Chapter 2

The Advanced HLA Typing Strategies for Hematopoietic Stem Cell Transplantation

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Additional information is available at the end of the chapter

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1. Introduction

The occurrence of graft rejection and/or graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT) is largely depended on whether the recipient and the donor have matched HLA types. Under normal circumstances, the individual with completely matched HLA antigens can be the donor. However, due to the high level of HLA polymorphism, the major obstacle in the allogeneic hematopoietic stem cell transplantation is to find a donor with HLA antigens that are a perfect match. This can prove to be quite problematic.

In 1954, an organ transplantation team led by Dr. Merri at Harvard University successfully completed a kidney transplantation between identical twins for the first time. From then on, the importance of histocompatibility in organ transplantation has been well recognized. The first human bone marrow transplantation between identical twins in 1957 provided a new approach for the treatment of leukemia and other hematologic malignancies. As a result, the basic research on HLA as well as the HLA typing techniques gained much attention over the next 20 years. The short-term survival rate of organ transplantation has been greatly improved since the 1980s due to the clinical application of immunosuppressive agents such as CsA. These successes,as well as the defects and limitations in serotyping and cellular typing of HLA, the clinical value of HLA typing has been largely ignored in the medical community.

With the advance of research in immunology and transplantation immunology, particularly in the structure and function of HLA in the 1990s, new technology for HLA typing has emerged and continues to improve. Terasaki and Opelz analyzed a large amount of organ transplantation cases performed in major transplantation centers around the world. The role, status and importance of HLA typing in hematopoietic stem cell transplantation have been recognized once again. Overall, HLA typing is required in hematopoietic stem cell

transplantation. HLA compatibility not only significantly reduces the incidence of acute rejection, but also significantly reduces the incidence of chronic rejection. HLA compatibility is one of the most critical factors that affect the long-term survival of the graft.

HLA loci are the most genetically variable gene loci in human. Two hundred and twenty four loci of HLA complex have been identified so far. Among these, 128 are functional loci that encode proteins, and 39.8% of HLA genes are related to the immune system, particularly those belong to class II loci. Almost all these genes display immune-related functions. Approximately 100 HLA genes loci have been cloned and named, and at least 18 of them have alleles. Since these loci have various amounts of alleles and each allele encodes a corresponding HLA antigen, the HLA complex has the most abundant genetic polymorphism in the human immune system.

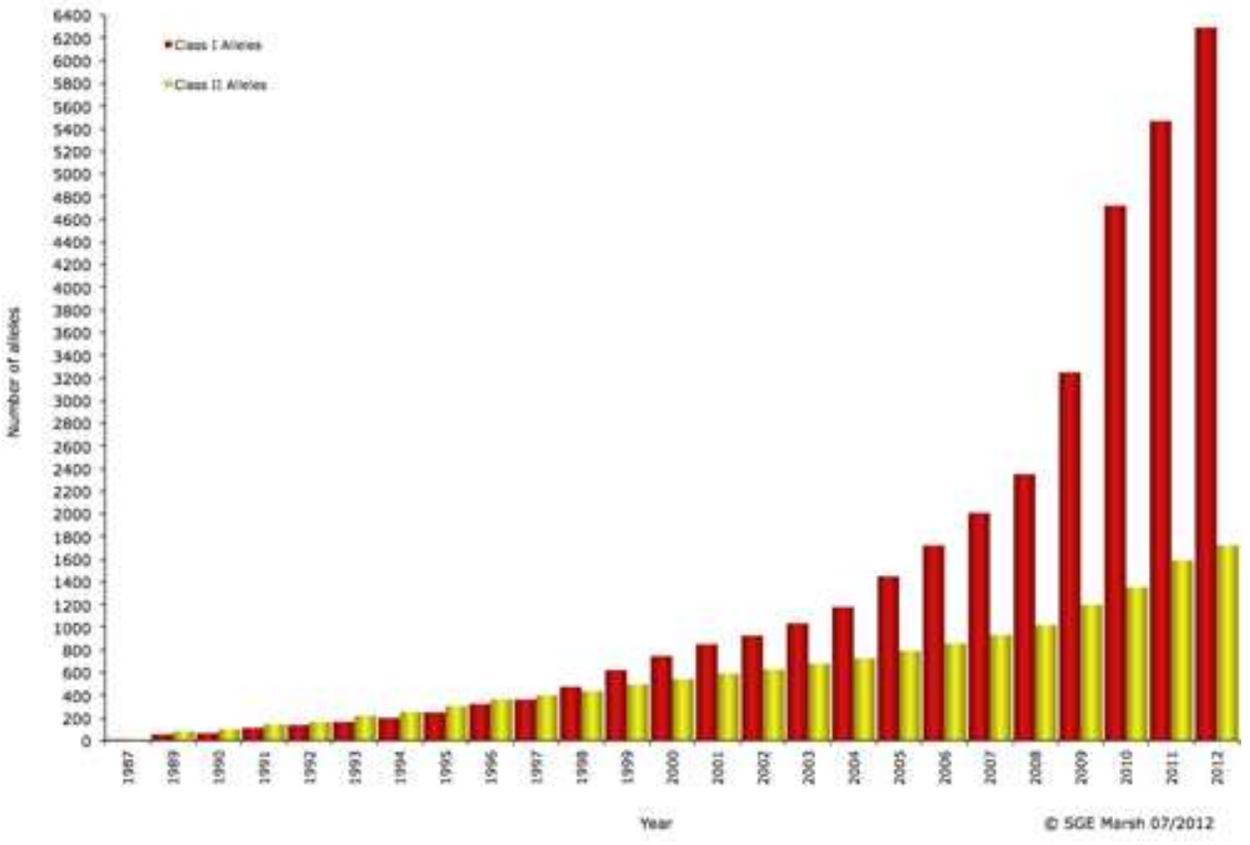

Figure 1. Increasing number of HLA alleles from 1987 to July 2012

Systemic investigations of the alleles in HLA loci began in 1987. There were just over 10 identified alleles at that time. The allele numbers in HLA-I and HLA-II loci were increased to 100 and 50 respectively in 1989. The allele number of HLA-I and HLA-II reached 1028 in 2000. As of July 2012, the total allele number of HLA loci has reached 8016. HLA-A, HLA-B and HLA-C loci have 2013, 2605 and 1551 alleles respectively. DRA site has 34 and DRB site has 1260 alleles. DQA1 and DQB1 sites have 47 and 176 alleles respectively, and DPA1 and

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DPB1 sites have 34 and 155 alleles, respectively (Fig 1). Theoretically, it is very difficult to find an unrelated donor with a perfectly matched HLA genotype (at the allele level) in the general population.

The polymorphism of HLA makes it difficult to find a match between unrelated donor and recipient in the allotransplantation. Currently, the most commonly used HLA typing in organ transplantations around the world is based on HLA-A, B, C and DR genes. There are up to 7400 alleles in these genes corresponding to more than 100 specific antigens. With the increasing number of patients who need hematopoietic stem cell transplantation, the lack of appropriate donors has become a significant challenge. Therefore, there is an urgent need to develop novel scientific, practical, and feasible HLA typing methods in the field of hematopoietic stem cell transplantation.

2. Principles for HLA typing strategy in allogeneic hematopoietic stem cell transplantation

The first successful human bone marrow transplantation between identical twins in 1957 has provided a new approach for the treatment of leukemia and other hematologic malignancies. After the successful hematopoietic stem cell transplantation between unrelated donor and recipient with matched HLA, a bone marrow donor registry was established in 1988 (National Marrow Donor Program, NMDP) in the USA. Later on, a public cord blood bank was established. According to the World Marrow Donor Association (WMDA), as of July 2012, the association has 68 bone marrow banks in 49 countries and regions. It also has 46 cord blood banks in 30 countries and regions. The registered bone marrow and umbilical cord blood donors have exceeded 20 million. Meanwhile, the technology of HLA typing has been transformed from simple serotyping to more accurate genotyping. Although there are hundreds of reports regarding the effect of HLA matching degree on the efficacy of hematopoietic stem cell transplantation, these results are not consistent due to the differences in sample size, disease type and stage, and HLA typing. In addition, the interpretation of HLA genotyping results and their biological significance is becoming increasingly complicated. It is challenging for the clinicians outside of the HLA field to select an unrelated donor with the best-matched HLA. To meet this challenge, WMDA, NMDP of the USA and European Federation of Immunogenetics (EFI) have provided guidelines for HLA typing.

2.1. Correlation between HLA allele and HLA antigen specificity

There is a fundamental difference in the result and biological significance between HLA serotyping and genotyping. In the HLA serotyping, HLA antibodies are used to identify the HLA antigens on the surface of lymphocytes. HLA antigens are proteins that can be recognized by the host immune system during blood transfusion, organ transplantation, as well as pregnancy. Specific antibodies against HLA antigens are the basis of the identification of the HLA antigens. The HLA antisera used in serotyping, regardless of whether

they are from the same species or different species, are all produced by immune stimulation with HLA antigens or peptides. In the HLA genotyping analysis, a specific HLA gene fragment is amplified *in vitro* from an individual's genomic DNA using synthetic oligonucleotide probes or primers. The genetic difference caused by variant HLA gene alleles is reflected by the variation in the DNA sequence. Therefore, HLA genotyping can identify all HLA alleles at the DNA level while HLA serotyping can only detect part of variants. The efficacy of bone marrow transplantation is closely related to the matching level of HLA between the donor and recipient. However, the HLA genotyping result does not directly reflect the antigen that causes immune rejection after the transplantation. Therefore, for the purpose of clinical relevance, the result of HLA genotyping should be converted to the HLA specificity. To this end, the NMPD and the University of California in Los Angels (UCLA) established the International Cell Exchange program, through which correlations between the HLA alleles and HLA antigen specificities are established by comparing a large amount of testing results worldwide. The dictionary of HLA alleles and their corresponding antigen specificities is under constant updating. As of 2008, 70% of HLA alleles have been correlated to HLA antigen specificities. The rest 30% alleles are rare alleles with a frequency less than 1 in 10,000. Therefore, their clinical values are relatively low. The HLA genotyping result can be easily converted to the HLA antigen specificity by using this HLA dictionary.

2.2. The number of donor with matched HLA gene types is much lower than that with matched HLA antigens

The criteria of matched HLA between the donor and recipient are different for the HLA genotyping and HLA serotyping in the bone marrow transplantation. From the HLA dictionary, one can tell that the HLA antigen specificity is unique, while a unique antigen may have one or more corresponding HLA alleles. For example, HLA-DR10 antigen only corresponds to HLA-DRB1*1001 allele, while HLA-DR11 antigen corresponds to 21 alleles such as HLA-DRB1*1101, 1102 and 1103. Therefore, the choice of donor for bone marrow transplantation may differ, depending on the method of HLA typing. For example, a donor and recipient listed in Table 1 may have matched HLA according to antigen specificity. However, their HLA genotypes may not match. Which method is more accurate for bone marrow transplantation is currently under investigation. Statistical analysis indicates that the chance of finding matched HLA genotypes in a random population is much lower than finding matched HLA antigens. For instance, as of February 2002, HLA-A, B and DR have 93 specific antigens. HLA-A, B and DR have 25, 50 and 18 loci respectively, which can generate 2.2 x 10^4 haplotypes. The genotype number of these haplotypes can be up to $2.5x10^{13}$. Currently, there are 2100 alleles have been identified in HLA-A, B and DRB1 genes. Their combination will yield 3.4 x10^7 haplotypes. As a result, the number of HLA-A, B and DRB1 genotypes in a population can be up to $5.78x10^{15}$, making it almost impossible to find the matched HLA genotype in a random population. In other words, the HLA genotypes of the donor and the recipient are always more or less mismatched in bone marrow transplantation. Because of this, the concept of permissible HLA mismatches has been introduced.

matching status		Recipient's HLA type				Donor's HLA type			
antigen	gene	Antigen		gene		Antigen		gene	
matched	matched	A2	A11	A* 0202	A*1101	A2	A11	A*0202	A*1101
		B60	B62	B*4001	B*1501	B60	B62	B*4001	B*1501
		DR4	DR8	DRB1*0402	DRB1*0801	DR4	DR8	DRB1*0402	DRB1*0801
matched	unmatched	A2	A11	A* 0202	A*1101	A2	A11	A*0205	A*1102
		B60	B62	B*4001	B*1501	B60	B62	B*4007	B*1504
		DR4	DR8	DRB1*0402	DRB1*0801	DR4	DR8	DRB1*0404	DRB1*0803
unmatched	unmatched	A2	A11	A* 0202	A*1101	A2	A30	A*0201	A*3001
		B60	B62	B*4001	B*1501	B61	B62	B*4002	B*1501
		DR4	DR8	DRB1*0402	DRB1*0801	DR4	DR11	DRB1*0401	DRB1*1102

Table 1. Examples of HLA antigen matching and allele matching between the recipient and the donor in bone marrow transplantation

2.3. Permissible HLA mismatches

In the case of permissible HLA mismatches, the donor and the recipient have mismatched HLA in a bone marrow transplant. However, the mismatch does not cause a significantly increased rate of GVHD or graft failure, and is acceptable for bone marrow transplantation. Results from retrospective analyses suggest that mismatched alleles in HLA class I antigens as well as alleles in HLA-DQ and DP loci have minimal impact on the efficacy of bone marrow transplantation.

2.3.1. HLA class I antigen or allele mismatch

Petersdorf *et al* had investigated the effect of matching level of HLA class I antigens and alleles on the success rate of bone marrow transplantation in 471 patients. The transplant failure rate is 0.7% in 280 cases with matched HLA-A, B and C genes, and is 0% in 47 cases with one of mismatched heterozygous HLA-A, B or C gene. However, the failure rate in 51 cases with one of mismatched HLA-A, B or C antigens is 14%, which is significantly higher than that in the control group. In 76 cases with 2 or more mismatched antigens or genes, the transplant failure rate is 17%. These results indicate that a single mismatched allele in the HLA class I gene does not increase the transplant failure rate, while a single mismatched antigen, or 2 or more mismatched antigens or genes can significantly increase the transplant failure rate. These results support Petersdorf's hypothesis that the immune response caused by mismatched HLA class I alleles is lower than that caused by mismatched antigens. Therefore, mismatched HLA class I genes are permissible in the bone marrow transplantation, as long as HLA antigens match. Rubinstein et al also believes that transplantation can be considered if there is only one mismatched allele. For example, the recipient's genotype is HLA-A*0202 while the donor's genotype is HLA-A*0203. This kind of mismatch does not increase the rate of immune rejection. Further analysis indicates that whether a single allele mismatch is allowed in the transplantation also depends on the type of corresponding mismatched amino acid and the position of that amino acid in the HLA class I antigen. HLA class I molecules consist of a covalently bound heavy chain molecule and a ▪ 2 microglobulin. The extracellular fragment of the heavy chain has 3 activity domains

(▪ 1, ▪ 2 and ▪ 3), and the ▪ 1 and ▪ 2 domains form the peptide-binding region. The complex of HLA and its bound peptide on the cell surface constitutes the ligand for the T-cell receptor (TCR), thereby inducing an immune response. If there is only one mismatched allele between the donor and recipient, the number of mismatched amino acids will be much lower, and may rarely involve the amino acids for TCR binding. On the other hand, if the donor and the recipient have a mismatched antigen, it may have many mismatched amino acids, and some of these amino acids may be involved in peptide binding and TCR binding. This may explain why the matching of HLA class I antigen is more important than the matching of genotype (Fig 2).

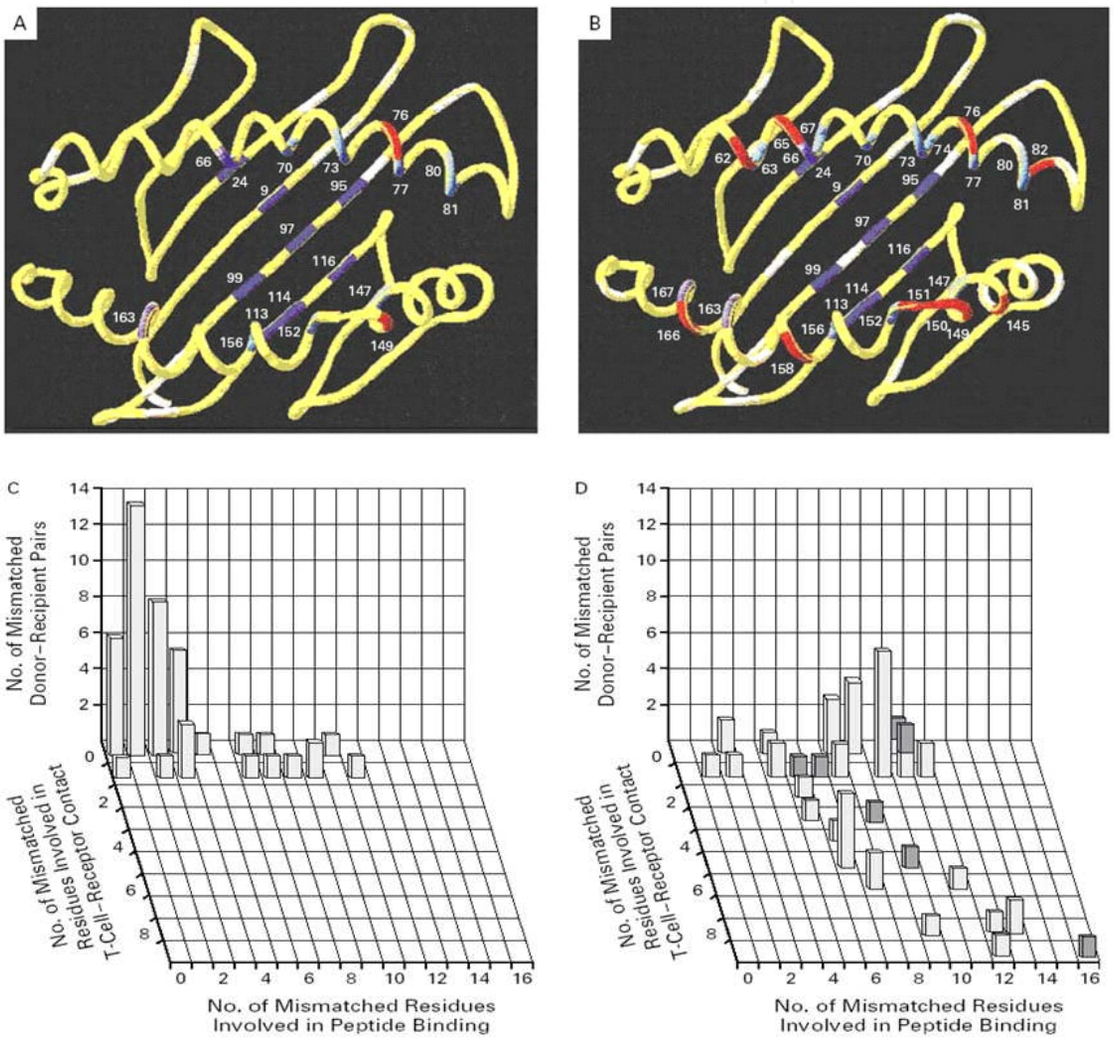

Figure 2. Spatial structure as well as the position and number of mismatched amino acid in the class I HLA with mismatched donor-recipient genotypes (A) or mismatched donor-recipient antigens (B). The position of amino acid residue is labeled according to its position in the whole protein. Amino acid residues with dark blue color are located in the ▪ -sheet and involved in peptide binding. Amino acid residues located on the ▪ helix are colored with light blue. Amino acid residues involved in TCR binding are in red. White amino acids are involved in neither TCR binding nor peptide binding, while gray amino acids are involved in both TCR and peptide binding. Number of mismatched amino acid involved in peptide binding and TCR binding in the class I HLA with mismatched donor-recipient genotypes (C) and mismatched antigens (D). In panel D, patients with a transplant failure are in dark gray block.

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Further analysis by Petersdorf *et al* shows that one mismatched HLA-A, B or C antigen causes 71% transplant failure in 7 HLA homozygous patients, but causes 7% failure rate in 98 heterozygous patients, suggesting that for HLA homozygous patients, when a matched donor is not available, the homozygous donor with other matched heterozygous alleles should be chosen. For example, the recipient's HLA type is HLA-A2, B44 and DR8/9. Donor 1's HLA type is HLA-A2, B51, B46 and DR8/9. Donor 2's HLA type is HLA-A2/11, B44, B46 and DR8/9. In this case, donor 1 is preferred.

2.3.2. HLA class II antigen or allele mismatch

HLA class II genes encode antigens such as HLA-DR, DQ and DP. In order to understand the importance of HLA class II genes in unrelated bone marrow transplantation, McGlave *et al* have investigated the effect of mismatched DR, DQ and DP alleles on the result of transplantation. Data from NMDP that contain 831 chronic myeloid leukemia (CML) patients received bone marrow transplantation between 1988 and 1997 were analyzed. 696 patients have matched HLA-A and B base on serotyping results. Among them, 565 (81%) have matched DRB1 genotypes. Data analysis shows that matched HLA-DRB1 alleles can significantly improve graft survival and patient survival. While, mismatched HLA-DQA1, DQB1, DPA1 and DPB1 genes do not significantly affect the GVHD incidence or transplantation result. This result suggests that matching HLA-DRB1 alleles is an important factor in bone marrow transplantation (Fig 3).

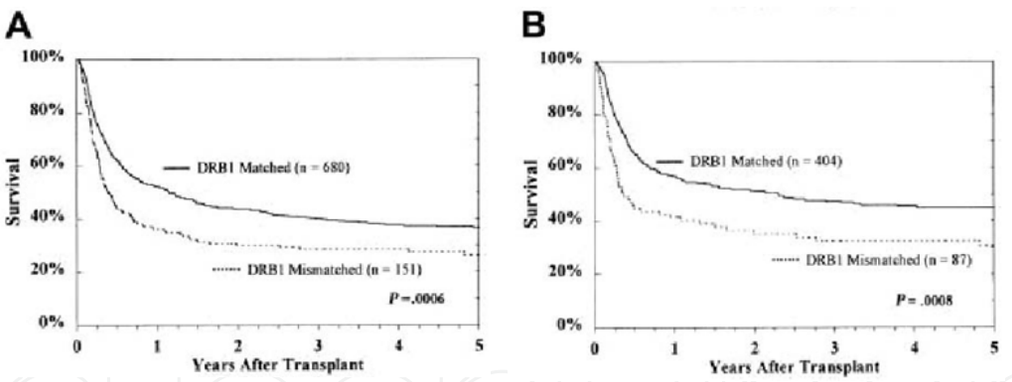

Figure 3. Effect of DRB1 matching level on patient's survival rate (A) CML patient, (B) Patients with matched HLA-A, B serotypes

2.4. HLA typing standard for hematopoietic stem cell transplantation

According to the guideline of World Bone Marrow Donor Association (WBDA) and European Federation for Immunogenetics (EFI), HLA typing of the donor in a large-scale bone marrow center is generally limited to 2 digits after the asterisk in the WHO HLA nomenclature, corresponding to the subtype of a specific HLA antigen. However, high-resolution HLA typing should be performed for recipients and donors with matched HLA. In addition, the typing of HLA class I genes should also include the locus C. Due to the increasingly recognized role of locus C in the immune rejection, the typing of HLA-C should be performed.

When choosing a donor, the HLA-DRB1 gene of the donor and the recipient should have 4 identical digits after the asterisk in the WHO HLA nomenclature.

Although most commonly used methods for HLA genotyping cannot cover all genes, it does not limit their applications in HLA typing for bone marrow transplantation. Among thousands of identified HLA alleles, most of them are rare alleles. Therefore, it is not necessary to type all HLA alleles. For instance, 244 expressing genes have been identified in DRB1 loci. Among them, 148 (60%) alleles have corresponding specific DR antigens, while 96 alleles (40%) do not. According to the NMDP, result of HLA-DRB1 typing in 65,752 donors shows that 86 alleles have 0 frequency and the frequency of another 105 alleles is lower than 0.0002. In addition, the total frequency of 10 alleles without corresponding antigens is 0.000084. Therefore, identification of the rest 43 DRB1 alleles will cover 99.6% of HLA-DR antigen specificities, which is sufficient for the screen of donor in hematopoietic stem cell transplantation.

3. PCR based HLA genotyping methods

The technology for HLA typing has evolved from the serological level to the cellular level, to the molecular level. Serotyping was the mainstream method for HLA type and has played a critical role in organ transplantations before 1990s. However, most HLA antisera are polyclonal and often have cross-reactions, making it difficult to distinguish antigens with subtle structural differences, and leading to misidentifications. Further more, many factors, such as a prolonged transportation time of the blood sample and excessive amount of immature cells, may affect the result of serotyping and cellular typing. These are the limitations of traditional HLA typing methods. The development of polymerase chain reaction (PCR) and its application in biomedical sciences has made the HLA typing at the DNA level possible. Therefore, using molecular methods to type HLA at the DNA level has gradually replaced serotyping and cellular typing. Commonly used DNA based HLA typing methods include PCR based sequence specific primers (PCR-SSP), and PCR based restriction fragment length polymorphism (PCR-RFLP), single-strand conformation polymorphism (PCR-SSCP), sequence-specific oligonucleotide (PCR-SSO) and single nucleotide polymorphism (PCR-SNP).

3.1. PCR-SSP (sequence specific primers)

To identify point mutations in a DNA molecule, Newton invented the amplification refractory mutation system (ARMS) for *in vitro* DNA amplification. The technique requires an allele sequence specific 3′ primer for the PCR amplification. Otherwise the PCR reaction will not be effective. This is because the Taq DNA polymerase used in the PCR reaction has 5′ to 3′ polymerase activity and 5′ to 3′ exonuclease activity but lacking 3′ to 5′ exonuclease activity. Therefore, the enzyme cannot repair the single mismatched nucleotide in the 3′ primer. In order to amplify the allele with a specific sequence, the primer with the corresponding sequence is designed. The conditions for PCR reaction are strictly controlled so that the amplification of the fragment with its sequence perfectly matching the primer is much more effective than the sequence with one or more mismatched nucleotide. One mismatched nucleotide between the 3′ primer and the template is sufficient to prevent the amplification.

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The PCR product is further analyzed by electrophoresis to determine whether the amplicon corresponds to the anticipated primer-specific product. Since the DNA sequence of HLA class I and class II genes are known, PCR primers can be designed based on the specific sequence of each allele for PCR-SSP genotyping.

The encoding allele sequences of various HLA antigens can be amplified with sequence specific primers. By controlling the conditions of PCR, a specific primer can only amplify its corresponding allele, and not other alleles. Therefore, the presence of a PCR product can be used to determine the presence or absence of a specific allele. The specificity of PCR product can be further determined by agarose gel electrophoresis. Fig 4 shows the principle of PCR-SSP.

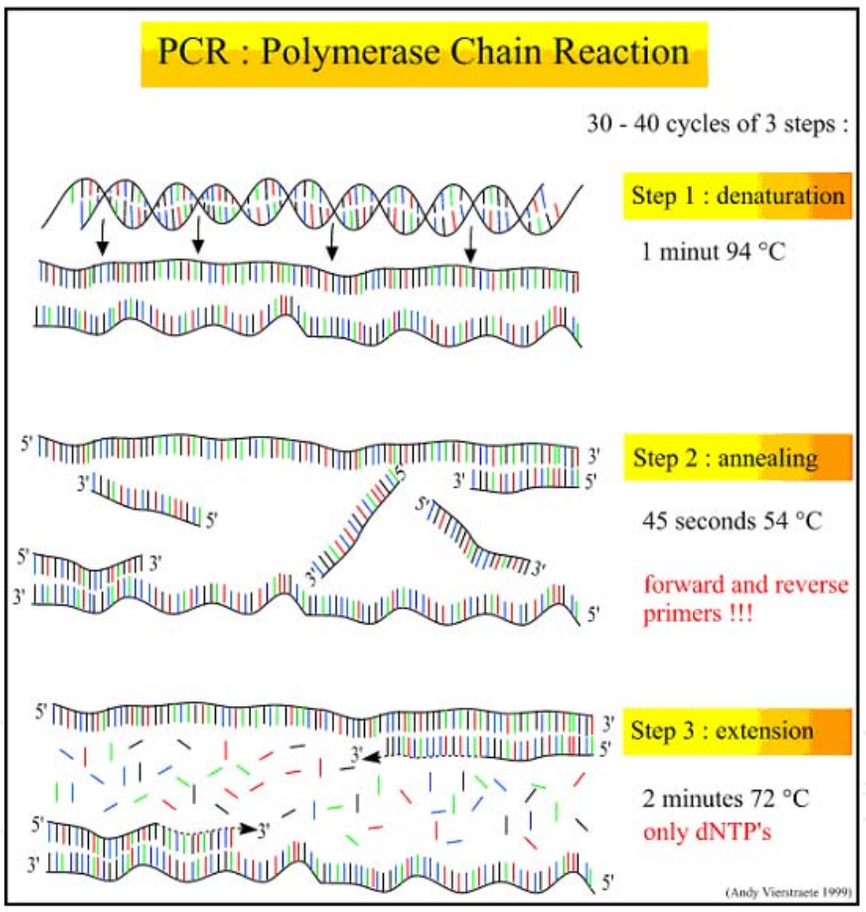
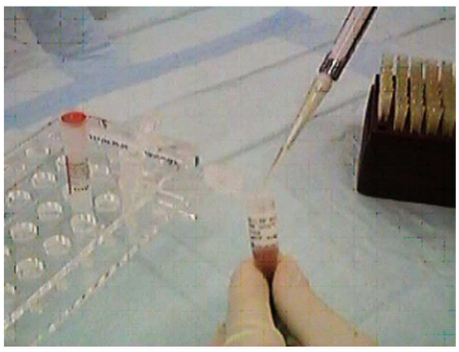


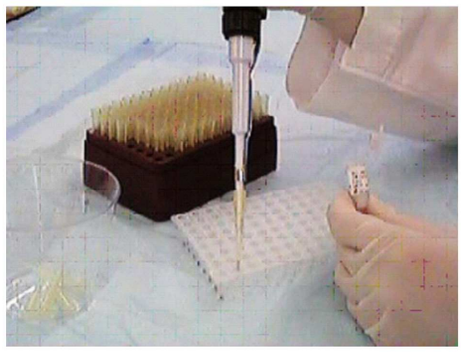
Figure 4. The diagram of PCR reaction

In the first step of PCR reaction, double-stranded DNA is denatured into single-stranded DNA. In the second step, specific primers anneal to the template DNA. In the third step, double stranded DNA is generated by TaqDNA polymerase by incorporating 4 types of dNTP into the newly synthesized DNA strand. After 30-40 cycles of amplification, the target gene is increased to 10^8 fold.

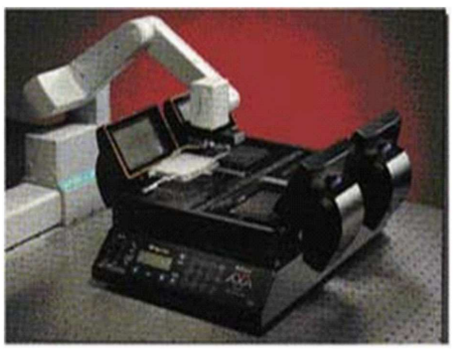
The main advantage of this method is that it is simple and fast, and the result is easy to interpret. The heterozygosity can be easily detected as well. Therefore, PCR-SSP is the currently most used method for HLA typing. There are several FDA approved high-resolution and low-resolution detection kits available for HLA class I and class II typing. Many clinical laboratories in China have been using this method for accurate pre-transplantation HLA typing. The procedure of PCR-SSP is shown in Fig 5. One disadvantage of this method is that it requires multiple primers in order to amply all relevant alleles.



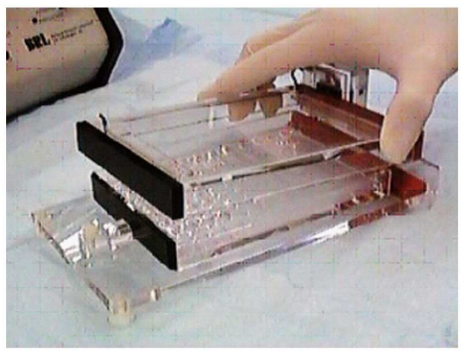
A. DNA polymerase and DNA sample are added to the tube containing PCR reaction buffer and dNTP.



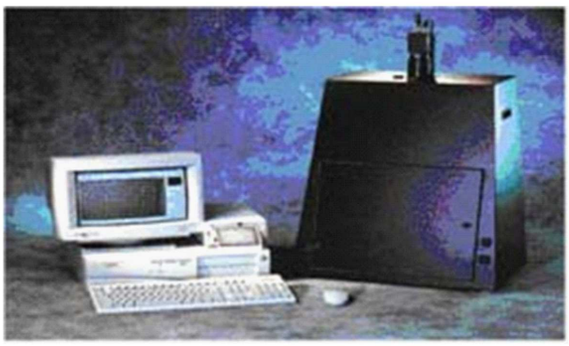
B. 10 ul of mixture of DNA and D-mix is added to the SSP kit. The negative control does not have this mixture.



C. PCR amplification



D. electrophoresis (2-4 min)



E. Automatic gel imaging system

F. Analysis of the result by software

Figure 5. The experimental procedure of PCR-SSP

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3.2. PCR-RFLP (restriction fragment length polymorphism)

Restriction endonucleases have unique recognition sites. Using computer analysis, restriction endonucleases that can recognize HLA sequence polymorphism are chosen to digest the PCR product. Because of sequence difference among the alleles, enzyme digestion will yield DNA fragments with unique patterns of length, which can be distinguished by electrophoresis.

Compared to serotyping, PCR-RFLP method is specific, simple and rapid and does not require probes. It can accurately detect single nucleotide difference and two linked polymorphic sites. The disadvantage of this method is that if the enzyme cannot completely digest the PCR product, the DNA fragments with similar lengths may be difficult to distinguish after electrophoresis. In addition, alleles need to have endonuclease recognition sites. Furthermore, PCR-RFLP cannot distinguish certain HLA heterozygosities. It requires multiple endonucleases for those alleles with high polymorphism such as HLA-DRB1, and may produce complicated restriction maps. For these reasons, this method is rarely used for HLA typing currently.

3.3. PCR-SSCP (single-strand conformation polymorphism)

Suzuki *et al* in Japan have found that a single-stranded DNA fragment has complex spatial conformation. The three-dimensional structure is generated by the intramolecular interactions among the base pairs. The changing of one nucleotide may affect the spatial conformation of the DNA strand. Single stranded DNA molecules have their unique size exclusion characters in polyacrylamide gels due to their molecular weights and three-dimensional structures. Therefore, they can be separated by non-denature polyacrylamide gel electrophoresis (PAGE). This method is sensitive enough to distinguish molecules with subtle structural differences, and it is called single-stranded conformation polymorphism (SSCP). The authors later applied SSCP in the detection of mutations in PCR products and developed PCR-SSCP technique, which has further improved the sensitivity and simplicity for mutation detection.

This method is simple, rapid, sensitive, requiring no special equipment, and is suitable for clinical applications. However, this method can only detect mutations. The location and the type of the mutation need to be determined by sequencing. In addition, the conditions of electrophoresis need to be tightly controlled. Furthermore, point mutations in certain locations may have no to little effect on the DNA conformation. Therefore, different DNA molecules may not be able to separate by PAGE due to these reasons and other factors. Nevertheless, this method has a relatively high detection sensitivity compared with other methods. It can detect mutations in unknown locations in the DNA molecule. Takao has demonstrated that SSCP can detect 90% of single nucleotide mutations in a DNA fragment smaller than 300bp. He believes that most known single nucleotide mutations can be detected by this method. Mutant DNA molecules can be separated and purified by PAGE due to the different migration rates, and the mutation can be eventually identified by DNA sequencing.

In SSCP analysis, the separation of single stranded DNA by non denature PAGE is not just based on their molecular weights and electric charges, but also on the retention force caused by their spatial conformations. Therefore, the migration rate of a DNA fragment does not

reflect its molecular size. Since the wild type and mutant DNA molecules may migrate very closely and are difficult to distinguish, it is generally required for DNA molecules to migrate for more than 16-18 cm in the gel. Mobility is calibrated using reference DNA as an internal control. Because of these reasons, this method cannot clearly determine the HLA genotype.

3.4. PCR-SSO (sequence specific oligonucleotide)

In PCR-SSO, specific probes are synthesized according to the sequence in the HLA polymorphic region. The target DNA fragment is amplified *in vitro* first. Then a specific probe will be hybridized to the PCR product under certain conditions based on base pair complements. The hybridized product can be detected by radioactive or non-radioactive signals. There are two types of SSOP method, direct hybridization and reverse hybridization. In the direct hybridization, the PCR product is fixed on the membrane while in the reverse hybridization, the probe is fixed on membrane. Figure 6 is the diagram of PCR-SSO.

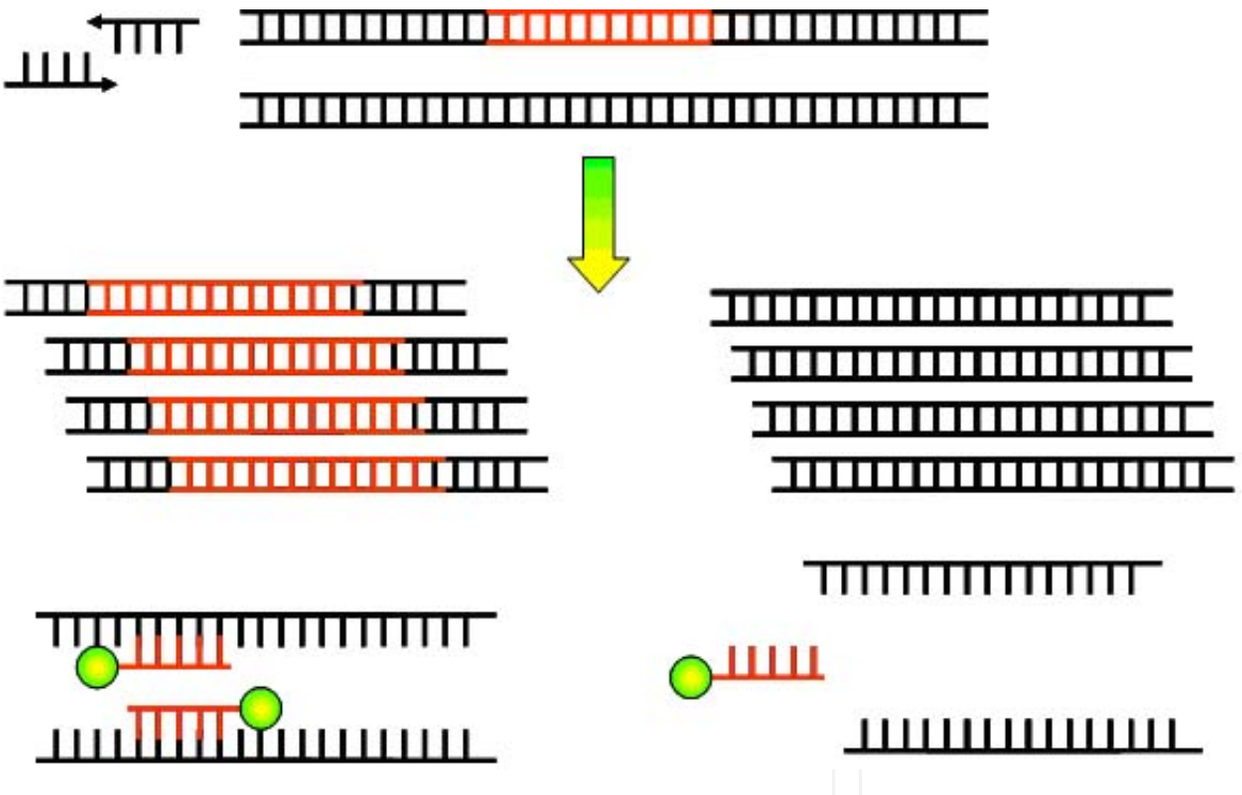

Figure 6. The diagram of PCR-SSO process

In 1986, Saiki *et al* were the first to report the analysis of DQA1 polymorphism using PCR and 4 allelic specific oligonucleotide (ASO) probes. MicKelson has typed the DR loci by serotyping and PCR-SSOP in 268 specimens. The success rate of serotyping is 91.0% while the success rate of PCR-SSOP is 97.0%. Overall, PCR-SSOP has a high success rate, a wide source of reagents, a high specificity and resolution. It can detect the difference of one nucleotide. In addition, PCR-SSOP can be used for a large number of samples with accurate and reliable results. However, this method is time consuming. It often takes a few days and

http://dx.doi.org/10.5772/53084

needs a large amount of probes. In addition, it is difficult to detect heterozygous alleles, particularly those of the complicated HLA-DRB1 genes.

Overall, PCR-SSO is an accurate HLA genotyping method, and can identify all known HLA alleles for accurate analysis of HLA polymorphism. HLA is a super gene family and new alleles are continuously been identified. SSO probes can only be designed based on the sequences of known alleles. Although PCR-SSO may discover new HLA polymorphism through its hybridization pattern, dot-hybridization often leads to false positives. In addition, when an allele is identified in the sample, it is difficult to determine whether the allele is homozygous or heterozygous. Therefore, the HLA allele frequency and haplotype frequency cannot be precisely determined by this method.

3.5. PCR-SNP (single nucleotide polymorphism)

Single nucleotide polymorphism (SNP) is the inheritable and stable biallelic single nucleotide difference. In the human genome, every 1000 base pairs have one to 10 SNPs. SNP may have some regulatory functions in gene expression and protein activity. High SNP density has been found in HLA class I genes with one SNP in every 400bp, setting the basis for high-throughput MHC-SNP analysis. Compared with other methods, SNP is less time consuming and with a low cost. Gou *et al* have developed a simple and effective oligonucleotide microarray to detect SNPs in the coding sequence of HLA-B locus. Based on the known polymorphism in the exon 2 and 3 of HLA-B genes, 137 specific probes were designed. In a double-blind experiment, these probes were used in the PCR-SNP analysis of 100 specimens from unrelated individuals. The result showed that this method could explicitly identify all SNPs in the HLA-B locus. Bu Ying *et al* have established a rapid, efficient, and cost effective SNP detection method using a single tube. In this method, 4 primers are used for the PCR amplification. Two primers are used to amplify the DNA fragment containing the SNP region, and the other two primers are SNP specific. The primer extension error is significantly reduced when 4 primers simultaneously carry out the PCR reaction, thereby the accuracy of SNP analysis is greatly improved. With the development of third-generation genetic markers, it is expected to find a series of single nucleotide polymorphisms in the HLA complex, and generate high-density SNP maps. In order to develop SNP technology into a simple and effective HLA typing method, production of high-density SNP maps in the HLA regions and development of HLA-SNP genotyping kits have been proposed in the 13th IHWC conference.

4. Reference-strand-mediated conformation analysis (RSCA)

Arguello *et al* devised the double-stranded conformation analysis (DSCA) technique in 1998 for the detection and analysis of gene mutations and complex polymorphic loci. Based on this technique, reference strand mediated conformation analysis (RSCA) has been developed. This is a major technical breakthrough in HLA typing. This technique combines sequencing and conformational analysis to overcome the limitations of the methods that just employ DNA sequencing or conformational analysis. The concept behind RSCA is that a fluorescent labeled reference strand is hybridized with the amplified product of a specific gene

to form stable double stranded DNA with unique conformation. After non-denature polyacrylamide gel electrophoresis or capillary electrophoresis, HLA alleles can be detected by laser scanning and computer software based analysis. Figure 7 is the basic procedure of RSCA.

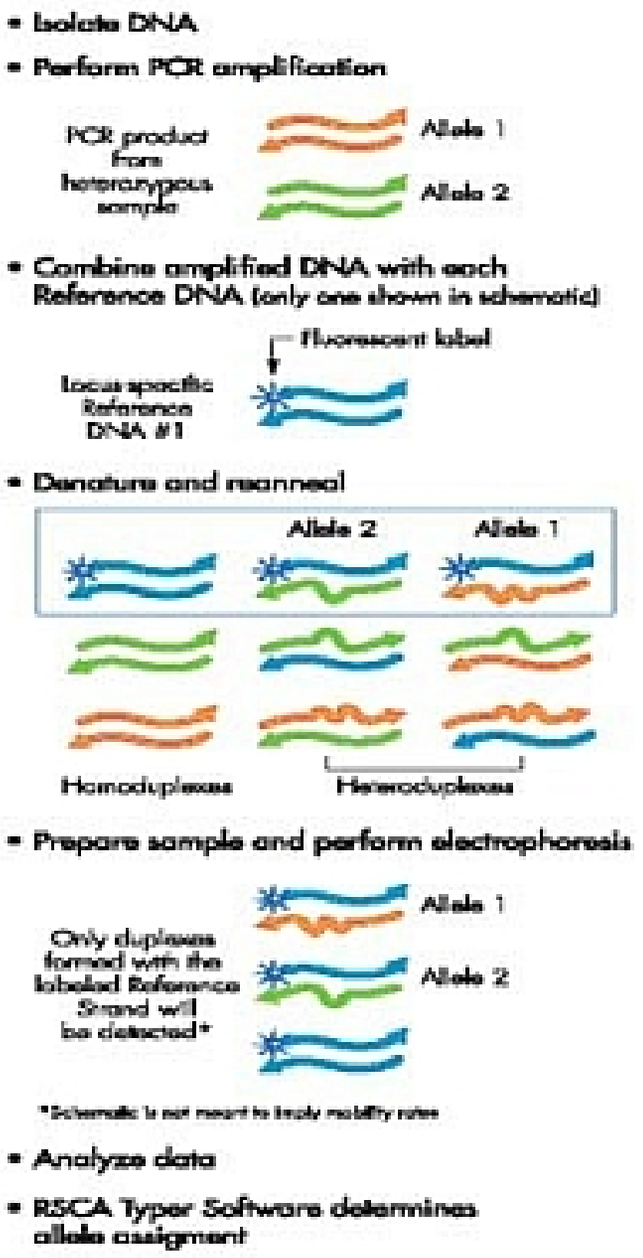

Figure 7. The basic procedure of RSCA

Compared with PCR-SSP, the most commonly used method of HLA genotyping, RSCA has the following advantages: (1) high resolution and sensitivity. RSCA is based on the differential migration rate of fluorescent-labeled double stranded DNA during the electrophoresis.

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Alleles with different sequences will produce DNA duplexes with different spatial structures after hybridization with their fluorescent labeled probes. Two alleles with one nucleotide difference will cause a change in the spatial structure of a hybridized duplex, resulting in an altered migration rate in electrophoresis. Therefore, RSCA can distinguish the alleles with a single nucleotide difference. For example, HLA-A*0207 and A*0209 alleles only differ from one nucleotide at the site 368 of exon 3. In this site, A*0207 has a G while A*0207 has an A. Likewise, HLA-A*0224 and A*0226 only differ from one nucleotide. These alleles all can be distinguished by RSCA. (2) high reproducibility. In RSCA, each lane in the non-denature polyacrylamide gel has markers and each gel has a DNA ladder. Therefore, the alteration caused by different gels or lanes can be eliminated. (3) new allele or mutation identification. RSCA is based on the electrophoretic mobility difference caused by different spatial structure of the duplexes after allele-FLR hybridization. New alleles or mutations will have electrophoretic mobility different from that of known alleles. (4) RSCA can be applied at a large scale with a low cost.

The disadvantages of RSCA are (1) time-consuming for a single sample; (2) requiring high quality samples; PCR-SSP requires 10-100ng/ml of DNA, which can be obtained with a regular DNA purification kit from patients even with a low amount of white blood cells. However, RSCA requires 50-100ng/ml of DNA. It may require an increased amount of blood sample for patients with low levels of white blood cell in order to obtain sufficient DNA; and (3) insufficient database.

5. Pyrosequencing: A high-resolution method for HLA typing

Pyrosequencing is a new HLA genotyping technology based on real time sequencing during DNA amplification. The reaction system contains 4 enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase), a substrate (APS: adenosine 5' phosphosulfate), fluorescein (luciferin), primers and the single stranded DNA template. After one type of dNTP (dATP, dTTP, dCTP and dGTP) is added to the reaction system, it will be incorporated into the newly synthesized chain if it is complementary to the nucleotide on the template. Incorporation of dNTP will generate the same molar amount of pyrophosphate (PPi). ATP sulfurylase converts APS and PPi into ATP, which provides energy for luciferase to oxidate luciferin and emit light. The amount of light signal is proportional to the amount of ATP. The optical signal is detected by a CCD (charge couple device) camera and generates a peak in the pyrogram. The principle of Pyrosequencing is shown in Fig 8.

The height of each signal's peak is proportional to the number of nucleotides incorperated. Unincorporated dNTPs and excessive ATP are converted to dNDPs, which are further converted to dNMPs by apyrase. The optical signal is quenched and the system is regenerated for the next reaction. The next dNTP can be added to the system to start the next reaction after the unincorporated dNTPs and excessive ATP are removed. The reaction cycle continues until the complementary DNA strand is synthesized. Under the room temperature, it takes 3-4 seconds from polymerization to light detection. In this system, 1 pmol of DNA will

generate $6x10^{11}$ pmol of ATP, which in turn yields $6x10^{9}$ pmol of photon with a wavelength of 560nm. The signal can be easily detected by a CCD camera. For the analysis of DNA with an unknown sequence by Pyrosequencing, a cyclic nucleotide dispensation order (NDO) is used. dATP, dGTP, dTTP and dCTP are sequentially added to the reaction. After one nucleotide is incorporated, the other three will be degraded by the apyrase. For the DNA with a known sequence, non-cyclic NDO can be used and will yield a predicted pyrogram. The sequence of the complementary DNA strand can be determined based on the NOD and peak value in the pyrogram.

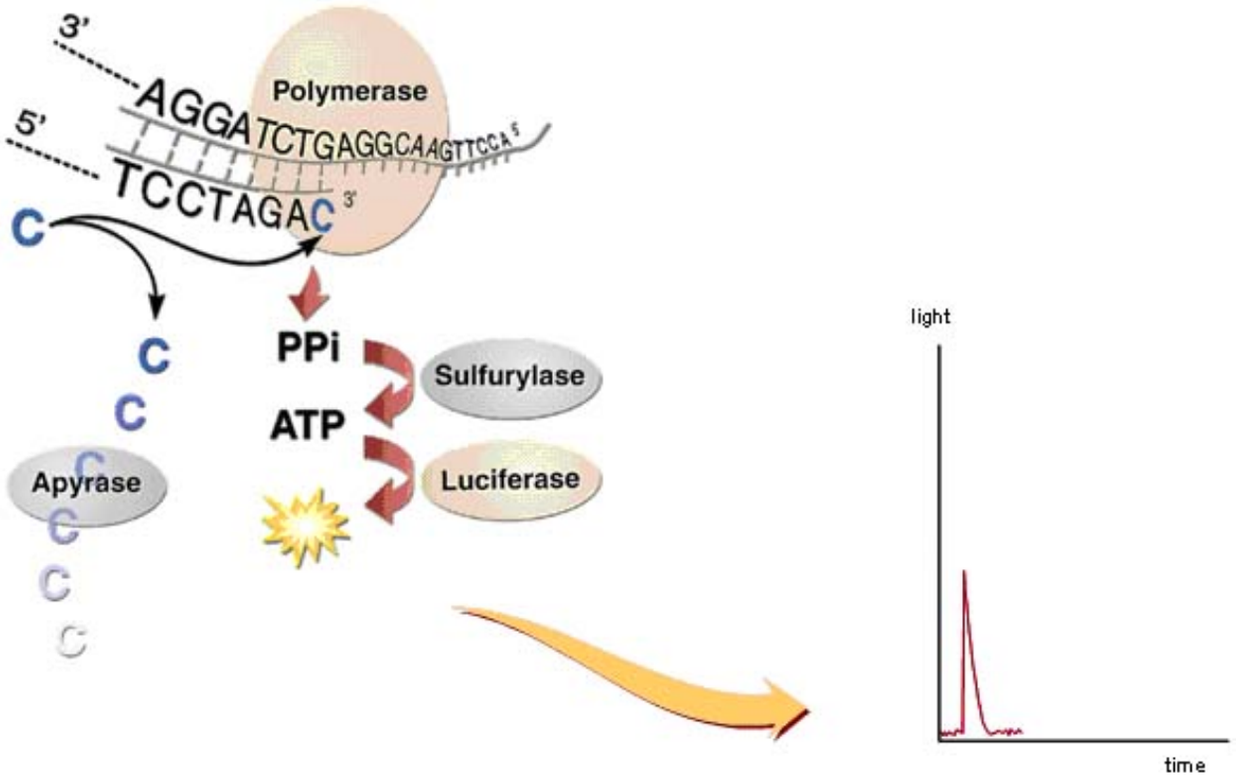

Figure 8. The principle of Pyrosequencing

Since nucleotides are differentially incorporated, Pyrosequencing can produce high-resolution results. Typing HLA-DRB1*04, 07 and DRB4* in the donor's DRB genes by Pyrosequencing not only yields the same result as using the SSOP typing kit, but also produces the result with a higher resolution. Compared with SSP, SSOP, direct or reverse hybridization, Pyrosequencing can be used to solve ambiguous allele combinations of HLA-DQ and HLA-A/B in a short time. The types of HLA-DQB1 and HLA-DRB alleles have been accurately determined by Pyrosequencing.

An inherent problem with this technology is the de novo sequencing of polymorphic region in heterozygous DNA, although polymorphism can be detected in most cases. When the nucleotide in the polymorphic region is altered, synchronized extension can be achieved by the addition of the substituted nucleotides. If there is a deletion or insertion in the polymorphic region,

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and the deleted or inserted nucleotide is the same as the adjacent nucleotide on the template, the sequence after the polymorphic region will be synchronized. However, if the deleted or inserted nucleotide is different from the adjacent nucleotide on the template, the sequence reaction can be out of phase, making the subsequent sequence analysis difficult. Another issue with this technology is the difficulty in determining the number of incorporated nucleotides at the homopolymeric region. The light signal will become nonlinear after the incorporation of more than 5-6 nucleotides. Studies on the polymerization efficiency of the homopolymeric region have shown that it is possible to incorporate less than 10 identical nucleotides in the presence of apyrase. However, it needs a specific software algorithm of signal integration to determine the precise number of incorporated nucleotides. For re-sequencing, the nucleotide is added twice to ensure complete polymerization in the homopolymeric region. Another limitation of this technology is the length of the sequencing.

6. Application of flow cytometry in HLA typing

Flow cytometry has failed to become a main method for HLA typing since it was applied to the field of immunology for the first time in 1977. This is mainly due to the large number of specific probes required for HLA typing. The flow analyzer LABScan100 that combines the flow cytometry and reverse SSO technology is trending to replace three conventional methods, SSO, SSP and SBT (sequence-based typing, direct sequencing), in HLA typing.

On a suspension platform, multiple types of color-coded beads conjugated with SSO probes specifically bind to the single stranded DNA. Each type of bead has its unique spectral characteristics due to the different amount of fluorescent dye conjugated to the beads. When beads pass through a flow cytometer, the difference in the light scattering pattern from various angles can distinguish HLA genotypes.

Currently, LabType ™ SSO is a relatively more mature technique compared with others in HLA typing. Its unique advantage is that thousands of molecules can be simultaneously analyzed in a matter of seconds. Therefore, this technique can be used for a large-scale analysis. Overall, this technique has the following advantages: (1) It has increased accuracy due to the automated detection system. (2) The workload and reagent consumption are reduced. One reaction tube can have 100 different SSO probes, thus greatly reducing the workload and reagent consumption. (3) It produces rapid and objective results. The ambiguous results can be avoided with Specialty Probe Technology ™ (SP Technology). (4) Unlike regular flow cytometry that requires fresh samples, this technique can examine the sample at any time upon request or retrospectively. DNA samples can be analyzed right after extraction or stored at –20°C for more than 1 year without affecting the results. (5) The technique can analyze multiple HLA loci with low, medium and high resolutions. (6) It can be used in laboratories with large or small samples. More than 100 probes can be put in one test tube for one sample or in a 96-well plate for 96 samples. The analysis of 96 samples takes less than 90 min after amplification. (7) The pollution to the environment and potential harm to the staff are reduced because electrophoresis is not required in this method.

7. Gene chip or DNA microarray

In gene chip or DNA microarray, large amount of probe molecules (usually $6x10^4$ molecules/cm^2) are attached to a solid surface. Labeled DNA samples are hybridized to the probes. The amount and sequence information of the target can be determined by the intensity of the hybridization signal. Gene chip or DNA microarray technology was first developed by Affymetrix in the USA, and has been improved significantly within a few years. The technology is based on the principle of reverse dot hybridization. Thousands of oligonucleotide probes representing different genes are spotted on a solid surface by a robot. These probes will bind to radioactive isotope or fluorescent dye labeled DNA or cDNA through complementary sequences. After autoradiography or fluorescence detection, signals are processed and analyzed by computer software. The intensity and distribution of hybridization signal reflect the expression level of the gene in the sample. The operation process of microarray is shown in Fig 9. Balazs *et al* spotted amplified DNA samples on silicon chips and compared the microarray results with PCR-SSO results in 768 specimens. It has been found that microarray has a high sensitivity and specificity. The consistent rate of genotyping results from microarray and PCR-SSO is 99.9%.

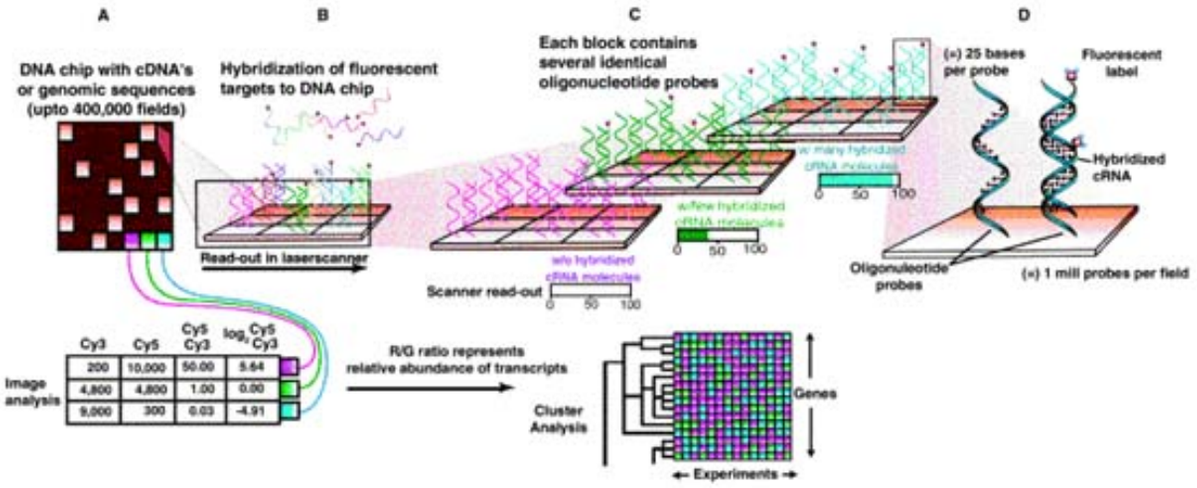

Figure 9. The procedure of gene chip/microarray analysis

Compared with existing genotyping methods, gene chip or microarray has the following advantages: (1) High intensity. The dot intensity on a chip can be higher than $6x10^4/cm^2$. Therefore, probes to thousands of HLA-A, B, C, DR, DQ and DP sequences can be spotted on a tiny chip of several square centimeters to obtain the information of individual HLA genes simultaneously. (2) High resolution. It can obtain information at the allele level. (3) Simple operation. The results are generated by fluorescence scanning instead of gel electrophoresis, which greatly simplifies the procedure and shortens operation time. (4) High sensitivity. Signals are amplified twice, first, PCR amplification of the template DNA and second, amplification of fluorescence signal. Therefore, the sensitivity is greatly improved. (5)High ac-

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curacy. The intensity of the fluorescent signal generated by the perfect pairing of the probe and the sample is 5 to 35 times higher than the signal generated by the probe and the sample with one or two mismatched nucleotides. Accurate detection of fluorescent signal intensity is the basis of the detection specificity. Studies have shown that the consistency between microarray and Sanger sequencing in the detection of mutations and polymorphism is 99.9%. (6) High efficiency. The whole process is highly automatic, which saves manpower and time for data analysis. Genotyping of genes such as HLA-A, B, DR and DQ in multiple samples can be done with one PCR reaction and hybridization on one chip. (7) High level of standardization. Using a variety of multi-point synchronized hybridization and automated analysis, the human error is minimized to ensure the specificity and objectivity. (8) Low cost. Since the chip fabrication and signal detection are all automatic, only a small amount of probes and samples are required. One chip can be used for the analysis of samples from multiple individuals, which further reduces the cost. The biggest drawback of microarray analysis is its expensive equipment, which prevents it from becoming widely used. Only institutions with a large program can afford the equipment.

8. DNA sequencing technology

For the analysis of gene structure, sequencing is the most direct and accurate method. In this case, the DNA fragment is amplified by PCR and followed by sequencing. The basic process of this method is shown in Figure 10. Since the entire nucleotide sequence of the amplified fragment is obtained, this is the most reliable and thorough genotyping method. It can not only identify the sequence and genotype, but also lead to the discovery of new genotypes. Currently, the newly identified HLA alleles can only be verified by sequencing. It has been reported that if the HLA type cannot be determined by serotyping or the results from PCR-SSP and PCR-SSOP are inconsistent, sequence-based typing (SBT) often can yield accurate and reliable results with a high resolution. Hurley *et al* have typed HLA alleles by PCR-STB in 1775 bone marrow transplant patients and unrelated donors in NMDP, USA. The study has found that the degree of HLA allele mismatching between the recipient and donor of bone marrow transplantation is much higher than previously thought after examining the antigen matching results of HLA-A, HLA-B and HLA-DR.

The advantage of SBT over PCR-SSP and PCR-SSOP is its ability to analyze the entire gene sequence including the non-polymorphic region. SBT can be used not only for DNA sequencing but also for cDNA sequencing to determine gene expression. With increasing popularity of DNA sequencing technology, the PCR-SBT method has gained much attention for genotyping. PCR-SBT has advantages over other typing methods in terms of accuracy, efficiency and the degree of automation. Specialized software and solid phase sequencing kits with automatic loading are available for HLA typing. In addition, the cost of DNA sequencing has been greatly reduced. Therefore, PCR-SBT is an ideal method for HLA typing in researches. With further decrease in the cost of automatic sequencing, this genotyping method will be widely used.

Currently, PCR-SBT is the gold standard of HLA typing. This method has several advantages: (1) It can accurately determine gene type in the exon 8 by a high-resolution sequencing, sufficient to meet the need in researches and clinics. (2) It can analyze more than 15,000 samples every month with high throughput detection. (3) Automated SOP and advanced data management system can reduce human error. (4) It has high quality assurance. Ten percent blind samples are used repeatedly as internal quality control and 100% accuracy is achieved for 10 consecutive times using UCLA external quality assurance samples. The results are confirmed by SSP. (5). It may lead to the discovery of new alleles. (6) HLA genotype can be updated by re-analyzing the sequence after the HLA database is updated.

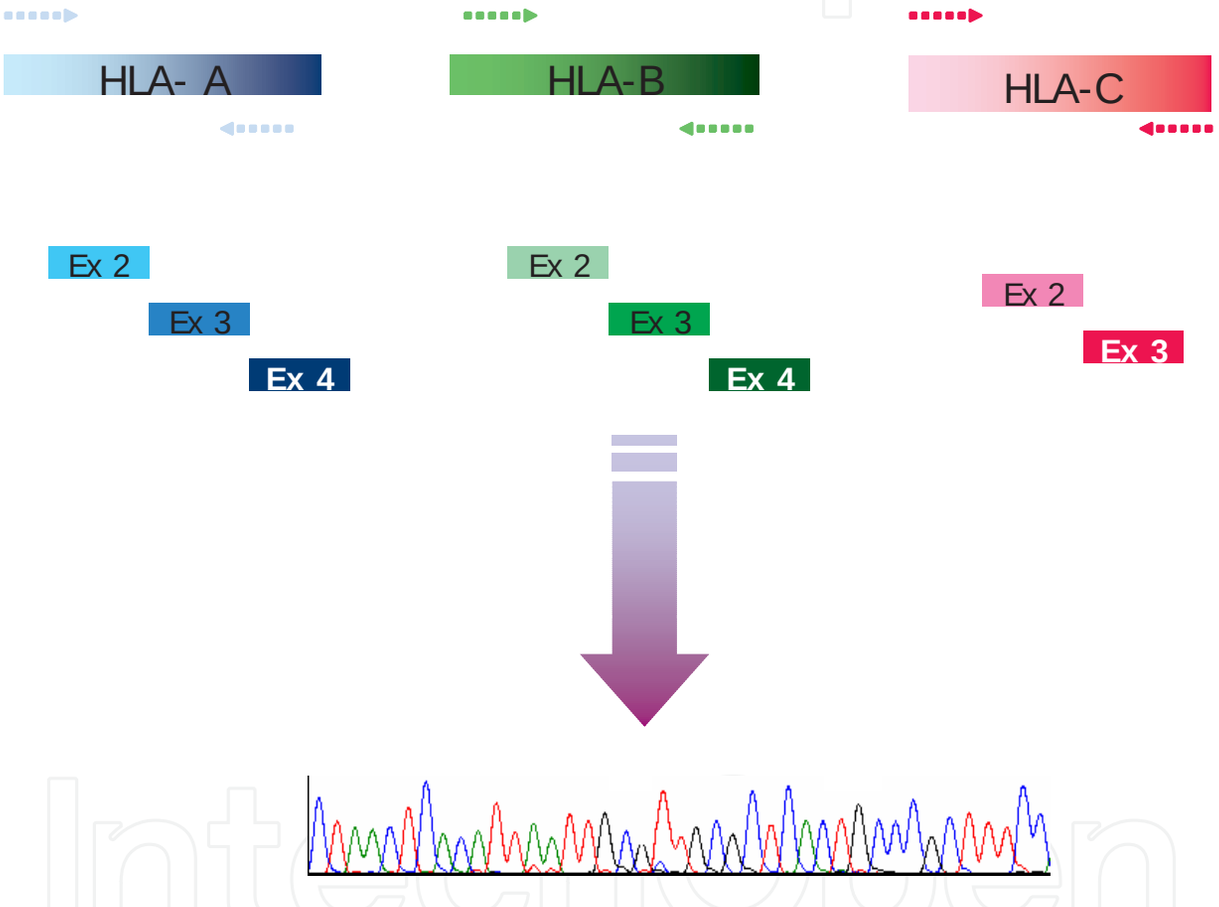

Figure 10. The diagram of DNA sequencing

9. Conclusion

Hematopoietic stem cell transplantation has become one of the most effective treatments for a variety of hematologic malignancies. However, graft-versus-host disease (GVHD) is still inevitable in some cases. This is mainly due to the difference in the major histocompatibility complex (human leukocyte antigen, HLA) between the recipient and the donor. Other known and unknown factors that may cause GVHD include minor histocompatibility anti-

gen (mHA) and tissue specific antigens. GVHD is the main cause of transplant failure in the allogeneic transplantation. Therefore, GVHD is the most significant challenge in allogeneic hematopoietic stem cell transplantation in clinics. It has been proven that whether the graft can survive largely depends on the degree of HLA matching between the recipient and the donor. Therefore, HLA typing of the recipient and the donor before the transplantation is particularly important.

Currently, PCR-SSP genotyping is a commonly used method for HLA typing in clinical laboratories worldwide. Like SSP method, PCR-SSP method depends on specific primers for genotyping. Although the process is simple and rapid, high-resolution genotyping requires a large number of sequence specific primers, which leads to a high cost and prolonged operation time. Similarly, SSO technique is based on the sequence-specific oligonucleotide probes. High-resolution genotyping by SSO significantly increases the cost and complexity. Therefore, it is rarely used for HLA typing today. PCR-SNP is a simple and fast method with a high resolution, and PCR-SNP is expected to become more popular in HLA typing as the technology continues to improve. Although RSCA and Pyrosequencing can achieve high-resolution results, their applications in HLA typing will be gradually eliminated as the technology of gene chip and sequencing continues to improve and the cost continues to decrease. HLA-chip genotyping is still largely dependent on the known sequence. It cannot identify new alleles with unknown sequence. At this moment, PCR-SBT technology has significant advantages over other HLA typing methods in terms of accuracy, efficiency and automation. There are specialized software and automatically loaded sequencing reagents for HLA typing by PCR-SBT. In addition, the operation cost has been greatly reduced. In conclusion, PCR-SBT technology with HLA-chip is the best method for HLA typing in research. With the reduction in the cost of automated nucleic acid sequencing, this genotyping method will be widely used in the field of basic research as well as in clinical transplantation.

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