pair size differences between the PCR products. Therefore, an accurate HLA haplotyping can be accomplished by running the fluorescently labelled amplicons of HLA microsatellites. The use of polymorphic microsatellites at the HLA locus allows not only

determination of the HLA haplotypes but also assessment of the allele drop out and the recombinations at this locus which leads to more accurate HLA typing in the single cells. This strategy prevents misdiagnosis and misinterpretation. Moreover, the contamination, aneuploidy, uniparental disomy or trisomy of chromosome six could be evaluated due to the analysis of the allele sizes. Finding of an extra peaks which are not inherited either of the parents could be accepted as a contamination from the outside. Another advantage of this strategy is efficient amplification of the microsatellites due to their small sizes (less than 300 bp). Therefore, amplification is more tolerant to the fluctuations in the PCR conditions and compared to the PCR-SSP strategy, more embryos could be analysed for the HLA compatibility which, in turn, resulted in availability of more embryos for the implantation.

Beside its advantages, it also brings up a molecular diagnostic level standardisation of the single cells. It eliminates the patient specific amplification procedures. Use of the microsatellites with high heterozygosity throughout the HLA locus could be applied to all patients irrespective of the HLA alleles in the patients. Moreover, it reduces the preparations and the laboratory work-up prior to clinical preimplantation genetic diagnosis cycle.

As the techniques developed in the single cells and the limitations of the SSP typing to perform a complete HLA typing have been fully understood, use of the microsatellites for the HLA typing has been prefered and accepted after the continuous studies in the single cells. We have had contributions to the validation of use of the microsatellites in the single cells for the selection of the HLA compatible embryos. Use of microsatellites in the single cells has been recently considered as the accepted procedure for the HLA compatibility worldwide.

6.5. Assessment of Preimplantation Genetic Diagnosis For Monogenic Disorders Combined with HLA Typing

The beta-globin gene was selected in order to analyze the mutations in the single cells, since the beta-thalassemia is one of the major genetic health concerns in Turkey. The genetic testing of the beta-globin mutations in the embryos prior to the implantation is

considered as a part of the preventive medicine due to the selection of healthy embryos for implantation. At this point, the preimplantation genetic diagnosis is a valuable alternative of prenatal diagnosis which could result in pregnancy termination due to the finding of an affected genotype in the fetus.

Since the beta-thalassemia and the sickle cell disease are very common in the Mediterrenean countries, the preimplantation genetic diagnosis has been the most commonly applied for these disorders coupled with the HLA match in order to prevent the birth of an affected child and to cure the affected child of the family after the HLA compatible stem cell transplantation. The preimplantation genetic diagnosis has been developing very rapidly and it has been soon available for more than two hundred genetic diseases in all around the world with the increased demand (Fragouli, 2007). In addition to the beta-thalassemia and the sickle cell disease, PGD has been applied to Fanconi anemia A and C, osteogenesis imperfecta, ataxia telengiectasia, griscelli, wiskott-aldrich syndrome and Peter's plus disease in our laboratory. PGD for fanconi anemia and wiskott aldrich syndrome were coupled with the HLA selection (unpublished data).

Preimplantation genetic diagnosis offers a great opportunity to couples at risk of having affected child by providing the genetic selection of unaffected embryos. PGD is a multidisciplinary procedure that requires strong collaboration of reproductive medicine and molecular genetics. Preimplantation of genetic diagnosis for hematopoietic disorders are generally coupled with the selection of the HLA compatible embryos whether the family has an affected child waiting for a stem cell transplantation from an HLA-compatible sibling. One of the best options to cure these diseases is provided by the stem cell transplantation from an HLA identical sibling since the transplantation from nonidentical individual is associated with high morbidity and poor survival. Since the PGD combined with the HLA selection is a complicated procedure, it is still very limited to few centers all around the world. It requires detailed preclinical work up in the samples of the family members and optimization of specific protocols for the HLA selection and mutation analysis for the single cell use. The first embryo selection for the HLA compatibility has been carried out in 2001 (Verlinsky *et al.,* 2001).

The major limitation of PGD is the limited amount of the sample and time to obtain a reliable and acceptable result. Two copies of DNA from a single cell is the only material to analyze the mutations and to determine the HLA compatibility. The time to get the results is less than twenty-four hours most of the time. That's why development of rapid, reliable and accurate molecular biology methods is of utmost significance for the single cell diagnostics. Misdiagnosis due to the allele drop out, recombinations, contamination and the preferential amplification are the major drawbacks and the limitations of single cell molecular biology. The methods have to be optimized and improved in order to prevent the misdiagnosis due to these drawbacks.

The HLA typing strategy used in the present study permits the rapid analysis of the HLA compatibility between the embryos. The preliminary work up for clinical PGD cycle was substantially reduced to a few days. The conditions are optimized to amplify more than ten regions simultaneously to get a reliable and accurate result.

More than one thousand (1180) embryos were analyzed which were collected from 107 cycles of 42 couples. In this study, couples were treated approximately two or three times in average. 58% of the cycles were resulted with at least one embryo transfer. A previous study has been reported analysis of 2205 embryos from the data collection of two European centers (Van de Velde *et al.,* 2009). In our data, approximately 8% of the analyzed embryos were transferred after the genetic analysis. The overall ratio has been reported as 11.3% for the combined results of two European PGD centers which is comparable with the present study (Van de Velde *et al.,* 2009). The overall implantation rate has been reported as 29.2% and our data revealed it as 20.2%. The overall live birth rate was determined as 9.3% in our study. However, it has been previously reported as 15.9%, 1,7 fold higher than the present data. (Van de Velde *et al.,* 2009). Since the previous parameters correlated to each other for both study, it is difficult to explain the difference in the birth rate. However, parameters produced from the IVF data demonstrate that IVF applications for PGD need to be improved, especially, when the live birth rate was concerned. Hormon stimulation, the quantity and quality of the oocytes retrieved, higher fertilization rates and better biopsy procedures may lead to the selection of higher number of HLA complatible and healthy embryos with the better quality which may result in higher implantation and live birth rate.

In this data, all the biopsied embryos were taken into the genetic analysis. Among them, 86% of the embryos were concluded with an acceptable PCR outcome. The reasons of failure in the single cells can be listed as the disruption of cell integrity or the loss of cell during the transfer from petri dish into the PCR plate, attachment of the cell to the inner surface of the transfer pipette, no cell nucleous, incomplete cell or nucleous lysis. The complete analysis of 1012 embryos resulted in 201 HLA compatible embryos, indicating that nearly 20% of the analyzed embryos were HLA compatible which was concordant with the expected ratio of 25%. The finding of 122 HLA compatible and healthy embryos constituted a ratio of 13% among the embryos with a conclusive result which was comparable with the expected ratio (18.75%).

In summary, preimplantation genetic diagnosis to the beta-globin mutations combined with the HLA match has been developed and validated as an advanced application of the single cell genetic testing. It provides the opportunity to cure the affected sibling by the stem cells from the umbilical cord blood and bone marrow of the HLA-identical sibling. Increasing demand for the PGD forces the scientists to develop, optimize and validate rapidly new applications and technologies to get an efficient, high quality and fast single cell diagnostics. The collaboration of the reproductive medicine and the molecular genetics has to be improved to increase the success of preimplantation genetic diagnosis. Technological improvements in the molecular biology will allow high-quality and faster techniques for preimplantation genetic diagnosis applications.

6.6. Assessment of Characteristics of Meiotic Recombination at the Major Histocompatibility Complex in Human Embryos

The allele drop outs, preferential amplification, inefficient amplification as wells as recombinations are determined for the accurate diagnosis of the unaffected embryos before implanting to the uterus. Preimplantation embryos from the families that have undergone PGD have provided a unique chance of sample size that can be utilised for accurate estimation of the recombination rates. The recombination frequencies of human embryos have been noted during the preimplantation genetic diagnosis (PGD) for the HLA selection (Verlinsky *et al.,* 2004, Van de Velde *et al.,* 2009). The frequency of recombinant embryos have been noted as 2% and 4% in the IVF generated and PGD-HLA tested embryos and

concluded as higher than the family or sperm studies. The recombination frequencies and the patterns in the human embryos were studied in detail and were found individual, gender and haplotype dependent recombinant MHC allele frequencies, and recombination susceptible regions in human embryos selected for the HLA by PGD. By microsatellite typing of 12.2 Mb extended HLA locus in the human embryos, 5.3% of the MHC alleles have been found to undergo recombinations. This corresponds to the recombination rate of 0.44 cM/Mb within the region and it is approximately half of the general recombination rate which is seen in the whole genome. The literature data about the recombination rates in human MHC have been summarized in table 6.1. The findings of this study are consistent with the results of the family studies (Martin *et. al.,* 1995) and the sperm typing studies (Cullen *et. al.,* 2002, Yu *et. al.,* 1996) which were 0.63 cM/Mb and 0.49 cM/Mb, respectively. However, it was not possible to compare neither the present data nor the sperm and the CEPH family data with the results of the PGD HLA tested embryos (Verlinsky *et al.,* 2004, Van de Velde *et al.,* 2009) because the data about recombinant allele rates, size of the tested region and any other recombination characteristics were not published.

Table 6.1. Literature data about recombination rates in human MHC.

Study	***Target Region***	***Size of the Region***	***Recombination rate (%)***	***Recombination rate (cM/Mb)***	***Characteristics***
Martin 1995	A-DPB1	3.3 Mb	2.1%	0.63 cM/Mb	Sex-averaged
Martin 1995	A-DPB1	3.3 Mb	1.85%	0.56 cM/Mb	Paternal
Martin 1995	A-B	1.34 Mb	0.31%	0.15 cM/Mb	Sex-averaged
Martin 1995	B-DRB1	1.2 Mb	0.94%	0.78 cM/Mb	Sex-averaged
Martin 1995	DRB1-DPB1	0.5 Mb	0.74%	1.48 cM/Mb	Sex-averaged
Yu 1996	MHC	20-25 Mb	5.1-11.2%	0.23-0.51 cM/Mb	Paternal
Cullen 2002	A-DPB1	3.3 Mb	1.62%	0.49 cM/Mb	Paternal
Verlinsky 2004	Not provided	Not provided	4.3%	-	Not provided
Van de Velde 2009	Not provided	Not provided	2%	-	Not provided
This study	xMHC	12.2 Mb	5.33%	0.44 cM/Mb	Sex-averaged
This study	xClass I	7.3 Mb	1.23%	0.17 cM/Mb	Sex-averaged
This study	xClass II	4 Mb	3.69%	0.92 cM/Mb	Sex-averaged
This study	Class III	0.99 Mb	0.41%	0.41 cM/Mb	Sex-averaged
This study	xMHC	12.2 Mb	8.47%	0.69 cM/Mb	Maternal

Table 6.1. Literature data about recombination rates in human MHC (continued).

Study	*Target Region*	*Size of the Region*	*Recombination rate (%)*	*Recombination rate (cM/Mb)*	*Characteristics*
This study	xClass I	7.3 Mb	2.46%	0.34 cM/Mb	Maternal
This study	xClass II	4 Mb	5.74%	1.44 cM/Mb	Maternal
This study	Class III	0.99 Mb	0.27%	0.27 cM/Mb	Maternal
This study	xMHC	12.2 Mb	2.19%	0.18 cM/Mb	Paternal
This study	xClass I	7.3 Mb	0.00%	0 cM/Mb	Paternal
This study	xClass II	4 Mb	1.64%	0.41 cM/Mb	Paternal
This study	Class III	0.99 Mb	0.55%	0.55 cM/Mb	Paternal

The study of 732 meioses from twenty-six couples revealed an average meiotic recombination frequency of 0.44 cM/Mb (5.33%) for 12.2 Mb extended HLA locus. The recombination rate showed variations between three regions of the HLA locus. The recombination rate for the class I, class II and the class III regions were respectively calculated 0.17 cM/Mb for 7.3 Mb region, 0.92 cM/Mb for 4 Mb region and 0.41 cM/Mb for 990 kb region. The low recombination rate in class I region of the human embryos is supportive of the strong linkage disequilibrium that explains the block conservation of the HLA class I haplotypes during the evolution. The 5.4 fold difference between two regions (class I and class II) of the MHC is consistent with the existence of cold and hot spots of recombination (Carrington, 1999, Cullen *et. al.,* 1997). The rate of recombination within the class III region (0.41 cM/Mb) was found half compared to a family study (Sanchez-Mazas *et. al.,* 2000). The difference may be attributable to the methods used where the family study was conducted by the SSP haplotyping that provides limited data compared to the microsatellite typing. Large pedigree studies have previously reported a sex-averaged recombination rate of 0.63 cM/Mb based on 952 informative meioses (Martin, *et al.,* 1995). Additionally, Cullen's study reported a recombination rate of 0.49 cM/Mb according to data obtained from the analysis of sperms from twelve donors (Cullen *et al.,* 2002). Although the general recombination rate of the extended HLA locus was two fold less than the genome average of 0.92 cM/Mb, the average recombination rate for the class II region is consistent with the genome average. The locations of recombination breakpoints have been mapped to a region covering the TAP2 gene between the DRB1 and the DPB1 at the class II HLA locus. The recombination hotspots have been previously mapped to the class II region since the molecular typings indicated a linkage equilibrium

between the DRB1 and the DPB1 (Cullen *et al.,* 1995, Cullen *et al.,* 1997). High resolution analysis of the TAP2 gene localized a recombination hotspot in the second intron of the TAP2 gene. The hotspot was abundant in sequence polymorphisms and flanked by DNA sequence which is less active in recombination. Therefore, the finding of increased recombination rate in the embryos at class II region compared to the class I and the class III region was concordant with the previous sperm typing and the family studies. The recombinations at the TAP2 region results in exchange of the DPA1 and the DPB1 alleles. As a result, the DPA1 and the DPB1 genes do not show strong linkage with the other class I (HLA-A, B and C) and the class II HLA (HLA-DQA1, DQB1, DRA, DRB1) genes in recombinant cases, resulting in generation of new HLA haplotypes. The recombination frequency of the class I has been found far below (5.4 fold) the genome average, indicating that the HLA class I genes are found closely linked to each other. Moreover, 67% of the recombination breakpoints at the extended class I region has been mapped 5.7 Mb region telomeric to the HLA-A gene. Therefore, it is expected that these recombinations would not affect the linkage of the HLA genes and the HLA haplotype. It seems that favorable interrelations and communications between specific alleles of the HLA genes may induce a particular pressure to keep them in close linkage and protect the certain haplotypes through the generations. The recombination frequency for the class III region has been calculated as 0.41 cM/Mb for a relatively short region (990 kb long) although only three recombinations have been mapped to this region. Due to the limited number of informative microsatellites at the HLA class III region for the single cell use, the fine map of the recombination breakpoints could not be established.

Individual disparity among the recombination rates have been noted previously in the family and the sperm studies (Yu *et al.,* 1996, Cullen *et al.,* 2002, Carrington, 1999) as two to six fold. The present study showed that individual differences in embryos could be as high as 14 fold (0.39 -5.46 cM/Mb) for the maternal alleles and 2.6 fold (0.37 - 0.96 cM/Mb) for the paternal alleles. Individual differences in the MHC sequence linked to the certain HLA haplotypes have been suggested to explain the variable vulnerability of each allele to the recombination (Cullen *et al.,* 2002). A potential genetic control may affect the recombination frequency within the extended HLA locus. Sequence motifs and polymorphisms may affect the activity of the recombination hotspots and the recombination mechanism.

This study showed a significant gender difference in the meiotic recombination activity at HLA locus. The recombination rate of 8.47% (0.69 cM/Mb) has been determined in the maternal chromosomes and 2.19% (0.18 cM/Mb) in the paternal chromosomes of the extended HLA locus which is about 12.2 Mb. The ratio of recombination frequency in the maternal chromosomes was determined 3.83 fold higher than that of the paternal chromosomes. The gender ratio (female:male) of the recombination rate has been reported as 2.3 fold according to the analysis of thirty recombinant chromosomes previously (Cullen *et al.,* 1997). The recombination frequency of the extended class II region in the maternal and the paternal chromosomes were found 1.44 cM/Mb and 0.41 cM/Mb in this study, respectively. The recombination rate of class II region in the maternal chromosomes were higher than the genome average, suggesting that the recombination hotspots of the class II region might be more active in the female meiosis. The recombination rates of the class I and the class III regions of the maternal chromosomes were found very low (0.34 cM/Mb and 0.27 cM/Mb, respectively), indicating that there is a strong linkage disequilibrium in these regions. Since we did not observe any recombination at the class I region of the paternal chromosomes in our samples, it is suggested that this situation is correlated with the low recombination frequency of the paternal chromosomes and strong linkage. The hotspot has been found to be much more active in female meiosis compared to the male meiosis which may explain the high recombination rate in females (Jeffreys *et al.,* 2000). Although a gender difference has been observed, the pattern of the recombination remained similar in both genders and was also condordant with the previous studies (Cullen *et al.,* 1997, Cullen *et al.,* 2002).

The data we described for the first time in human embryos contribute to the sperm studies and the family studies and thus may provide clues in better understanding of the linkage disequilibrium, MHC evolution and the selection process in this locus. The location of the recombination hotspots and the sequence of the MHC may explain the maintenance of the linkage disequilibrium patterns and the recombination rates. Preferred haplotypes may either confer selective advantage under the certain environment or may be subjected to different recombination rates (Traherne, 2008, Vandiedonck and Knight 2009). The low recombination rate may reflect the maintenance of haplotypes through the selection. Most of the disease associations in the MHC haplotype as whole is implicated in

certain disease associations like type I diabetes, rheumatoid arthritis, myasthenia gravis, systemic lupus erthematosus and IgA deficiency.

7. CONCLUSION

Single cell molecular biology provides analysis of the genomic DNA in a single cell to determine the genetics of cell clonality, genetic anticipation and DNA polymorphisms. This thesis focused on genetic analysis of mutations in single blastomeres. For this purpose, conventional molecular biology techniques were used for single cell analysis. The beta-globin gene and HLA locus were selected to study in the single cells. Both genetic regions were amplified by the multiplex PCR. Conventional PCR, fluorescent multiplex PCR and real-time PCR were used to amplify the HLA alleles, microsatellites and the beta-globin gene. In this thesis, real-time PCR and melting curve analysis has been applied for the first time for detection of the HLA alleles by using allele specific hybridization probes. Another application of real-time PCR in this study was detection of the most common beta-thalassemia mutations in the single cells. Sequencing was used for both detection of the beta-thalassemia mutations and determination of the rare beta-thalassemia mutations. Due to drawbacks of conventional HLA typing in single cells which were mentioned in previous sections, HLA haplotyping was futher developed and was achieved by utilizing the microsatellites located in the extended HLA locus. Moreover, use of short tandem repeats enabled to determine the meiotic recombination rate at the HLA locus in the single cells. This strategy brought up use of the multiplex fluorescent PCR and capillary electrophoresis. Single cell genetic testing was applied to preimplantation genetic diagnosis in order to select disease free and HLA compatible embryos. This study shows that multiple genetic conditions can be analysed in blastomeres biopsied from six-eight cell embryos by using PCR-based amplification strategies and following different detection methods. The strategy used in this study can be adapted for other PGDs to analyze other genes and genetic conditions in the embryos.

The significance of genetic testing in a single blastomere is that not only it helps prevention from the birth of an affected child or abortions due to the prenatal diagnosis results, but also it provides saviour siblings that are stem cell sources for the affected children. In addition to PGD, the single cell genetic testing can be used for noninvasive prenatal diagnosis of fetuses for certain genetic conditions. Few fetal cells circulating in the maternal blood could be picked up and genetically analysed. Moreover, the single cell

molecular biology has also a promising niche in the research of tumor biology which includes the study of circulating tumor cells and cells obtained from pathology samples. Genetic analysis of a single cell becomes significant with respect to the prevention from genetic disorders prior to pregnancy, prenatal diagnosis without an invasive procedure such as CVS or amniocentesis and early diagnosis of cancers in the limited pathology samples.

Development of technologies and methods for investigation of the single cell have found its major ground in the field of stem cell and embryo molecular biology (Tang *et al.,* 2010, Guo *et al.,* 2010). The human embryos have given us the chance to characterize the human embryo meiotic recombinations at the HLA locus and compare with the data obtained only from the sperm and the family studies. High inter-individual variations, gender dependent recombination rates and the presence of cold / hot recombination regions in the human embryos may have implications for better understanding of the linkage disequilibrium, MHC evolution and the selection process in this locus. The results also draw attention to respect the recombination rates and regions in designing and interpretation of the HLA preimplantation genetic diagnosis.

In recent years, with the improvements in the biotechnology, whole genome amplification (WGA) methods have become an encouraging alternative of polymerase chain reaction in the single cells. Instead of using multiplex PCR, WGA could be preferred at the initial step. This will allow amplification of the whole genome and therefore, enables access to more genomic regions for analysis. Although PCR gives better results when the selected genetic regions are targeted, WGA in combination with the microarrays is preferred for analysis of multiple genetic variations in the single cells.

Beside the genomic studies, understanding gene expression become attractive in the single cells. Determination of the quantitative transcriptomics in a single cell is based on the technological improvements in the quantitative real-time RT-PCR method. The single cell gene expression profiling has a potential to understand molecular mechanisms futher. New technologies specialized for the single cell molecular biology have been developing recently. These technologies are capable of monitoring the single cell dynamics, interactions and the gene expression profiling. Use of highthroughput sequencing provides to observe genome dynamics at the single cell level.

The single cell genetic testing has potential applications from embryo to cancer cell diagnostics. The methods have to be developed in order to study more mutations and genes simultaneously in the single cells from different sources. Moreover, the methods should be less laborious and more rapid, technically. The major problem of the current methods is the occurence of allele drop out which leads to misdiagnosis and elimination of potentially healthy embryos. Therefore, the methods to be developed should prevent the allele drop out in the single cell diagnostics. Whole genome amplification in combination with the microarray technologies are promising methods which allow simultaneous analysis of more sequence variations and genes. Applications of high resolution melting curve analysis in the single cells will provide sensitive, highthroughput and rapid mutation analysis.

The single cell transcriptomics and the proteomics are future goals to understand the cellular physiology, gene expression, cell signaling and cell response to a specific stimulus. The studies on single cell gene expression are concentrated in the development of techniques (Hartshorn *et al.,* 2005, Hartshorn *et al.,* 2007, Bengtsson *et al.,* 2008). The gene expression profiles of the single cells presently are studied in order to develop techniques with a high sensitivity and precision rather than to reveal a gene expression profile under a certain condition or stimulus . Therefore, genes with high expression profile are preferred in the single cell studies in order to optimize the techniques. A single cell gene expression study consist of five steps which are cell collection, cell lysis, cDNA synthesis, amplification of cDNAs by real time PCR, and data analysis (Ståhlberg and Bengtsson, 2010). Moreover, sequencing based gene expression profiling at single cell resolution has been recently developed (Lao *et al.,* 2009). mRNA sequencing for whole transcriptome analysis showed that thousands of genes expressed two or more transcript variants in a single cell. It is expected that the single cell sequencing assays will provide understanding of the transcriptome complexity in different cell types. Commercial kits and instruments have been developing for single cell use. The development of microfluidic technology and its combination with the single cell assays will provide novel insights to understand gene regulation and cell biology. Improvements in the single cell biology will enable molecular biologists to ask fundamental biological questions previously not possible.

APPENDIX A: CONGRESS ABSTRACTS

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