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# Genetic Diversity and Disease Susceptibility

*Edited by Yamin Liu*





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# GENETIC DIVERSITY AND DISEASE SUSCEPTIBILITY

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Edited by **Yamin Liu**

## **Genetic Diversity and Disease Susceptibility**

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# Meet the editor



Yamin Liu is an assistant professor and clinical pharmacist in the pharmacology department, Zhongda Hospital, Southeast University, China. She specializes in cardiovascular pharmacology, genomic medicine, and pharmacogenomics. She received her PhD in Clinical Pharmacy at the China Pharmaceutical University. She had three years of postdoctoral research experience in the USA, where she conducted research on mechanisms linked to cardiovascular disease. She has published over 20 papers in local and international peer-reviewed journals in the fields of pharmacy, cardiology, and genomics. As a principal investigator, she has received several funds from the National Natural Science Foundation of China, the Science Foundation of Jiangsu, China, and Jiangsu Pharmaceutical Association.





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## Preface

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This book is a self-contained collection of scholarly papers targeting the audience of academics, scientists, physicians, pharmacists, practicing researchers, and postgraduate students whose work relates to genetic polymorphism and its application in individual-specific treatment. This book intends to provide readers with a comprehensive overview of genetic diversity and disease susceptibility, pharmacogenomics, ongoing advances in technology, and analytic methods in this field. It is divided into three sections with a total of eight chapters. These chapters are written by several authors: researchers, scientists, and experts in specific research fields related to genetic polymorphism. The editor would like to take this opportunity to thank all the authors for their valuable contributions. In fact, these chapters not only cover the overview of genetic polymorphism but also provide genetic diversity and disease susceptibility, as well as pharmacogenomics, which will help to optimize drug therapy, with respect to the patient's genotype, and will allow clinicians to determine the most effective and least toxic personalized therapy.

The first section contains two chapters related to genetic polymorphism, which describe polymorphisms at DNA level, type of polymorphisms, common DNA-based molecular markers, and the major technique for DNA-based molecular marker detection.

The second section consists of four chapters that focus on genetic diversity and disease susceptibility, which include gene polymorphism in cancer, immunosuppressants in solid organ transplantation, and small populations.

The third section includes two chapters on pharmacogenomics, which characterize the pharmacogenetics of cardiovascular disease and immunopharmacogenomics in cancer, describing their population distribution and summarizing current knowledge on their biological plausibility. Clinical relevance and current guideline recommendations are also discussed.

**Yamin Liu**

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# Genetic Polymorphism

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# Polymorphisms

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Wasana Sukhumsirichart

Additional information is available at the end of the chapter

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## Abstract

Polymorphism or variation in DNA sequence can affect individual phenotypes such as color of skin or eyes, susceptible to diseases, and respond to drug, vaccine, chemical, and pathogen. It occurs more often than mutations (frequency  $\geq 1\%$ ). The common polymorphism is single nucleotide polymorphism (SNP) which is a single base change in a DNA sequence that occurs most commonly in the human genome. SNPs have been used as molecular markers in a wide range of studies. Genome-wide association studies (GWAS) searches for SNPs that occur more frequently in person with a particular disease than in person without the disease and pinpoint genes or regions that may contribute to a risk of disease. This topic describes about polymorphisms, SNPs, GWAS, linkage disequilibrium (LD), minor allele frequency, haplotype, method for SNP genotyping, and application of SNPs and genome-wide association study in human diseases and drug development.

**Keywords:** drug development, genome-wide association studies, human diseases, polymorphisms, single nucleotide polymorphism, SNPs

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## 1. Introduction

Phenotype of living organism is controlled by DNA. Variation in DNA sequence or polymorphism may make individual difference such as the differences in phenotype, risk of various diseases, and response to drugs, vaccine, chemical, and pathogen. Polymorphisms commonly occur in nature and related to biodiversity, genetic variation, and adaptation. It helps to maintain variety of forms in a population living in a varied environment [1]. It is preserved by frequency-dependent selection. The polymorphisms in this topic focus on human polymorphisms related to diseases and drug respond. Because the Human Genome Project had been completed, a large number of polymorphisms among the population have been found [2–4]. The most abundant type of the variations is single nucleotide polymorphisms (SNPs), with

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more than 9 million reported in public databases [5–7]. In this chapter, the definition of several terms such as polymorphism, minor allele frequency (MAF), allele frequency, haplotype, and linkage disequilibrium (LD) is clarified. Moreover, SNPs, genome-wide association study (GWAS), methods to detect SNPs and application of SNPs in association with diseases and drug development are mainly discussed topics.

## 2. Polymorphisms

Genetic polymorphism, the definition by Cavalli-Sforza and Bodmer, is the occurrence in the same population of two or more alleles at one locus, each with appreciable frequency, where the minimum frequency is typically taken as 1% [8]. An allele is one of the variant forms of a gene at specific locus on a homologous chromosome. The different forms of the polymorphism (alleles) are observed more often in the general population than mutations. The most common polymorphism in the human genome is the single-nucleotide polymorphism (SNP) [9].

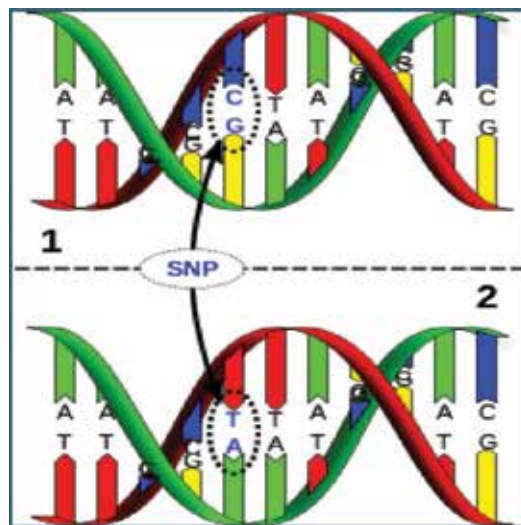
### 2.1. Single nucleotide polymorphism (SNP or snip)

SNPs are popular molecular genetic markers in disease genetics studies and pharmacogenomic research. It is a single base change in a DNA sequence, with a normal alternative of two possible nucleotides at a given position. This variation occurs at a specific position in the genome and has allele frequency of 1% or greater [10]. Around 325 million, SNPs have been identified in the human genome, 15 million of which are present at frequencies of 1% or higher across different populations worldwide [11]. An example for SNP is shown in **Figure 1**. It demonstrates that at a specific position of human genome when compared between two individuals and two DNA sequences. The DNA sequence of a person 1 has C nucleotide which is similar to most of the other person (majority group), whereas the DNA sequence of a person 2 has T at this position which is minority group of population. It is said that there is an SNP at this specific position between allele C or T.

The majority of SNPs have two alleles, which represent a substitution of one base for another. The SNP occurs at each allele of an individual may be different. If the SNP occurs more frequently in the general population, it is called “major” allele. In contrast, if the frequency of the SNP exist is rare in the population, it is designated the “minor” allele. Since human have two copies of chromosome or diploid, therefore, an individual can have various genotypes such as homozygous of major or minor alleles, or heterozygous of major and minor allele [9]. Many SNPs are correlated with one another, so it is difficult to distinguish the SNP that affects the phenotype from the several SNPs associated with it [12].

SNPs are identified and characterized by sequencing the same genomic region in several populations [13, 14]. The sample size of the population being resequenced is important. In general, larger sample sizes are needed to identify SNPs on the lower end of the minor allele frequency spectrum. The minor allele frequency (MAF) refers to the frequency at which the less common allele occurs in a given population. By using population genetics theory prediction for a SNP detection rate of 99%, a SNP with a minor allele frequency of 5% or greater needs 48





**Figure 1.** Single nucleotide polymorphism (SNP). ([http://en.wikipedia.org/wiki/Single-nucleotide\\_polymorphism](http://en.wikipedia.org/wiki/Single-nucleotide_polymorphism)).

chromosomes, whereas a SNP with a minor allele frequency of 1% or greater requires 192 chromosomes for the verification of genotype of SNP [15].

Currently, the genotyping in a large scale of SNPs can be performed by automated machines, which facilitate the genetic association study using DNA-based marker. Human Genome Project rank SNP discovery and characterization as high priorities [16, 17] and encourage public and private sections [4, 18, 19] to push an effort toward these objectives.

#### *2.1.1. Effects of SNPs location*

The location of SNPs may affect gene products and others. The SNPs within a gene may alter protein structures. The SNPs in the regulatory region outside a gene may affect when and how the gene is turned on, which affects the quantity of the protein produced. They also affect gene splicing, transcription factor binding, or the sequence of non-coding RNA. The SNPs that are not within the proximity of a gene may be used as genetic markers for locating disease-causing genes (**Figure 2**).

#### *2.1.2. Types of SNPs*

As described earlier, the SNPs may fall within coding sequences of gene, or non-coding regions of gene, or in the intergenic regions (regions between genes). The SNPs in the coding region of gene are divided into two types: synonymous and nonsynonymous SNPs. The synonymous SNPs do not change the amino acid sequence of protein or not affect the protein function. The nonsynonymous SNPs are divided into two types: missense and nonsense. A missense SNP, single nucleotide change results in a codon that codes for a different amino acid, resulting in protein nonfunction. For nonsense, a point mutation in a sequence of DNA that changes to a stop codon results in a nonfunctional protein product. SNPs that are in non-coding regions

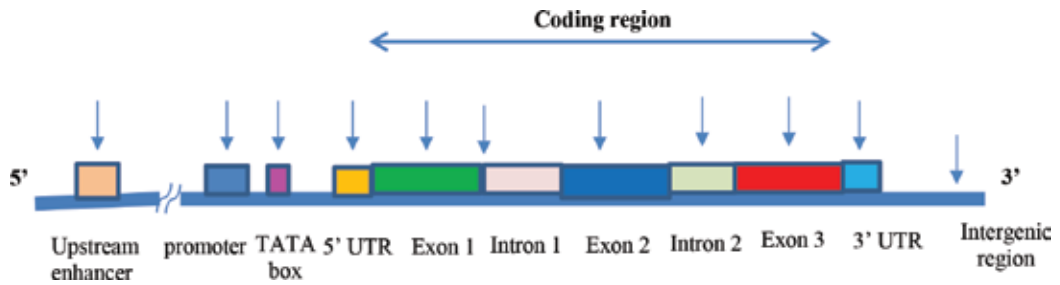


Figure 2. SNP location.

of gene, or in the intergenic regions may affect gene splicing (SNPs at intron region), transcription factor binding (SNPs at 5' untranslated region), messenger RNA degradation, or the sequence of non-coding RNA. The type of SNPs located upstream or downstream from the gene that affect gene expression is referred to an expression SNP (eSNP).

### 3. Association study

By comparing the patterns with patterns obtained by analyzing the DNA from a group of individuals affected and unaffected by the disease is called as an 'association study'. This study demonstrates the linking between the polymorphism and diseases or drug response.

#### 3.1. Minor allele frequency

Minor allele frequency (MAF) refers to the frequency at which the less common allele occurs in a given population. MAF is widely employed in GWAS for complex traits [20]. SNPs with a minor allele frequency of 5% or greater were targeted by the HapMap project.

#### 3.2. Allele frequency and genotype frequency

Allele frequency is the relative frequency of an allele at a particular locus in a population.

Genotype frequency in a population is the sum of the individuals with the same genotype divided by the total number of individual in that population. In addition, the genotype frequency, in population genetics, means the proportion or frequency of genotypes in a population ( $0 < f < 1$ ). The genotype frequency may also be used in the genomic profiling to predict someone is having a disease [21] or even a birth [22] defect. It can also be used to determine ethnic diversity.

#### 3.3. Haplotypes

Haplotypes are a combination of alleles at different markers along the same chromosome that is inherited as a unit. Each haplotype is a combination of major and minor alleles along

the chromosomes, and each individual is represented twice to account for the maternal and paternal contributions [9]. The fundamental difference between haplotypes and individual genotypes at SNPs is that the alleles are assigned to a chromosome.

Haplotypes inform about the exchange of DNA during meiosis or recombination, which is useful for locating the mutation that are associate with diseases by using linkage method. It has an effect on linkage disequilibrium.

### 3.4. Linkage disequilibrium (LD)

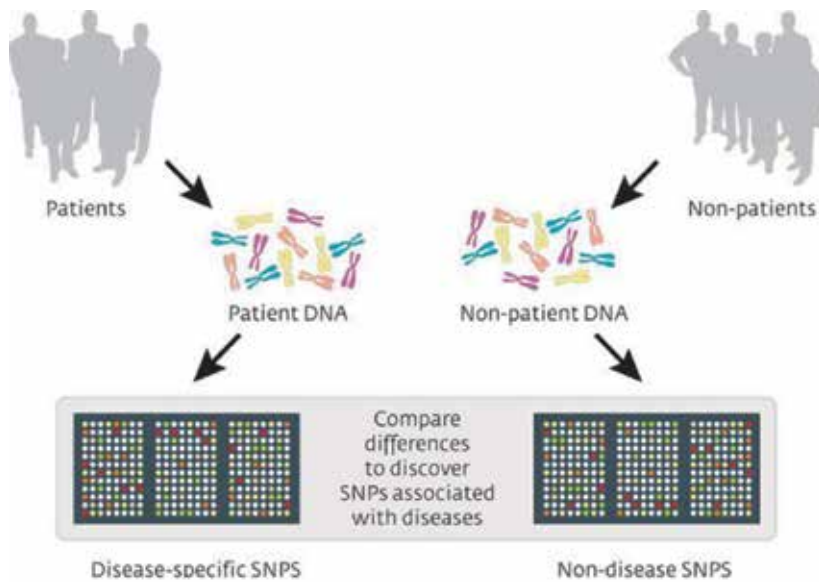
In population genetics, linkage disequilibrium (LD) is the non-random association of alleles at different loci in a given population that may or may not be on the same chromosome. Loci are said to be in linkage disequilibrium when the frequency of association of their different alleles is higher or lower than what would be expected if the loci were independent and associated randomly [23]. LD can detect differences between the SNP patterns of the two groups and reveal which pattern is most likely associated with the disease-causing gene or response to certain drugs.

LD is an important concept in genetic studies that aims to identify and localize genes related to disease susceptibility. LD is commonly used to indicate that two genes are physically linked. It is defined as the difference between the observed frequency of a particular combination of alleles at two loci and the frequency expected for random formation of haplotypes from alleles. The frequency of a particular allele at a given locus will be independent of alleles at other linked loci. LD plays a crucial role in the current methods for mapping complex disease or trait-associated genes or plays a key role in health and disease. The level of linkage disequilibrium is influenced by a number of factors such as genetic linkage, selection, the rate of recombination, the rate of mutation, genetic drift, non-random mating, and population structure.

### 3.5. Genome-wide association studies (GWAS)

GWAS identify the common disease-causing variants by using high throughput genotyping equipment to examine hundreds of thousands of common SNPs and compare these common genetic variants in large numbers of affected cases (patient) to those in unaffected controls (non-patient) to determine whether have an association with disease (**Figure 3**) [24, 25].

In most chromosome regions, there is strong association among SNP, therefore, only a few SNPs in each region are selected to be sequenced to predict the alleles of the remaining SNPs in that region. An accurate mapping of LD pattern among SNPs which differ across ancestral groups is required for selecting the best tag SNPs. The precise LD maps are needed to help genetic association studies and stimulated the developing of human haplotype map [26, 27]. GWAS pinpoint genes that may contribute to a risk of developing disease. The data derived from GWAS inform about disease etiology, therapeutic targets, and gene function [28].



**Figure 3.** Genome-wide association studies (GWAS). (<http://mmg-233-2014-genetics-genomics.wikia.com/wiki/File:GWAS.jpg>).

## 4. Method for detection of SNPs

SNP genotyping strategies typically involve allele-discrimination and allele-detection. The other methods based on physical properties of DNA [29].

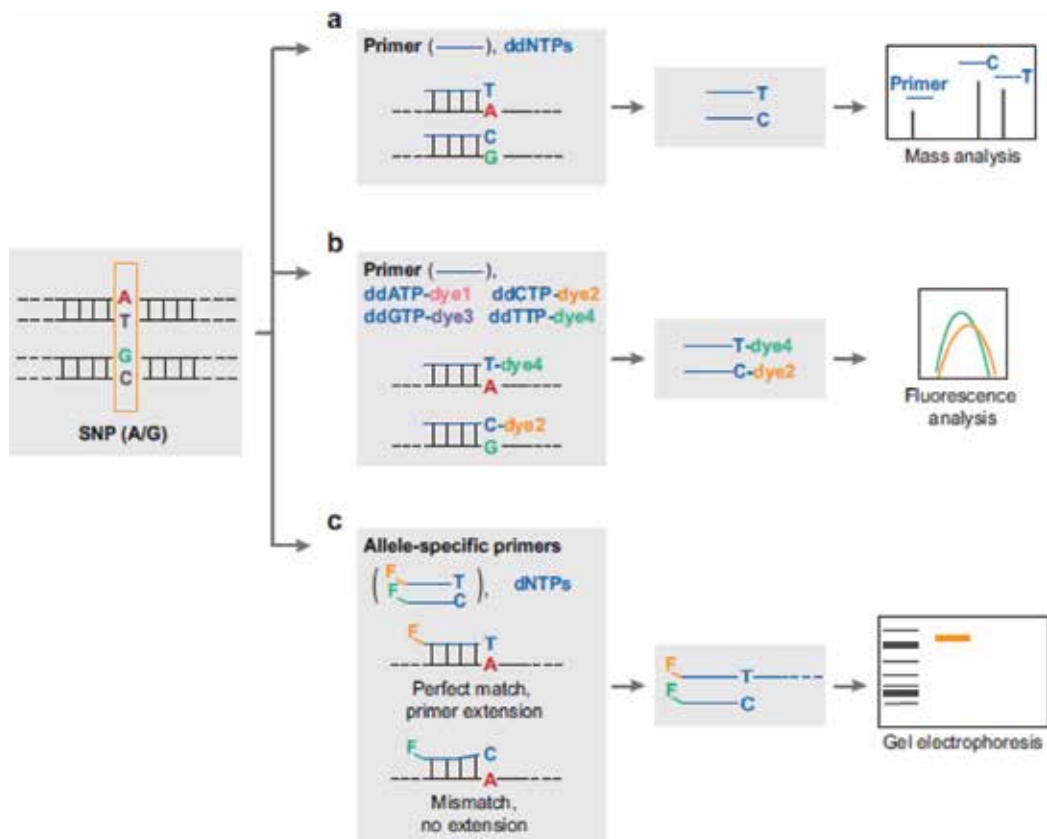
### 4.1. Allele-discrimination

#### 4.1.1. Primer extension

These approaches involve allele-specific incorporation of nucleotides in primer extension reaction with a DNA template, utilizing enzyme specificity to accomplish allelic discrimination. In the reaction of a common primer extension (CPE) protocol, a designed primer is annealing with its 3' end near a SNP site and nucleotides are added by polymerase enzyme [30]. The extended nucleotide is examined by either mass or fluorescence to verify SNP genotype. Because of the simplicity in primer and assay design, it can detect multiple SNPs at the same time, therefore, several SNP genotyping use CPE [29]. CPE-based methods that use MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) for the discrimination of alleles include PinPoint assay [31, 32], MassEXTEND™ [33, 34], SPC-single base extension (SBE) [35], and GOOD assay [36]. In the reaction of these methods, SNP-specific primers are simultaneously extended with numerous nucleotides using PCR products as a template yielding extended products of dissimilar masses. The genotypes of SNPs of the products are examined by mass analysis (**Figure 4a**). The PinPoint assay performs the simplest technique by using dideoxynucleotides (ddNTPs) for single base extension (SBE)

of primer [31, 32]. The CPE approaches that use fluorescence-based detection involve SBE of primer with fluorescently labeled ddNTPs (**Figure 4b**). Specific primer extension (SPE) methods use two primers that have the same nucleotide sequences except specific allele/base at their 3' end. The primers can extend if the nucleotide at their 3' end of the primers perfectly binds with the SNP of complementary template, then allelic discrimination can be examined by mass different using gel electrophoresis (**Figure 4c**).

MassEXTEND™ procedure uses a mixture of dNTPs and ddNTPs for primer extension and discriminate the allele by mass detection. It provides medium-to-high-throughput genotyping

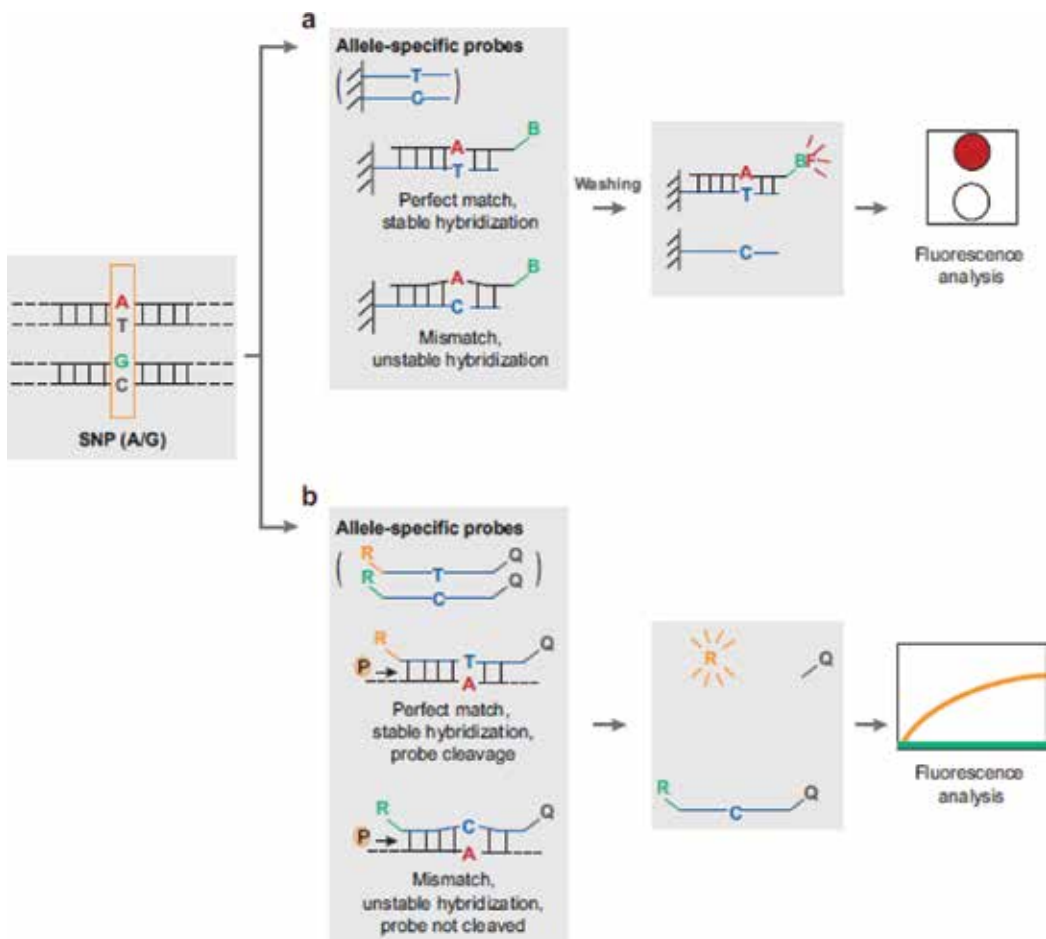


**Figure 4.** Primer extension approaches for SNP genotyping. (a) Mass spectrometry-based detection. This method uses a primer that anneals one base upstream of the SNP site followed by its extension with ddNTPs. Extension products are detected by mass spectrometry, and the difference between mass of extension product and primer identifies incorporated nucleotide(s) and therefore the SNP genotype. (b) Fluorescence detection using capillary electrophoresis. This technique uses a primer that anneals one base upstream of the SNP site after that its extension with ddNTPs that are labeled with different fluorescent tags. Products are detected by fluorescence after capillary electrophoresis and the color of dye indicates incorporated base(s). (c) Allele-specific primers with detection of PCR products. This method uses two allele-specific primers that anneal with their 3' ends at the SNP site and a common reverse primer (not shown) for PCR reaction. The amplification occurs when the forward primer perfectly matches to the SNP at its 3' end and the genotype can be determined by gel electrophoresis of PCR product [29].

that can be used to genotype a group of SNPs in candidate genes, SNPs for GWAS and SNPs in a large sample set [33, 34].

#### 4.1.2. Hybridization

Hybridization methods use differences in thermal stability of double-stranded DNA to separate between perfectly matched and mismatched target-probe pairs for succeeding allelic discrimination. Hybridization methods have been applied on high-throughput platforms using microarrays (**Figure 5a**). In the GeneChip® array technology (Affymetrix, CA) protocol, probe array is synthesized in an ordered fashion on a solid surface using 25-bases oligonucleotides with specific allele by photolithography [37].



**Figure 5.** Hybridization-based approaches for SNP genotyping. (a) Target hybridization to a probe array. This method uses allele-specific probes attached to a solid surface for hybridization with tagged-targets containing SNPs. The surface is washed to remove mismatched targets, then the genotype of the perfectly matched target-probe pairs is detected by fluorescence. (b) TaqMan® assay. The assay uses two allele-specific probes with different dyes, reporter (R) and quencher (Q), at either end with a mismatch at the SNP site. The perfectly matched probe is cleaved during PCR amplification of the SNP-containing region, release its reporter, and the SNP genotype indicates by fluorescence analysis [29].

The DNA fragment containing SNPs are amplified from genomic DNA. The PCR products are cleaved, tagged, and hybridized to the probe array under stringent conditions, then wash, and label with fluorescent. The genotypes of SNPs are examined from the fluorescence signal based on probe-target hybridization. Numerous probes that differ at a single base are used to confirm each SNP to increase genotyping accuracy. In this method, a single array contains millions of probes and be used for parallel genotyping of  $10^4$  to approximately  $10^5$  SNPs [38]. The TaqMan® genotyping assay (Applied Biosystems, CA) combines the procedure of hybridization and 5' nuclease activity of polymerase coupled with fluorescence detection (**Figure 5b**). It employs four oligonucleotides containing two allele-specific oligonucleotide probes that have a single base mismatch and a pair of PCR primers neighboring the SNP covering region [29, 39].

#### 4.1.3. Ligation

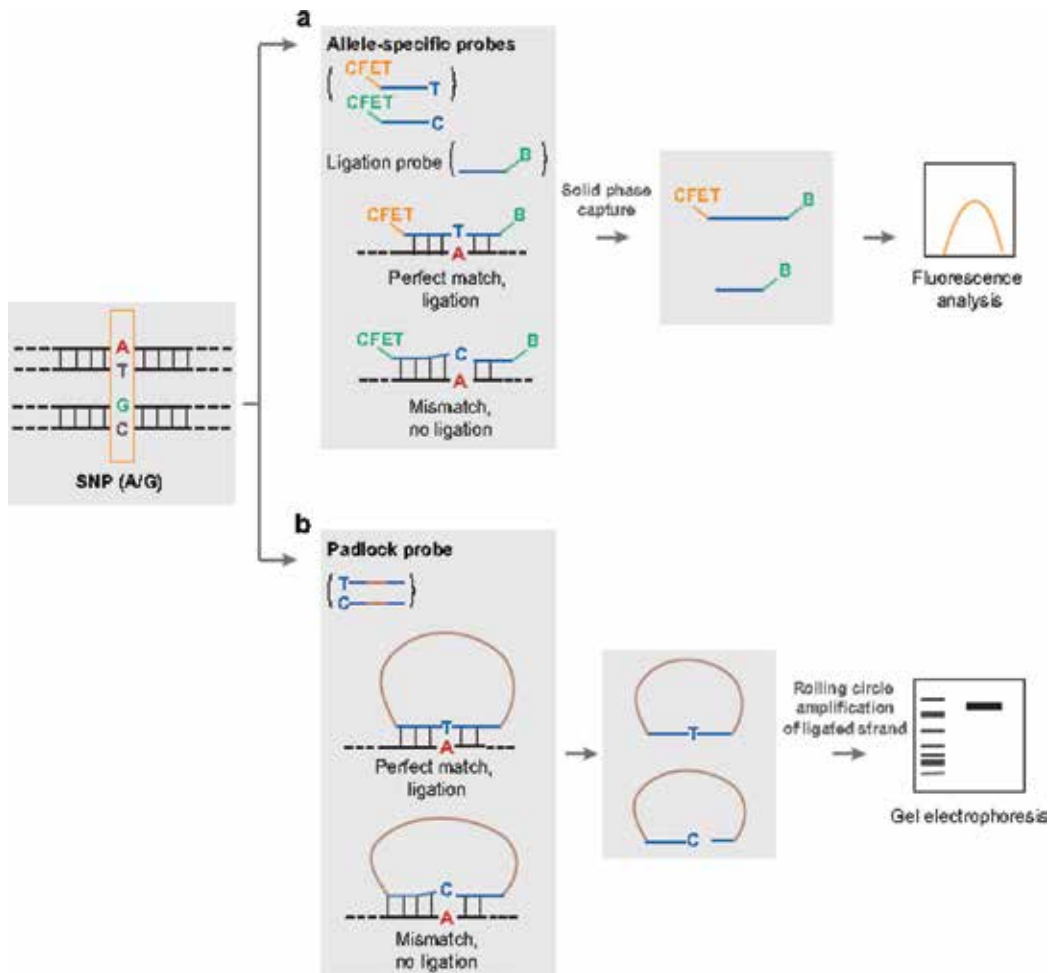
Ligation method discriminates the allele by using specificity of ligase enzymes. When two oligonucleotides (allele-specific probes and ligation probes) hybridize to single-stranded template DNA with perfect complementarity, they are nearby to each other, then ligase enzymes join them together to form a single oligonucleotide. Normally, three oligonucleotide probes are used in ligation assays, two probes are allele specific that bind to the template at the SNP site and ligation probe [40]. Combinatorial fluorescence energy transfer (CFET) tags have been used with ligation for SNP genotyping [41]. CFET tags are composed of fluorescent dyes that can transfer energy when they are in close proximity. For SNP genotyping, two probes of allele specific are labeled with CFET tags and common probe is labeled with biotin (**Figure 6a**). After the ligation reaction, product is separated using the biotin-streptavidin interaction. Genotyping is carried out by using capillary array gel electrophoresis based on tag fluorescence. In Padlock technology, a linear oligonucleotide probe is used with its ends designed to mimic the allele-specific probe and common probe for ligation at the SNP site (**Figure 6b**).

#### 4.1.4. Enzymatic cleavage

The method is based on the capacity of enzymes to cleave DNA by recognition of specific sequences and structures. Difference between alleles can be discriminated when SNPs are located in an enzyme recognition site and affect the enzyme activity.

The ability of restriction enzymes has been used for detection of genetic variation by the method of restriction fragment length polymorphism (RFLP) [42]. These enzymes recognize specific sequences in double-stranded DNA and cleave both strands at a specific site in the sequence or near it to generate smaller DNA fragments (**Figure 7a**). For SNP genotyping, the PCR product containing the SNP is incubated with appropriate restriction enzyme and separated by gel electrophoresis. The SNP genotype is simply determined from sizes of the digested products. This method does not need any probes but it has limited amount and a number of SNPs.

The Invader® assay (Third Wave™ Technologies, WI) utilizes structure-specific cleavage by a flap endonuclease enzyme (**Figure 7b**). It uses three probes for genotyping a SNP, two allele-specific probes and a third common probe (invader) [43].



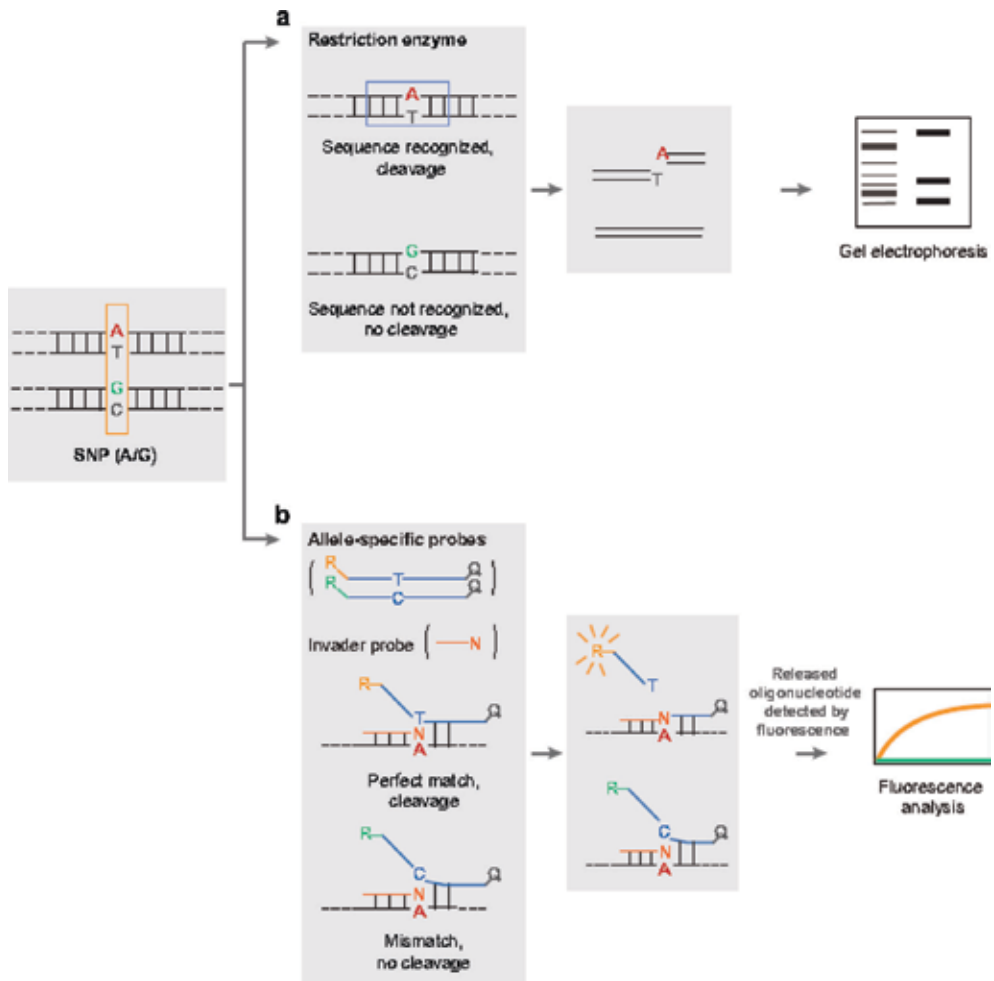
**Figure 6.** Ligation-based SNP genotyping approaches using ligase enzymes. (a) Detection using CFET labels. This protocol uses two allele-specific probes that have different CFET labels and a biotin-tagged common probe that hybridizes adjacent to them. Allele-specific probe and common probe bind to the template containing SNP. If the sequences are perfectly matches at SNP site, the two probes are ligated. The ligated product is proved by isolation using the biotin tag on the common probe and the fluorescence signal from the CFET label to indicate SNP genotype. (b) Ligation of padlock probe. This method uses two allele-specific probes that hybridize to target DNA with their ends aligned end-to-end at the SNP site. One end of the probe is allele-specific that is ligated to the other end only when there is a perfect match at the SNP site, leading to circularization of the probe. After rolling circle amplification using a specific prime, the product is analyzed by gel electrophoresis [29].

## 4.2. Allele detection methods

### 4.2.1. Mass-based detection

For mass analysis of oligonucleotides, MALDI-TOF MS is a widely used method. It involves the use of a small organic molecule termed matrix that absorbs energy from a laser source of certain wavelength for ionization. When analytes are mixed and cocrystallized with matrix, they are ionized in the form of intact molecules owing to transfer of energy from the matrix molecules [44, 45]. **Figure 8** demonstrates a multiplex SNP genotyping by primer extension and MALDI-TOF MS detection.

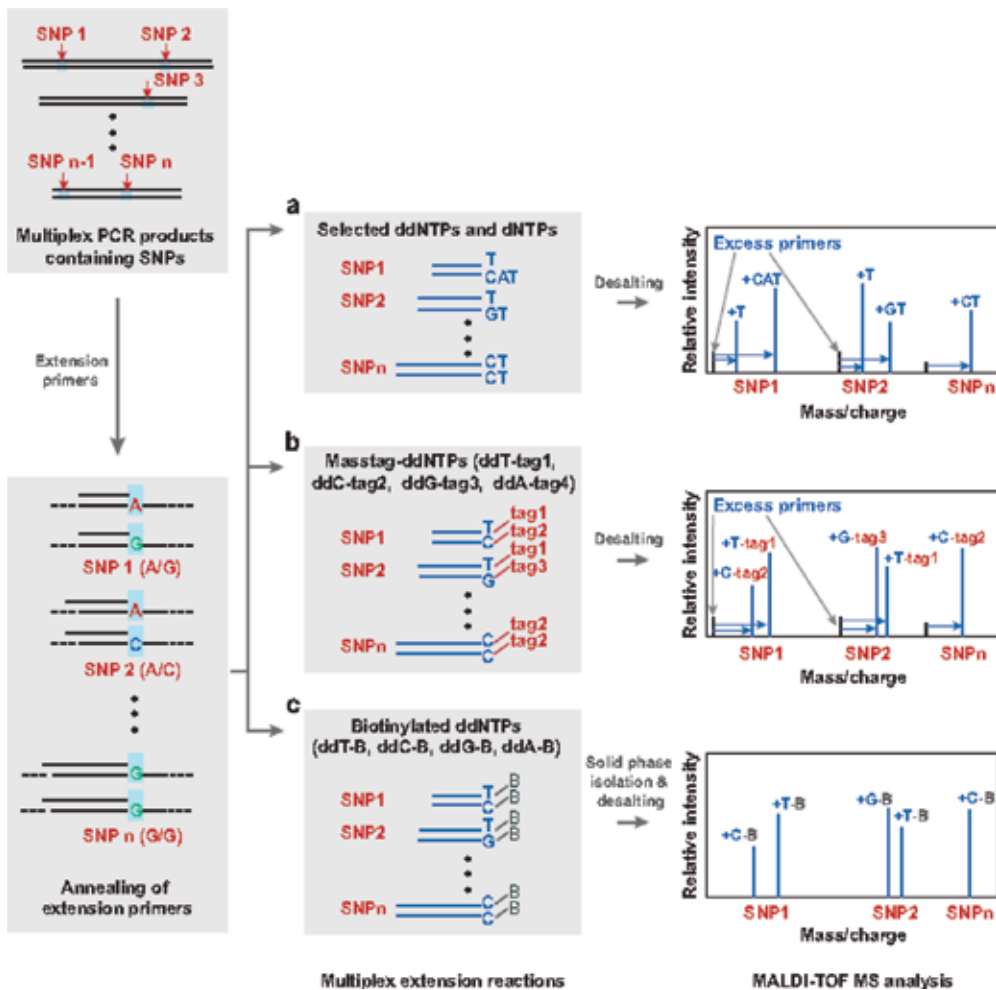




**Figure 7.** Enzymatic cleavage for SNP genotyping. (a) Digested with specific restriction enzyme. This method uses a restriction enzyme that cleaves only one of the alleles. The digested products are run on agarose gel electrophoresis, and the SNP genotype is determined based on the size and number of DNA fragments. (b) Invader® assay. This assay uses two allele-specific probes with different dyes, reporter (R) and quencher (Q), at either end or one common invader probe. The allele-specific probe and invader probe hybridize with target DNA to form a three-dimensional structure at the SNP site which recognized by cleavase enzyme. The allele-specific probe that complementary to the SNP is cleaved by the enzyme and releases its reporter dye in which SNP genotype can be discriminated by fluorescence analysis [29].

#### 4.2.2. Fluorescence signal-based detection

Monitoring fluorescence signal is widely used in genotyping technologies because its operation is simple and detection is fast with high sensitivity. Fluorescence detection is used for direct sequencing (DS) using capillary array electrophoresis. Fluorescence polarization (FP)-based detection uses the change in polarization of plane polarized light by a fluorescent dye molecule owing to change in its molecular weight under conditions of constant temperature and solvent viscosity [46]. FP has been coupled with other SNP genotyping techniques including TaqMan® and Invader® [47]. TaqMan® assay is a single-step assay that uses fluorescence-based detection.



**Figure 8.** Multiplex SNP genotyping by primer extension and MALDI-TOF MS [29]. First, genomic DNA is amplified in multiplex PCR reaction, and the PCR products are used for a primer extension reaction with a collection of primers specific to each SNP site. The mass of the primer indicates SNP, and the mass difference between primer and its extension product(s) tells identity of incorporated nucleotide(s) and the genotype. (a) MassEXTEND<sup>™</sup> method in which primers are prolonged with a specific mixture of dNTPs and ddNTPs by one or more bases, specific to each allele. (b) Modified PinPoint method using mass-tagged ddNTPs for primer extension. This increases mass difference between extension products of alleles of a SNP, when compared with regular ddNTPs, increasing the accuracy of genotyping. (c) SPC-SBE method relating the production of primer extension products with biotinylated ddNTPs for isolation on a solid surface. This excludes excess primers from the mass spectrum, permitting the analysis of more products at the same time [29].

It is well suited for low-to-medium throughput genotyping applications but is currently limited to genotyping of one SNP per assay.

#### 4.2.3. Chemiluminescence

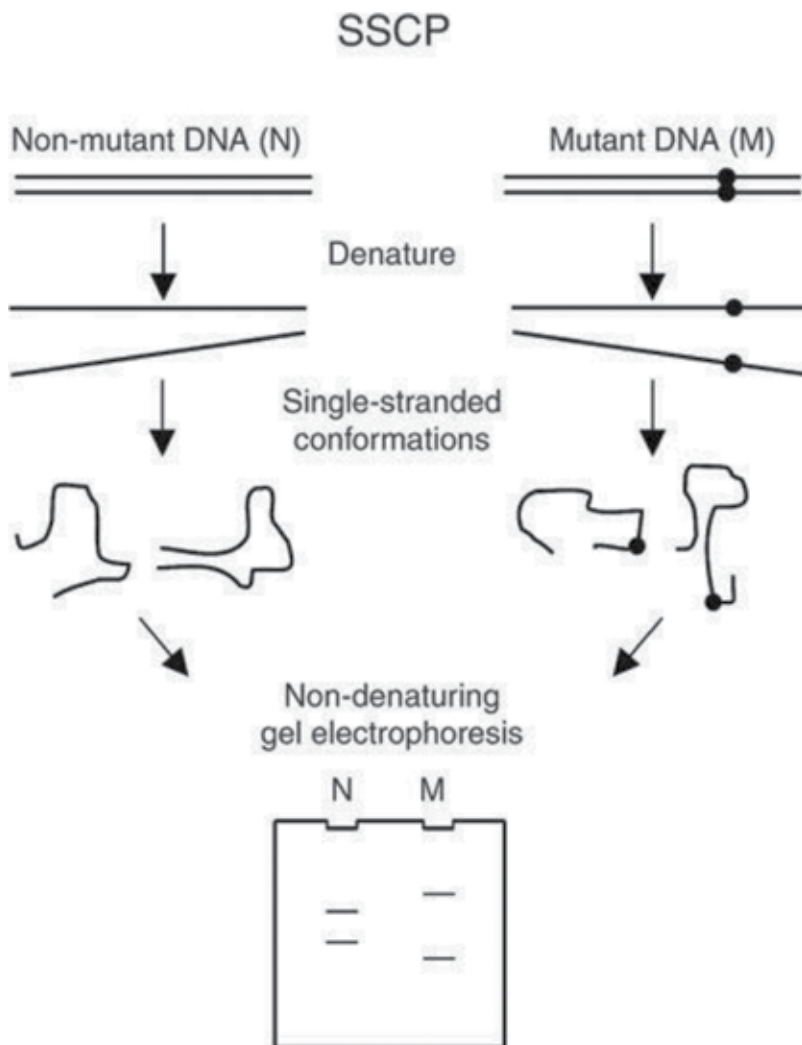
Chemiluminescence has several advantages as a detection technique, such as high signal-to-noise ratio, rapid detection, and feasibility for automation. Pyrosequencing<sup>™</sup> (Biotage, Sweden) employs chemiluminescence-based detection for SNP genotyping using a cascade

of enzyme reactions [48]. Pyrosequencing™ is an approach that combines sequencing-by-synthesis with chemiluminescence detection. In SNP genotype, it provides sequence information on the region surrounding the SNP site.

### 4.3. The other methods

#### 4.3.1. Single-strand conformation polymorphism (SSCP)

SSCP discriminates the allele by using secondary structure of single-stranded DNA. The single-stranded DNA molecules which differ at a single base run on a non-denaturing gel electrophoresis display different mobility based on their native conformations (**Figure 9**) [49].



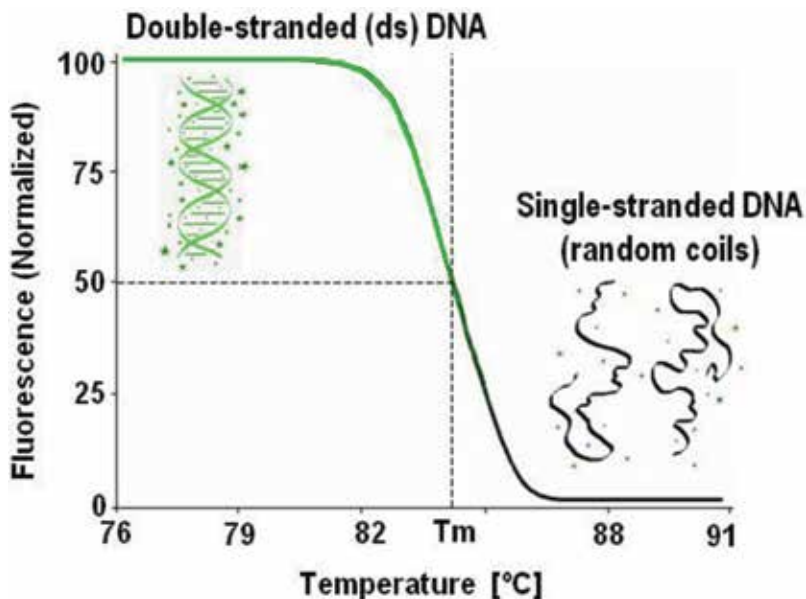
**Figure 9.** Single-strand conformation polymorphism (SSCP) (<https://media.nature.com/m685/nature-assets/nprot/journal/v1/n6/images/nprot.2006.485-F1.jpg>).

#### 4.3.2. High resolution melting analysis (HRM)

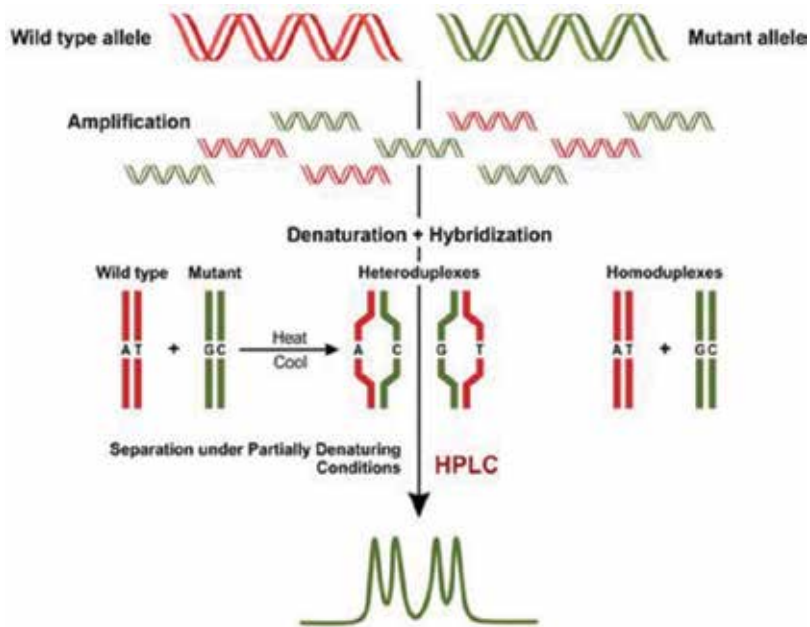
This method, the fragment cover SNP is amplified by real-time PCR and followed by HRM. The HRM is a technique for the detection mutations and SNPs, which based on analysis of melting curve when double-strand DNA (dsDNA) separate into single-strand DNA (ssDNA) during increased temperature from around 50°C up to around 95°C. At some point during this process, the melting temperature of the amplicon is reached and the two strands of DNA separate. This can visualize the melting behavior of the product through a fluorescent dye [50]. The fluorescent dye binds to double-strand DNA during the amplification resulting in an increase of fluorescence (**Figure 10**). This method is simple, cost-effective, fast and able to accurately genotype many samples rapidly. It also reduces the need to design multiple pairs of primers or purchase expensive probes.

#### 4.3.3. Denaturing high performance liquid chromatography (DHPLC)

It is a method for screening DNA samples for SNPs and inherited mutations. The analysis begins with a PCR amplification, followed by a step of denaturation-renaturation to create hetero- and homoduplexes from the two populations in the PCR. The heteroduplexes with mismatch pairing and homoduplex can be detected on reversed-phase chromatography of denaturing high performance liquid chromatography (DHPLC). The heteroduplexes thermally that less stable than their corresponding homoduplexes will be resolved by chromatography when subjected to a sufficiently high temperature. This mismatch will decrease the interaction with the column and a reduced retention time compared to the homoduplexes (**Figure 11**).



**Figure 10.** High resolution melting (HRM) analysis ([https://www.dna.utah.edu/Image/Hi\\_Res%20Melting\\_Normalized.JPG](https://www.dna.utah.edu/Image/Hi_Res%20Melting_Normalized.JPG)).



**Figure 11.** Denaturing high performance liquid chromatography (DHPLC) ([http://www.tankonyvtar.hu/en/tartalom/tamop425/0011\\_1A\\_Molekularis\\_diagnosztika\\_en\\_book/images/image047.png](http://www.tankonyvtar.hu/en/tartalom/tamop425/0011_1A_Molekularis_diagnosztika_en_book/images/image047.png)).

## 5. Application of SNPs

### 5.1. SNPs as biological markers of human diseases

Most SNPs are not responsible for a disease state. They serve as biological markers for pinpointing a disease on the human genome map. SNPs occur on average once every 200 base pairs [18, 51–53] in the human genome. Common SNPs (a minor allele frequency range from 5% to >20%) can explain a proportion of common human disease. Most SNPs do not occur in the coding region of genes or even in genes [4]. Nonsynonymous SNPs that suspect in causing a human disease do not account for all SNPs that can cause disease or susceptibility to disease. Other functional SNPs associated with human disease or susceptibility to disease includes SNPs located in promoters [54, 55], introns [56], splice sites [57], and intragenic region [56, 58]. Even synonymous SNPs have been involved in functional consequences via unknown mechanism [59].

### 5.2. SNPs and drug development

Variants of genes encoding drug metabolizing enzymes or drug targets have been studied in association with personal drug responses. SNPs are popular molecular markers in such pharmacogenomics studies. Using SNPs to study the genetics of drug response will help in the creation of personalized medicine or the most appropriate drug for an individual and could be determined in advance of treatment by analyzing a patient's SNP profile. SNPs may

be associated with the absorbance and clearance of therapeutic agents. The association of different SNPs with a wide range of human diseases such as cancer, infectious diseases autoimmune, neuropsychiatric and others can be used as targets for drug therapy [60].

## 6. Studies of SNPs in association with human diseases

### 6.1. Cancer

Cancer is a disease that involves abnormal cell growth. There are several kinds of cancers. Gemignani et al. studied polymorphisms in dopamine receptor gene, *DRD2*, in association with colorectal cancer risk utilizing the APEX system which is primer extension assays that use fluorescence and mass detection [61]. They genotyped seven SNPs of *DRD2* in 370 case and 327 control samples and found three of the seven SNPs to be highly associated with colorectal cancer, related to reduced levels of D2 dopamine receptor. Hartikainen et al. investigated genes associated with breast cancer in an eastern Finnish population [62]. Ten SNPs were genotyped in the 22q12-q13 region from DNA of 497 patients and 458 control subjects using the TaqMan® assay. The SNP, rs733655, in matriptase-2 gene (*TMPRSS6*) was found to have a strong association with risk of breast cancer. Ribas et al. genotyped 899 SNPs in 175 candidate cancer genes from Spanish population using BeadARRAY™ and SNPlex™ methods to evaluate applicability of HapMap data (for subjects of European ancestry) to cancer research. They found that allele frequencies and haplotype distributions obtained in the study were consistent with the HapMap data [63]. Shatalova et al. examined SNPs in sulfotransferase 1A1 (*SULT1A1*) and UDP-glucuronosyltransferase 1A1 (*UGT1A1*) genes in 119 patients with breast cancer and 121 controls using Pyrosequencing™ [64]. They found only *UGT1A1* gene to be associated with risk of breast cancer. Copson et al. genotyped a T/G SNP (SNP309) in the *MDM2* gene in 116 patients with pathogenic mutations in *BRCA1* gene and 102 healthy controls [65]. Results suggested that the *MDM2* SNP309 locus does not have a significant association with accelerated cancer development in carriers of known pathogenic mutations of *BRCA1*.

### 6.2. Schizophrenia

Schizophrenia is a severe psychiatric disorder characterized by hallucinations, delusions, cognitive deficits, and apathy, with a lifetime prevalence of ~1%. Epidemiologic studies on twins indicate that schizophrenia has a complex genetic background with heritability estimated at 73–90%.

Arinami et al. genotyped 5861 SNPs of 602 individuals from 236 Japanese families using the BeadARRAY™ Linkage Panel (IV) for the genome-wide linkage analysis [66]. They found a strong association of schizophrenia to the region 1p21-p13 and implied that schizophrenia might have common susceptibility loci across populations with different ethnicity-specific effects.

Panichareon B et al. used GWAS-discovered SNPs of Europeans ancestry in *OPCML* gene and investigated SNPs in Thai schizophrenia patient by using polymerase chain reaction (PCR) and high-resolution melting (HRM) analysis. The results of this study found a strong

association between an intronic SNP (rs1784519) and the risk of schizophrenia in a Thai population [ $p = 0.00036$ , odds ratio for the minor A allele: 2.11(1.57–2.84)] [67].

### 6.3. Dyslipidemia

Dyslipidemia is an abnormal of lipid and/or lipoproteins in the blood. It is a major risk factor of coronary heart disease and atherosclerosis. A genome-wide association study (GWAS) examined the concentrations of HDL-C and triglyceride in European ethnic and identified the SNP at 15 loci which associated with HDL-C levels (such as, APOA1/C3/A4/A5 gene cluster) and SNPs at 12 loci associated with triglycerides (such as APOB, APOE gene) [68]. Thongket et al. examined SNP in apolipoprotein E receptor 2 gene using real-time PCR and HRM analysis and found that the rs2297660 showed strong association with risk of dyslipidemia in Thai population [69].

### 6.4. Diabetes mellitus

Diabetes mellitus (DM) is a group of metabolic disorders. Untreated diabetes patient can cause many complications. Acute complications can include diabetic ketoacidosis, hyperosmolar hyperglycemic state, or death [70]. SNPs in the gene encoding aldose reductase (*AKR1B1*) were studied for their association with diabetic nephropathy by Wolford et al. They genotyped eight SNPs of *AKR1B1* gene in two different case control sets by Pyrosequencing™ method and found that common *AKR1B1* SNPs were unlikely to be major determinants of diabetic nephropathy [71]. Altshuler et al. genotyped SNPs from 3000 individuals to investigate the association of two polymorphisms, a missense variant in *PPARG* (Pro12Ala) and a silent C/T polymorphism in exon 22 of *ABCC8*, with type 2 diabetes using FRET and FP methods. It was found that only Pro12Ala showed a significant association with decreased risk of type 2 diabetes [72].

## 7. Conclusion

Polymorphism is a variation in DNA sequence that may affect individual phenotypes. It occurs more often in the general population than mutations (frequency  $\geq 1\%$ ). The majority of variation is single nucleotide polymorphism (SNP) which is a single base change in a DNA sequence that occurs at a specific position in the genome. SNP may locate within coding, or non-coding, or intergenic regions of genes. Most of SNPs have two alleles, for an individual SNP, one is major allele and the other is the minor allele based on their observed frequency in the general population. Genome-wide association studies (GWAS) search for SNPs that occur more frequently in person with a particular disease than in person without the disease and pinpoint genes that may contribute to risk of disease. Linkage disequilibrium (LD) is commonly used to indicate that two or more genes are physically linked. It plays an important role in health and disease. Most SNPs are not responsible for a disease state but serve as biological markers for various complex diseases such as cancer, diabetes, dyslipidemia, schizophrenia, and so on. There are several methods for analyzing SNPs such as MALDI-TOF MS, GeneChip® array, pyrosequencing, DHPLC, HRM, RFLP, and so on.

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# DNA Polymorphisms: DNA-Based Molecular Markers and Their Application in Medicine

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Salwa Teama

Additional information is available at the end of the chapter

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## Abstract

DNA polymorphisms are the different DNA sequences among individuals, groups, or populations. Polymorphism at the DNA level includes a wide range of variations from single base pair change, many base pairs, and repeated sequences. Genomic variability can be present in many forms, including single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs, e.g., mini- and microsatellites), transposable elements (e.g., Alu repeats), structural alterations, and copy number variations. Different forms of DNA polymorphisms can be tracked using a variety of techniques; some of these techniques include restriction fragment length polymorphisms (RFLPs) with Southern blots, polymerase chain reactions (PCRs), hybridization techniques using DNA microarray chips, and genome sequencing. During the last years, the recent advance of molecular technologies revealed new discoveries of DNA polymorphisms. DNA polymorphisms are endless, and more discoveries continue at a rapid rate. Mapping the human genome requires a set of genetic markers. DNA polymorphism serves as a genetic marker for its own location in the chromosome; thus, they are convenient for analysis and are often used as in molecular genetic studies.

**Keywords:** copy number variations, genetic polymorphism, microsatellite, minisatellite, molecular markers, single nucleotide polymorphisms (SNPs), variable number tandem repeats (VNTRs)

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## 1. Introduction

Genetic polymorphism is the existence of at least two variants with respect to gene sequences, chromosome structure, or a phenotype (gene sequences and chromosomal variants are seen at the frequency of 1% or higher), typical of a polymorphism, rather than the focus being on rare variants [1].

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The human genome comprises 6 billion nucleotides of DNA packaged into two sets of 23 chromosomes, one set inherited from each parent. The probability of polymorphic DNA in humans is great due to the relatively large size of human genome. Genomic variability includes a wide range of variations from single base pair change, many base pairs, and repeated sequences [2].

Single nucleotide polymorphisms are the most common type of genetic variations in humans [3], due to their abundance across the human genome; single nucleotide polymorphisms (SNPs) have become important genetic markers for mapping human diseases, population genetics, and evolutionary studies. SNPs have become very important since technologies for DNA sequencing have become feasible and widely available. Advance continues at a rapid rate [4].

A major step forward in genome identification is the discovery of about 30–90% of the genome which is constituted by regions of repetitive DNA which are highly polymorphic in nature [5]. Polymorphic tandem repeated sequences have emerged as important genetic markers and initially, variable number tandem repeats (VNTRs) were used in DNA fingerprinting. In recent years, evidence has been accumulated for the involvement of VNTR repeats in a wide spectrum of pathological states [6].

Throughout the past years, scientists have believed that genes strictly came in two copies in a genome. However, with the recent advancement in molecular technology, discoveries have revealed substantial segments of DNA, ranging in size from thousands to millions of DNA bases that could vary in copy number. Such copy number variations (or CNVs) encompass gene copies, newly discovered CNVs are important sources of genomic diversity [7, 8].

The development and use of DNA-based molecular markers is one of the most significant developments in the field of molecular genetics that facilitate the study of genetic variations in health and diseases [5].

This chapter reviews the DNA-based genetic markers and their application in medicine, with a particular emphasis on common DNA-based genetic markers, including single nucleotide polymorphisms and short tandem repeats (STRs).

## 2. Polymorphisms at DNA level

Genomic variability at DNA level can be present in many forms including: single nucleotide polymorphisms, variable number of tandem repeats (e.g., mini- and microsatellites), transposable elements (e.g., Alu repeats), structural alterations, and copy number variations. It can occur in the nucleus or mitochondria. Two major sources: (1) mutations that may result as chance processes or have been induced by external agents such as radiation and (2) recombination. Once formed, it can be inherited, allowing its inheritance to be tracked from parent to child [3].

The genomes of humans may be divided into different parts based on known functional properties; the coding and noncoding regions mostly do not code for protein [2, 9]. The coding

regions contain DNA sequences which determine primarily the amino acid sequences of the proteins for which they code. Noncoding DNA generally containing DNA sequences with no function has not yet been discovered or possibly no function exists [10]; such sequences may be either single copy or exist as multiple copies called repetitive DNA [10]. Indeed, regions of DNA that do not code for proteins tend to have more polymorphisms. Recently, there has been substantial progress in understanding genome content which centered on discovered protein-coding genes which considered a functional DNA sequence moving away from discoveries of many repeat families, and various copy number variations encompass gene copies leading to dosage imbalance that plays an important role in genome structure, evolution, and diversity [11, 12]. "The Human Genome Project has revealed that humans have only 20,000–30,000 structural genes (protein-coding genes) (International Human Genome Sequencing Consortium, 2004)" [13].

### **3. Type of polymorphisms**

#### **3.1. Single nucleotide polymorphisms**

Single base change is "high-density natural sequence variations in human genome" [14]. SNPs are mostly formed when errors occur (substitution, insertion and deletion). SNPs are prominent sources of variation in human genome and serve as excellent genetic markers. Some regions of the genome are richer in SNPs than others. SNPs may occur within gene sequences or in intergenic sequences. SNPs mostly are located in noncoding regions of the genome and have mostly no direct known impact on the phenotype of an individual but their role till now remains elusive, and depending on where SNPs occurs, it might have different consequences at the phenotypic level [3].

#### **3.2. Insertion/deletion polymorphisms**

It is a type of DNA variation in which a specific nucleotide sequence of various lengths ranging from one to several 100 base pairs is inserted or deleted. Indels are widely spread across the genome. Some authors consider one base pair as SNPs or repeat insertion/deletion as indels.

#### **3.3. Polymorphic repetitive sequences**

DNA repeats can be classified as interspersed repeats or tandem repeats. This can comprise over two-thirds of the human genome [15]. Interspersed repeats are dispersed across the genome within gene sequences or intergenic and include retro (pseudo) genes and transposons. Tandem repeats or variable number tandem repeats ( $\geq 2$  bp in length) that are adjacent to each [16] can involve as few as two copies or many thousands of copies. Centromeres and telomeres largely comprise tandem repeats. Despite increasing evidence on the functionality of DNA repeats, their biologic role is still elusive and under frequent debate [11]. Tandem repeats are organized in a head-to-tail orientation; based on the size of each repeat unit, satellite repeats can be further divided into macrosatellites, minisatellites, and microsatellites [17]. Some of these repeats are

described as follows: macrosatellites, with sequence repeats longer than 100 bp, are the largest of the tandem DNA repeats, located on one or multiple chromosomes [11], minisatellites, stretches of DNA, are characterized by moderate length patterns, 10–100 bp usually less than 50 bp [9, 18], and microsatellites also known as short tandem repeats (STRs) repeat units of less than 10 bp, [3].

### **3.4. Structural and copy number variations**

Structural and copy number variations (CNVs) are another frequent source of genome variability [6, 19, 20]. The term CNVs therefore encompasses previously introduced terms such as large-scale copy number variants (LCVs) [19], copy number polymorphisms (CNPs) [20], and intermediate-sized variants (ISVs) [21]. Some currently used terms are structural variations; a genomic alteration (e.g., an inversion) that involves segments of DNA > 1 kb, copy number polymorphisms; a duplication or deletion event involving >1 kb of DNA [22], intermediate-sized structural variant; and a structural variant that is ~8–40 kb in size, this can refer to a CNVs or a balanced structural rearrangement (e.g., an inversion) [21].

## **4. Common DNA-based molecular markers**

The development and use of molecular methods for the detection of DNA molecular markers is one of the most significant progresses in the field of molecular genetics. Mapping the human genome requires a set of genetic markers to which we can relate the position of genes. Some of these markers are genes, others SNPs and VNTRs. Molecular markers can be used to mark in genomes for various purposes such as mapping human diseases, pharmacogenetics, and human identification.

### **4.1. Single nucleotide polymorphisms**

Single base pair change leads to single nucleotide variant, probably accounting for many genetic conditions caused by single gene or multiple genes. SNPs represent the major source of human genomic variability. Due to the lack of knowledge on exact SNP number, it is difficult to give a direct estimate of the number of the SNPs in the human genome but in different public and private data bases, more than 5 million have been recorded and about 4 million validated [23]. “The data from the Human Genome project revealed that that human nucleotide sequence differs every 1000-1500 bases from one individual to another” [24]. “The SNP Map working group observed that two haploid genomes differ at 1 nucleotide per 1331 bp”. Over 60,000 however are within genes and some of them associated with diseases [2].

Single nucleotide polymorphisms within protein-coding regions either synonymous polymorphisms; those that do not have any effect on the organism and are said to be selectively silent as the substitution causes no amino acid change in the protein produced (silent mutation) or nonsynonymous substitution results in change in encoded amino acids either missense mutation; change the protein through codon alteration or nonsense mutation results in a chain termination codon [3].



Single nucleotide polymorphisms within a coding sequence cause genetic diseases including sickle cell anemia. SNPs responsible for a disease can also occur in any genetic region that can *eventually* affect the expression activity of genes, for example, in promoter regions. SNPs in the noncoding region of the gene, though their effect is still debatable, most of the genome mostly consists of regulatory elements that control gene expression, but these regions have remained largely unexplored in clinical diagnostics due to the high cost of whole genome sequencing and interpretive challenges. Clinical diagnostic sequencing currently focuses on identifying causal mutations in the exome, where most disease-causing mutations are known to occur.

Another important group of SNPs is the one that alters the primary structure of a protein involved in drug metabolism; these SNPs are targets for pharmacogenetics studies.

However, some SNPs are not causative, some SNPs are in close association with, and therefore segregate with, a disease-causing sequence so, the presence of SNP correlates with the presence or an increased risk of developing the disease; these SNPs are useful in diagnostics, disease prediction, and other applications [3].

Single nucleotide polymorphisms can be used as genetic markers for constructing high genetic maps and to carry out association studies related to diseases because of their abundance and the availability of high throughput analysis technologies. SNPs have become an important application in the development and research of genetic markers [14].

There are numerous strategies that can be implemented to new single nucleotide variant (SNVs) discoveries; the most common and well-known method is by direct sequencing and in comparison to a public or other sequence data base [25, 26] or locus specific amplification of target genomic region followed by sequence comparison [27, 28]; prescreening prior to sequence determination is needed. SNV detection encompasses two broad areas: (1) scanning DNA sequences for previously unknown polymorphisms and (2) screening (genotyping) individuals for known polymorphisms. Scanning for new SNVs can be further classified to two different types of approaches, the first one being the global (or random approach) and the other one being the regional (targeted approach) [14]. There are certain methods which have been developed for using SNVs randomly in the genome; “such as representation shotgun sequencing [14, 29], primer-ligation-mediated PCR [14, 30] and degenerate oligonucleotide-primed PCR” [14, 31].

Haplotypes are groups of SNPs that are generally inherited together. Haplotypes can have stronger correlations with diseases or other phenotypic effects compared with individual SNPs and may therefore provide increased diagnostic accuracy in some cases [32].

#### **4.2. Microsatellites (short tandem repeats)**

Microsatellites are short tandem repeats (STRs), repeat units, or motifs of less than 10 bp; because of high variability, microsatellite loci are often used in forensics, population genetics, and genetic genealogy. Significant associations were demonstrated between microsatellite variants and many diseases [15].

Depending on the search algorithm, there are approximately 700,000–1,000,000 microsatellite loci which are 2–6 bp long in the human reference genome [33, 34]. Di- and tetra-nucleotides constitute about 75% of microsatellites, with the remaining loci containing tri-, penta, and hexanucleotide. Within genes, STRs are nonrandomly distributed across protein-coding sequences, untranslated regions (UTRs), and introns. STRs containing dinucleotide repeat units that are much more abundant in the regulatory or UTR regions than in other genomic regions. In the coding regions of the genes, repeats mostly have either trimeric or hexameric repeat unit, likely as a result of selection against frameshift mutations [34, 35]. “The mutation rates of STRs often lie between  $10^3$  and  $10^6$  per cell generation which is 10- to  $10^5$ -fold higher than the average mutation rates observed in nonrepeated regions of the genome” [36, 37].

“Polymorphism of tandem repeats within protein-coding regions reveals that tandem repeat variation is an important source of variation in many proteins, many of this variation is of significant impact on protein function. Tandem repeats has been associated with a number of diseases and phenotypic conditions, changes in the protein products of genes, leading to diseases, other tandem repeat polymorphisms in noncoding regions are known to modify function through their impact on gene regulation”. “These polymorphisms can arise from events such as unequal crossover, replication slippage or double-strand break repair” [38].

Variations in the STR length play a significant role in modulating gene expression and STRs are likely to be general regulatory elements; regulatory STRs manifest significant polymorphism because of their high intrinsic mutation rate [15].

There are examples for distinctive phenotypic changes and diseases that are directly associated with the increases or decreases of microsatellite repeat arrays; for example, considering Huntington disease gene, triplet nucleotide mutations, the mutation that causes the disease, is an expansion of CAG repeats from the normal range of 11–14 copies to abnormal range of at least 38 copies. The extra CAG repeats that causes extra glutamine is produced [9] and there are more than 40 neurological diseases in humans, such as spinocerebellar ataxia with polyglutamine tracts, which are caused by microsatellite motif length changes in trinucleotide arrays [39].

Testing candidate genes for polymorphisms in exons, promoters, splice sites, or other regulatory regions will have to be done using SNP testing, because it is the most common polymorphisms and more likely responsible for phenotypic variations. For complex phenotypic traits and candidate loci, single-loci SNP analyses present less information due to the bi-allelic nature of the markers, as compared to the multi-allelic microsatellites. However, performing haplotype frequency may improve the accuracy [40]. Recently, polymorphic tandem repeated sequences and copy number variations have emerged as important sources of genomic diversity that facilitate the study of genetic variations in health and diseases.

## 5. The major technique for DNA-based molecular marker detection

Different forms of DNA-based molecular markers can be tracked using a variety of techniques. Some of these techniques include RFLPs with Southern blots and polymerase chain reactions (PCRs). Recently great advances in methodology for DNA polymorphisms detection using

real time PCR, hybridization techniques using DNA microarray chips, genome sequencing each technique has its own advantage and disadvantage.

### **5.1. Restriction fragment length polymorphism with southern blot**

DNA digestion with restriction enzyme endonuclease cuts DNA at a specific sequence pattern known as a restriction endonuclease recognition site. Thus, the alleles differ in length and can be distinguished by gel electrophoresis, which can arise from a number of genetic events including point mutation in restriction sites, mutation that creates a new restriction site, insertion, deletion, and repeated sequences. The first polymorphic RFLP was described in 1980. RFLPs were the original DNA targets used for human identification, parentage testing, and gene mapping.

The method of hybridization of DNA with probes is called Southern blotting, after the name of the inventor, Southern [41]. RFLP requires relatively large amounts of DNA. Hence, it cannot be performed with the samples degraded by environmental factors and also takes longer time to get the results [42, 43]. PCR-RFLP is now replaced to avoid using Southern blot.

### **5.2. Polymerase chain reaction**

In-vitro amplification of particular DNA sequences with the help of specifically chosen primers and DNA polymerase enzyme is done. The amplified fragments are separated electrophoretically and detected by different staining methods. Real-time PCR useful modification of PCR can detect polymorphisms by various methodologies using real-time PCR chemistries, for example, TaqMan assay or molecular beacons.

### **5.3. Genomic array technology**

Genomic array technology is a type of hybridization analysis allowing simultaneous study of large numbers of targets or samples. In 1987, macroarray evolved into the microarray. Tens of thousands of targets can be screened simultaneously in a very small area. Automated depositing systems (arrayers) can place thousands of spots on glass substrate of the size of a microscope slide (chip) with spotting representative sequences of each gene in triplicate, simultaneous screening of the entire human genome on a single chip. This technique facilitates the process of identifying specific homozygous and heterozygous alleles, by comparing the disparity of hybridization of the target DNA with each redundant probe. Microarray is also used to characterize genetic diversity and drug responses, to identify new drug targets, and to assess the toxicological properties of chemicals and pharmaceuticals [44].

### **5.4. Sequencing**

Since technologies for rapid DNA sequencing have become available they are now widely used. There is a great progression for the detection of single nucleotide variants (SNVs) by direct sequencing, but intermediate-sized (from 50 bp to 50 kb) structural variants (SVs) remain a challenge. Such variants are too small to detect with cytogenetic methods but too large to reliably discover with short-read DNA sequencing. Recent high-quality genome

assemblies using long-read sequencing have revealed that each human genome has approximately 20,000 structural variants, spanning 10 million base pairs, more than twice the number of bases affected by SNVs. New long-read sequencing approaches are needed to meet this challenge, as short-read sequencing technologies only detect 20% of the SVs present in the human genome [45–48].

## 6. The major application for DNA-based genetic markers

DNA-based molecular markers are such powerful tools for mapping human diseases and discover many multifactorial diseases and disorders.

### 6.1. Mapping human diseases and risk prediction

Genetic mapping and linkage: The mapping of the human genome has made possible to develop a haplotype map in order to better define human SNV variability. The haplotype map or HapMap will be a tool for the detection of human genetic variation that can affect health and diseases [23]. The HapMap project is far more useful because it will reduce the number of SNVs required to examine the entire genome for association with a phenotype or diseases from the 10 million SNPs that are expected to exist to approximately tag 500,000 SNPs [38]. The first large-scale effort to produce a human genetic map was performed mainly using RFLP; other several projects are underway to identify more markers in humans and to make this data publicly available to scientists worldwide. Many groups that are involved in these massive efforts through DNA polymorphisms discovery resource include the SNP consortium (TSC) <http://snp.cshl.org> [49, 50]. The reason for the current enormous interest in SNPs is the hope that they could be used as markers to identify genes that predispose individuals to common, multifactorial disorders by using linkage disequilibrium (LD) mapping.

“The HapMap Project (<http://hapmap.ncbi.nlm.nih.gov/>), and other approaches, such as genome wide association studies, have been widely reported for complex polygenic diseases, with some interesting novel genes affecting disease susceptibility now identified. Genome Wide Association; the GWAS has now been used for a large range of traits and diseases e.g. baldness and eye color” [51, 52].

### 6.2. Quantitative trait loci mapping, candidate genes, and complex traits

The identification of genes affecting complex trait is a very difficult task. For many complex traits, the observable variation is quantitative, and loci affecting such traits are generally termed quantitative trait loci (QTL). (SNVs) can be used as genetic markers for constructing high-density genetic maps and to carry out association studies related to complex traits and diseases [14].

### 6.3. Pharmacogenetics

Individual response to a drug is governed by many factors such as genetics, age, sex, environment, and disease. The influence of genetic factors on the response of a drug is a known fact.

Polymorphic STRs, together with SNPs and CNVs, can explain variability in response to pharmacotherapy because of their prevalence in the human genome and their functional role as regulators of gene expression and its applications. Pharmacogenetics is the study of the influence of genetics factors on drug response and metabolism. The science of pharmacogenetics when applied can be used to evade adverse drug reactions, predict toxicity and therapeutic failure, and refine therapeutic efficiency and improve clinical outcomes [53].

## 7. DNA fingerprinting and human identification

Establishing an individual's identity is one of the uses of DNA sequence information that highlights uniqueness of a particular sample [5], also known as genetic fingerprinting; DNA typing and DNA profiling are molecular genetic methods that enable the identification of individuals using hair, blood, semen, or other biological samples, based on unique patterns in their DNA. This uniqueness in each individual is the basis of human identification at the DNA level, forensic identification, determination of genetic variation, determination of family relationship, and one important instance is identifying good genetic matches for organ or marrow donation. When first described in 1984 by British scientist Alec Jeffreys, the technique used was minisatellites; these sequences are unique to each individual, with the exception of identical twins. Different DNA fingerprinting methods exist, using either restriction fragment length polymorphism (RFLP) or PCR or both. More than 200 RFLP loci have been described in human DNA. Initially, forensic medicine used minisatellite testing; however, this method requires a large amount of material and yield low-quality results especially when only little amount of materials are available. Nowadays, in most forensic samples, the study of DNA is usually performed by microsatellite analysis. The most useful microsatellite for human identification is those with a greater number of alleles, smaller size, higher frequency of heterozygotes (higher than 90%), and low frequency of mutations [43]. Among others, the microsatellite DNA marker has been the most widely used, due to its easy use by simple PCR, followed by a denaturing gel electrophoresis [40]. Each person has some STRs that were inherited from the father and some from mother, useful in paternity testing but however no person has STRs that are identical to those of either parent. The uniqueness of an individual's STR provides the scientific marker of identity and hence is helpful in forensic identification [54]. Genomic and mitochondrial are two types of DNA which are used in forensic sciences. The genomic DNA is found in the nucleus of each cell in the human body and represents a DNA source for most forensic applications. Mitochondrial DNA (mt DNA) is another source of material that can be used; various biological samples such as hair, bones, and teeth that lack nucleate cellular materials can be analyzed with mt DNA [43, 55].

### 7.1. Sex-chromosome STR testing

"Majority of the length of the human Y chromosome is inherited as a single block in linkage from father to male offspring as a haploid entity. DNA genetic markers on the human Y chromosome are valuable tools for understanding human evolution, migration and for tracing relationships among males" [43, 56]. "Chromosome X specific STRs is used in the

identification and the genomic studies of different ethnic groups worldwide, because the small size of X-chromosome STR alleles; about 100–350 nucleotides, it is relatively easy to be amplified and detected with high sensitivity” [43].

## 7.2. DNA typing and engraftment monitoring

DNA typing becomes the method of choice for engraftment monitoring, donor cells are examined by following donor polymorphisms in the recipient blood and bone marrow. Although RFLP can efficiently differentiate donor and recipient cells, the detection of RFLP requires the use of southern blot methods, which is too labor intensive and has limited sensitivity for this application, in comparison with small minisatellites or microsatellites that are easily detected by PCR amplification, because of increased rapidity and the 0.5–1% sensitivity achievable with PCR. Sensitivity can be raised to 0.01% using Y-STR, but this approach is limited to that transplant from sex mismatched donor recipient pairs preferably from a female donor to a male recipient [2].

Nowadays, DNA fingerprinting is used as a tool for designing “personalized” medical treatments for cancer patients.

## 8. Conclusion and future perspectives

Single nucleotide polymorphisms (SNPs) have become an important application in the development and research of genetic diseases or other phenotypic traits. Haplotypes are groups of SNPs that are generally inherited together. Haplotypes can have stronger correlations with diseases or other phenotypic effects compared with individual SNPs and may therefore provide increased diagnostic accuracy in some cases.

Polymorphic tandem repeated sequences have emerged as important genetic markers and initially, variable number tandem repeats (VNTRs) were used in DNA fingerprinting; in recent years, evidence has been accumulated for the involvement of VNTR repeats in a wide spectrum of pathological states.

The new global CNV map will transform medical research in four main areas: detection for genes underlying common diseases, study of familial genetic conditions, exclude variation found in unaffected individuals, helping researchers to target the region that might be involved and the data generated will also contribute to a more accurate and complete human genome reference sequence used by all biomedical scientists. Currently, approximately 2000 CNVs have been described; there could be thousands more CNVs in the human population. About 100 CNVs were detected in each genome tested with the average size being 250,000 bases (an average gene is 60,000 bases). With advanced molecular technologies more CNVs will be discovered and more DNA samples from worldwide populations are examined.

Recently, there has been substantial progress in understanding genome content which centered on protein-coding genes which considered a functional DNA sequence moving away for many discoveries, many repeat families, and various copy number variations that play

an important role in genome structure, evolution, and diversity. Additional efforts are being placed to develop strategies that would overcome the obstacles in alignment next-generation sequencing data. "Future precision medicine efforts will direct to connect genotypes to phenotypes and distinguish common, from rare or potentially disease linked variants. New long-read sequencing approaches are needed to meet this challenge."

Other important applications of genetic polymorphism knowledge are improving health care through gene therapy, discovery of new drugs and drug targets, and upgradation of the discovery processes with advanced technologies.

Advances in molecular technologies, DNA sequencing technology, and microarray, coupled with novel, efficient computational analysis tools, have made it possible to analyze sequence-based experimental data, more discoveries, and development at a rapid rate.

## Conflict of interest

The author declares that there is no conflict of interest.

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# Genetic Diversity and Disease Susceptibility

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# Genetic Diversity in Small Populations

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Arne Nils Linløkken

Additional information is available at the end of the chapter

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## Abstract

The chapter focuses on animal populations of low genetic diversity, among which some have low population size and are, or have been, threatened by extinction. Genetic diversity is regarded as a must for a species to be able to adapt to environmental challenges, but despite this, several species, also among advanced animal groups like birds and mammals, seem to thrive well with low genetic diversity. Some species are assumed to have done so for thousands of years. Other species have low genetic diversity resulting from heavy bottleneck events, in some cases very close to extinction, caused by human activities. Although some species live with surprisingly low genetic diversity, being prone to further loss of genetic variation, this may be retarded due to sexual selection and fitness superiority of heterozygotes. Simulations with population size  $N = 25$  showed that a homozygote fitness of 0.75 compared to fitness = 1.0 of the heterozygote resulted in exclusion of a  $p = 0.10$  frequency allele in <10% of 50 simulation over 50 generations, whereas fitness 1.0 of all genotypes resulted in exclusion of the  $p = 0.10$  allele in 78% of 50 simulations.

**Keywords:** allele exclusion, genotypes, heterozygote fitness, population size, selection

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## 1. Introduction

Genetic diversity is a crucial characteristic of any population or species, as genes coding for causative traits are tools by which the populations are equipped to adapt to environmental challenges [1–3]. High genetic diversity, therefore, is assumed an advantage or even a must, for species survival when environmental factors are changing, like climate change, new species appear, and among those new parasites and diseases. The development at present, with steadily decreasing number of species, often caused by human activity [4] imposes a responsibility on human society to take countermeasures. This is necessary also for our own welfare and prosperity.

In the conservation of populations and species, the preservation of natural habitats, for example wooded areas, of sufficient size should always be the first priority, though it is not always possible, and it may already be too late. One could claim, in a conservation perspective, that the population or the species, first of all is a gene pool, and that the preservation of a population handles about preservation of the gene pool as the most inalienable. The genetic diversity should therefore be explored and described as soon as possible in any population, but primarily for those already known to be threatened. Alleles may go extinct, especially low frequency alleles in small populations, and new alleles are added by a certain mutation rate, not necessarily keeping up with the loss rate.

Several molecular biological methods are available, and the choice of method is a matter of discussion and depends on the purpose. Amplified fragment length polymorphism (AFLP) [5] randomly amplified polymorphic DNA (RAPD) [6], restriction fragment length polymorphism (RFLP) [6], microsatellite analysis (MS), also denoted simple sequence repeat (SSR) [7] and single nucleotide polymorphism (SNP) [8] are conducted on selected marker loci. The latter two are favorites, and MS loci are polymorphic, that is, one locus may exhibit several different alleles, commonly 3–15, whereas SNP loci, like RFLPs, are biallelic. MS analysis includes usually 10–20 marker loci, sometimes more, whereas SNP analysis includes several thousand loci. Microsatellite loci are noncoding and therefore neutral, though the loci may be linked to coding loci and apparently be under selection, if the linked locus is under selection. SNPs may be located in coding loci and consequently be under selection. The SNP assays have an advantage due to being easier to standardize across detection platforms and laboratories than the MS method.

To describe the genetic variation of a population and relatedness, or lack of such, between individuals and populations, the MS method is well suited due to its high variability. Allele frequencies may be compared between populations and genetic structure within groups of populations, for example, in metapopulations, may be explored. Number of alleles (allele richness, when adjusted for sample size) per analyzed locus is an important index together with the fraction of heterozygote genotypes, that is, observed ( $H_o$ ) and expected (assuming Hardy Weinberg equilibrium) heterozygosity ( $H_e$ ), often referred to as genetic diversity, the inbreeding ( $F_{IS}$ ) and outbreeding ( $F_{ST}$ ) coefficient. Estimates of effective population size  $N_e$  [9] may also be conducted based on linkage disequilibrium, heterozygote excess, and others [10, 11].  $F_{ST}$  is one of several indices of genetic differentiation between populations. Microsatellites are well suited for that kind of studies due to the high variability.

The value of such indices depends on the markers, that is, marker set chosen, so the comparison between populations should be based on the same marker set. The same applies to SNP analysis, but SNPs are advantageous when the aim is to focus on important traits to explore selectivity and fitness among individuals and populations [12–14]. In a breeding context, for improved growth and survival of economical important species, to secure survival and fertility of populations and species, wild or domestic, the preservation of certain alleles or combination of such, can be monitored. There is a potential for selection of mates when animals are bred in captivity by conducting genetic screening of parental generation before fertilization to strengthen or weaken specific traits [15].



## 2. A short review

### 2.1. Low genetic diversity, but still successful

Though genetic diversity is assumed to be a prerequisite of success, there are several known examples of viable and apparently successful species with low genetic variability, like the African Cheetah (*Acinonyx jubatus*), with expected heterozygosity  $H_E < 0.0153$ , showing no characters of inbreeding, like reduced fertility, survival or fluctuating asymmetry, in the wild [16]. There are problems with reproduction in captivity, that is, in zoos [17], but this may be due to management as reproduction of cheetahs in North America was improved by changed husbandry [18–20], though this could potentially be due to limited adaptability as a consequence of low genetic diversity.

Mauritius kestrel (*Falco punctatus*) of the Mauritius Islands was characterized as one of the rarest bird species in the world when only one pair was left in 1974, after deforestation and invading species. After careful breeding, by picking naturally laid eggs in nest in the wild, for hatching and breeding chicks for stocking, the endemic species now counts several hundred pairs [21, 22]. The population appears viable, though the genetic variability is low with heterozygosity  $H = 0.10$ , as compared with historical  $H = 0.20$  (from up to 170 years old museum skins) and  $H = 0.59–0.70$  in continental kestrel species [21].

Another example of successful species with low genetic diversity is two species of albatross, the wandering albatross (*Diomedea exulans*) with a circumpolar distribution in the Southern Sea, breeding on six islands in numbers of tens of thousands, and the Amsterdam albatross (*Diomedea amsterdamensis*) breeding on the Amsterdam Islands in the Indian sea. The Amsterdam albatross was down in only five breeding pairs due to introduction of cattle, cats and ship rats [23]. The two species are supposed to have developed from a common root 840,000 years ago, and this time span includes repeated glaciations, and the low genetic diversity with  $H \leq 0.08$  may have existed before the deviation [24]. Both seem successful in their natural environment, though, the question is what will happen if the species encounter a new environment? Nevertheless, it is questioned whether their low genetic diversity has ever been a potential problem?

In Australia, with its distinctive fauna, the duck-billed platypus (*Ornithorhynchus anatinus*), representing the primitive mammal order Monotremata, is one of the most special. If any species deserves special attention, this is one of them. The distribution is limited to South and East Australia, and the populations are small. Reserves are established and platypuses have also been stocked to establish new populations [25], the last mean of conservation action, next to breeding in captivity. Two island populations are described by Furlan et al. [25]: one natural occurring population on King Island and a stocked population on Kangaroo Island. The King Island population has low genetic diversity due to low population number, whereas the stocked population has quite high genetic diversity due to admixture of specimens from different populations. Though the genetic diversity generally is low,  $H_o = 0.026–0.55$ , in platypus populations, they survive.

In North America, the black-footed ferret (*Mustela nigripes*) has been present from pleistocene (> 11,700 years ago) when they immigrated from Asia over the Bering strait [26]. The species was extinct in the wild, after the close to extinction of its main prey the prairie dog (*Cynomys* sp), followed by plague, when a breeding program started in 1985, based on 18 individuals, of which seven reproduced in captivity [27]. The expected heterozygosity dropped to  $H_e \leq 0.11$  in some populations after bottleneck events in the 1970s, but the populations now seem to reproduce without noticeable effects of inbreeding.

## 2.2. How to keep a small but diverse gene pool

The species described above, all with low genetic diversity in at least some populations, still seem viable, but a crucial question is whether the low diversity populations are sustainable. Can they meet environmental changes to come? The lower the diversity and population size  $N$ , the higher the risk of loss from genetic drift following bottleneck events, and after generations, fixation of the most frequent allele at a locus may be expected, when loss rate exceeds mutation rate. Experiments have demonstrated lower fitness of low diversity specimens of, for example an estuarine crustacean (*Americamysis bahia*) showed reduced fitness (fertility, survival) in populations with low genetic diversity compared to populations of high diversity, and this was most pronounced in stressful environments [28]. Closely related mates may lead to inbreeding depression with loss of low frequent alleles. Nevertheless, inbreeding in wild populations of moderate size is not necessarily harmful, as it may lead to exclusion of recessive harmful alleles, purging, and result in a population that is more adapted to its environment [29, 30]. The effect, or cost, of inbreeding in wild populations is difficult to observe, and unfit combinations may be excluded in all stages of life, from pre-zygotic to reproductive phase [31].

Salmonid fishes are commonly bred in fish farms for food production and for stocking in rivers and lakes to improve fishery. Major economic interests are involved, and considerable effort is spent on research. Lehnert et al. [32, 33] found that sperm competition and cryptic female choice (CFC) help to maintain allele richness in Chinook salmon (*Oncorhynchus tshawytscha*). An assessment of genetic variation within metapopulations of steelhead trout (*Oncorhynchus mykiss*) related to climate and landscape showed that climate variation induced genetic variation [34], and the genetics of river living salmonids is affected by dams as obstacles to migration [35]. Several studies have showed genetic differentiation between wild and hatchery stocks, though of common origin, indicating serious effects of breeding based on forced, artificial mating, avoiding natural sexual selection [36, 37].

Human interventions of different kinds affect populations and their genetic diversity and structure, and the effect within a given time span is impossible to predict. Nevertheless, the loss or exclusion of alleles from a population is in any circumstances worrisome when it is due to human action. Conservation of metapopulations, consisting of small and moderately sized (effective population size  $N_e < 50$ ) populations with some possibility of admixing, is one way to secure allele preservation. To explore this, natural metapopulations may be studied. Linløkken et al. [38] found in a study of brown trout (*Salmo trutta*) in nine tributaries to Lake Mjøsa in central Norway, that effective population size was positively related to habitat length (size). A bit unexpected, the heterozygosity based on MSs, was not correlated with

effective population size (mostly < 90) and was the highest in the middle-sized habitats. There was significant inbreeding coefficient  $F_{IS}$  in some of them, and the observed heterozygosity was in most cases lower than the expected. The low observed heterozygosity indicated inbreeding, which may lead to allele loss, but the lack of correlation between heterozygosity and  $N_e$  may suggest that other mechanisms worked. It could be due to increased fitness of heterozygotes, compared with the homozygotes, acting as a mechanism to slow down allele exclusion in populations. A conflicting interpretation of observed heterozygote excess is that heterozygote excess may indicate a recent bottleneck event [39].

Experiments with fruit flies (*Drosophila melanogaster*) demonstrated excessive heterozygosity, and this was explained by associative overdominance, that is, though the markers are noncoding loci, they are linked to causative loci that are under selection. Higher fitness of heterozygotes compared with homozygotes at the linked loci will retain the allele exclusion [40]. Noncoding or neutral markers may also be linked to (hitchhiking with) causative loci where coding alleles are removed by selection, called purging, excluding harmful recessive alleles. The reduction of hitchhiking non-coding alleles is called background selection [40].

### 3. On population size and heterozygosity of brown trout

#### 3.1. Heterozygote excess in small populations of brown trout

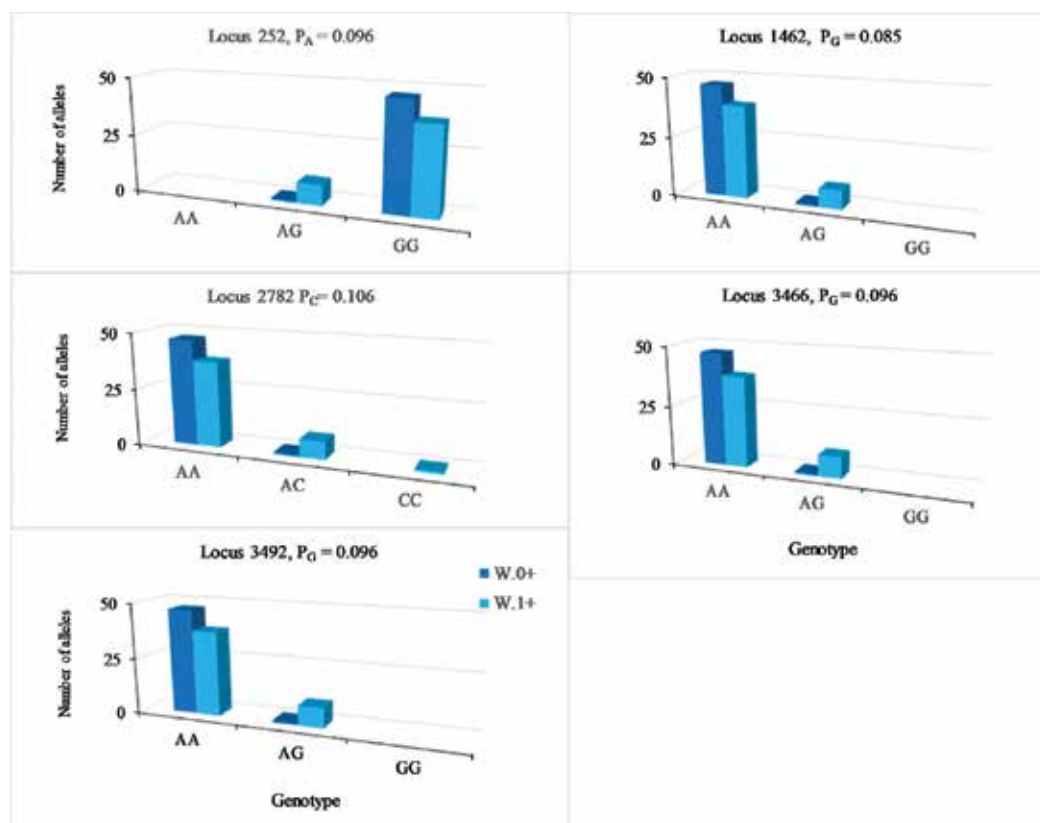
A small tributary to the Lake Savalen in Central Norway serves as spawning area for brown trout of the lake. The number of breeders (effective population size of one cohort,  $N_b$ ), based on linkage disequilibrium in 10 MS loci, was estimated to  $N_b = 38$  for young of the year (0+) in autumn, and  $N_b = 35$  for 1-year (1+) old fish in June the subsequent year (i.e., of the same cohort) [41]. The observed heterozygosity based on the same MSs, was  $H_o = 0.69$  for 0+, and increased to  $H_o = 0.78$  for 1+, and both were significantly higher than the expected heterozygosity ( $H_e = 0.67-0.72$ ). This corresponded to  $H_o = 0.333$  for both 0+ and 1+ and  $H_e = 0.323$  and 0.325, respectively, based on SNPs. For both marker types, the deviation from Hardy-Weinberg equilibrium was significant, and this excess of heterozygotes is interesting. When comparing wild 0+ and 1+ and a group of hatchery brown trout, all of the same cohort, Linløkken et al. [41] found that allele frequencies were changed from October to June in the subsequent year and was even more differentiated in the hatchery group.

By analyzing biallelic markers, that is, with two possible homozygotes and one heterozygote, like in SNPs, this is simpler to explore than in cases of the poly-allelic microsatellites. Outlier  $F_{ST}$  analysis of 3871 SNP loci detected 421 (10.8%) loci as candidates of selection, and among those, 34 loci showed significant mean length differences between genotypes in the 1+ wild fish group. In 30 of these loci, the largest genotype was significantly more frequent in the 1+ than in the 0+ group, indicating positive selection of large specimens, and 19 (63%) of these large genotypes were heterozygotes. This indicated that the differentiation between fry and the yearlings was in part due to size selective mortality, disfavoring the smallest specimens of fry through increased autumn to spring mortality. At five loci, only one of the homozygotes was recorded in the 0+ group (Figure 1). The heterozygote was significantly more frequent in

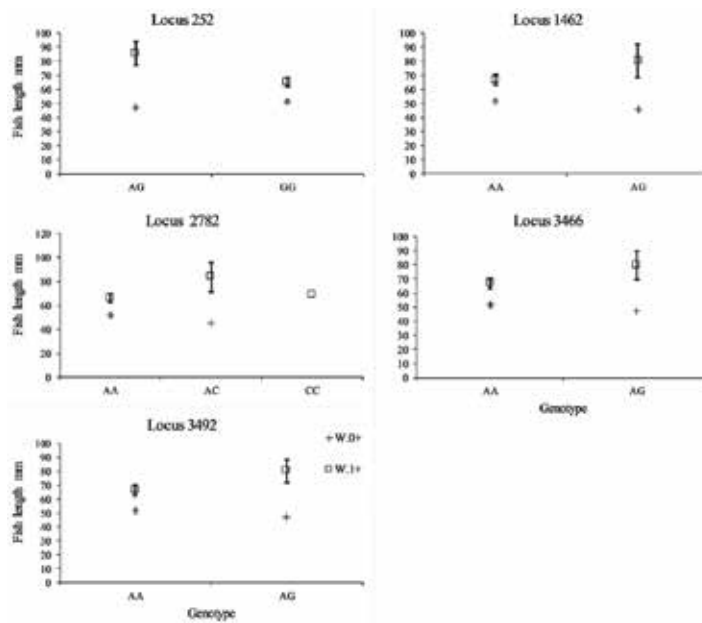
the 1+ than in the 0+ group (Fisher exact test,  $P < 0.05$ ) and was larger than the homozygote, different from in the 0+ group (**Figure 2**) (t-test,  $P < 0.05$ ).

### 3.2. Simulating the fate of a low frequency allele at biallelic loci

The low allele frequency of **Figure 1** ( $p =$  approximately 0.10) was used to simulate allele exclusion by means of the Allele Simulator software (available on the web: <http://popgen-simulator.pitt.edu/graphs/allele>), choosing population size  $N = 25, 50,$  and  $100,$  and performing 50 replicates of 50 simulations over 50 generations (corresponding to 150–250 years with maturation at 3–5 years of age). To explore the effect of allele frequency on exclusion rate, 50 simulations with  $N = 25$  and allele frequency  $p = 0.01, 0.05, 0.10, 0.25,$  and  $0.50$  were conducted. Fitness was set to 1.00 for all genotypes, and the proportion of exclusion showed a curved decrease by increasing  $p$  and resulted in 97% exclusion with  $p = 0.01,$  being reduced to 90% with  $p = 0.05,$  further to 78% with  $p = 0.10$  and to 23% with  $p = 0.50$  (**Figure 3**). This suggests that with  $N = 25,$  the probability of retaining a  $p = 0.01$  allele in 50 generations, without any heterozygote superiority, is close to null.

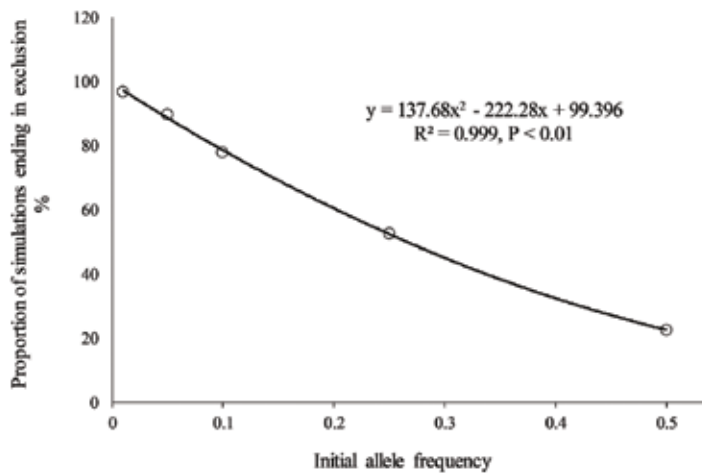


**Figure 1.** The distribution of genotypes of five SNP loci (numbers refer to Linlökken et al. [36]) in young of the year (W.0+,  $N = 48$ ) and 1-year-old (W.1+,  $N = 47$ ) brown trout of the same cohort and population.  $P$  denotes the observed frequency of the low frequency allele at the five loci.

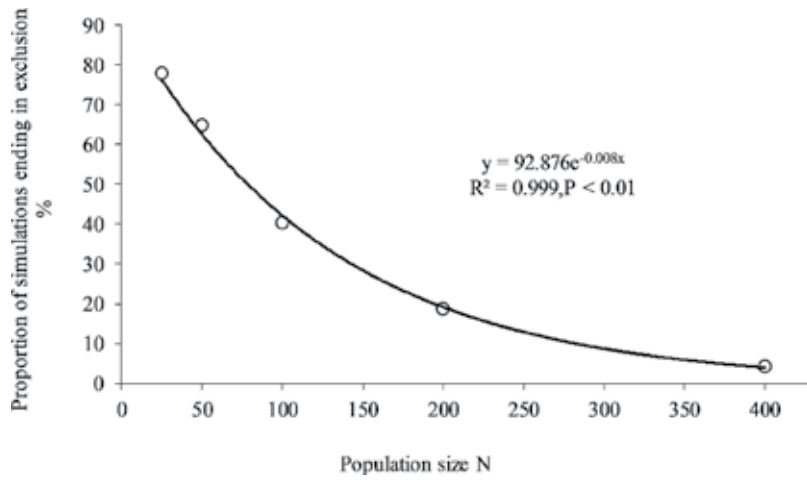


**Figure 2.** Mean lengths of young of the year (+) and 1-year-old brown trout (□) of different genotypes at loci at which mean length of heterozygotes were larger than that of homozygotes, and heterozygotes were more frequent in the 1-year-old group (W.1+) than in the young of the year group (W.0+) (Figure 1). Vertical lines show 95% confidence limits.

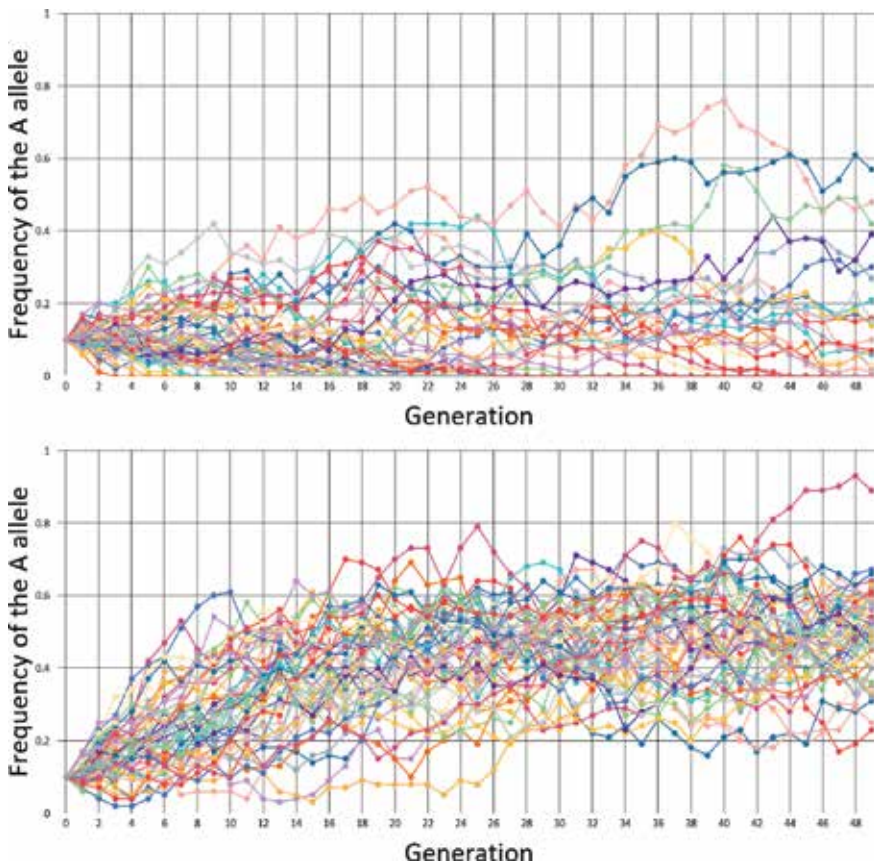
The initial allele frequency was then set to  $p = 0.10$ , and 50 simulations were run with fitness = 1.00 of all genotypes and  $N = 25, 50, 100, 200,$  and  $400$ . The proportion of exclusion decreased exponentially by increasing  $N$ , and less than 50% of the simulations ended in exclusion when  $N > 77$ , and 62% of the simulations ended with exclusion with  $N = 50$  (calculated from the regression, Figure 4). With  $N = 400$ , only 4% of the simulations resulted in exclusion.



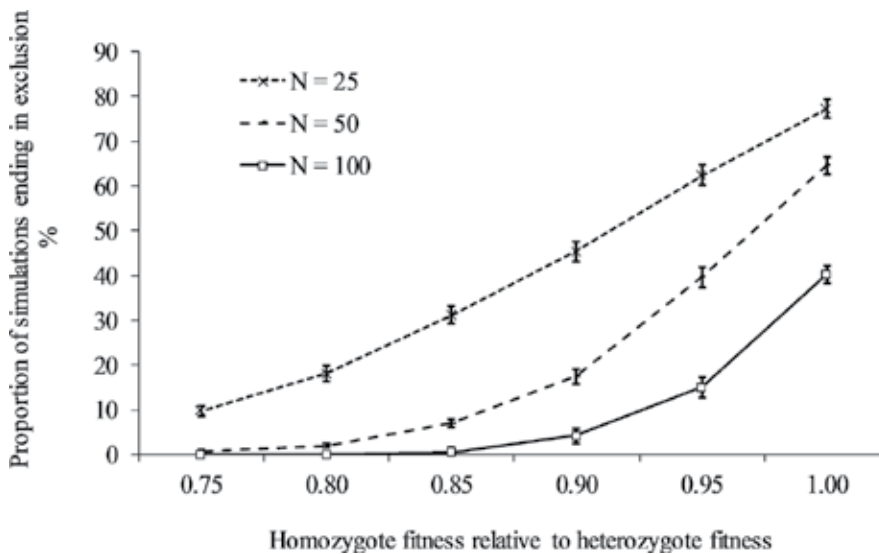
**Figure 3.** The proportion of 50 simulations that led to extinction during 50 generations in a population of  $N = 25$  as a function of the initial frequency of the allele.



**Figure 4.** The proportion of 50 simulations that led to extinction during 50 generations of an allele with initial frequency  $p = 0.10$  as a function of population size  $N = 25, 50, 100, 200,$  and  $400$ .



**Figure 5.** Proportion of 50 simulations of the frequency of an allele with initially  $p = 0.10$  during 50 generations, population size  $N = 50$  with fitness = 1 for all genotypes (upper panel), and with fitness = 1.0 of the heterozygote and fitness = 0.80 for both the homozygote (lower panel).



**Figure 6.** Proportion of 50 simulations of the frequency of an allele with initial frequency  $p = 1.0$  that led to extinction within 50 generation with fitness = 1 of the heterozygote and fitness 0.75–1.0 of the two homozygotes and population size  $N = 25, 50,$  and  $100$ .

To explore the effects of relative heterozygote fitness, the fitness of the heterozygote was set to 1.0, whereas the fitness of the two homozygotes was set equal, varying from 0.75 to 1.0, that is, the heterozygote fitness was similar or higher than that of the homozygotes. The simulations (**Figure 5**) showed that when fitness was equal for all genotypes, exclusion of the  $p = 0.10$  allele decreased from 78% with  $N = 25$  to 65% of the simulations with  $N = 50$  and further to 40% with  $N = 100$  (**Figure 6**). With fitness 0.90 of the homozygotes, less than 50% of the simulations ended with exclusion with  $N = 25$ , corresponding to less than 20% with  $N = 50$ , and less than 5% ended in exclusion with  $N = 100$ . Less than 10% of the simulations led to exclusion with homozygote fitness = 0.75 and  $N = 25$ , less than 1% led to exclusion with  $N = 50$ , and null simulations ended with exclusion with  $N = 100$ .

#### 4. Conclusion

Many animal species, among them representatives of advanced groups like birds and mammals, thrive well despite low genetic diversity, that is, apparently with a limited toolbox for evolutionary adaptation to new environments. Nevertheless, when genetic diversity is low, it is important to retain the alleles that still exist to avoid fixation at all loci. In small populations, like  $N = 25$ , the exclusion rate is quite high for alleles of frequency  $p = 0.10$ , and it increased inversely with the allele frequency and population size, according to the simulation experiments. This will, to some extent, be compensated for by mutations and introgression from migrants. The exclusion rate was reduced when heterozygote fitness exceeded that of the homozygotes, as was expected, and the increased heterozygote fitness helps effectively to retard the exclusion rate of alleles. As an example, young of the year and 1-year-old brown trout suggested positive selection of heterozygotes during the first winter, possibly due to faster growth and increased survival of large specimens.

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## Conflict of interest

There is no conflict of interest.

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# Toll-Like Receptors Gene Polymorphism and Susceptibility to Cancer Development

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## Abstract

Toll-like receptors (TLRs) play an important role in immune-surveillance and responses towards pathogenic and non-pathogenic microorganisms. They act as innate immune sensors against endogenous and exogenous danger signals by recognizing the pattern recognition molecules (DAMPs and PAMPs) and drive an adaptive immune response through their signaling pathways, which leads to NF- $\kappa$ B and IRF3 transactivation and induces different inflammatory cytokine genes. TLRs polymorphisms were investigated in various cancer types studies. However, precious studies have reported that the Polymorphisms on TLR1-TLR10 cluster have been associated with increased risk of prostate cancer. However, it has known that TLRs genetic variation is associated with increased the susceptibility to gastric cancer. A same synthetically meta- analysis also confirmed the association of TLRs with increased the gastrointestinal cancer but with decreased prostate cancer risk. Our previous studies have demonstrated a strong link between TLRs polymorphisms and colon cancer and breast cancer in Saudi Arabia population. Similar studies were analyzed with Korean patients with papillary thyroid cancer and their clinic-pathologic features in age matched controls by using direct sequencing. The general objective of this chapter was to investigate the role of different TLRs (i.e., TLR2, TLR4, and TLR6) polymorphisms and their association with cancer development.

**Keywords:** toll-like receptors, TLRs signaling pathway, polymorphism, cancer

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## 1. Introduction

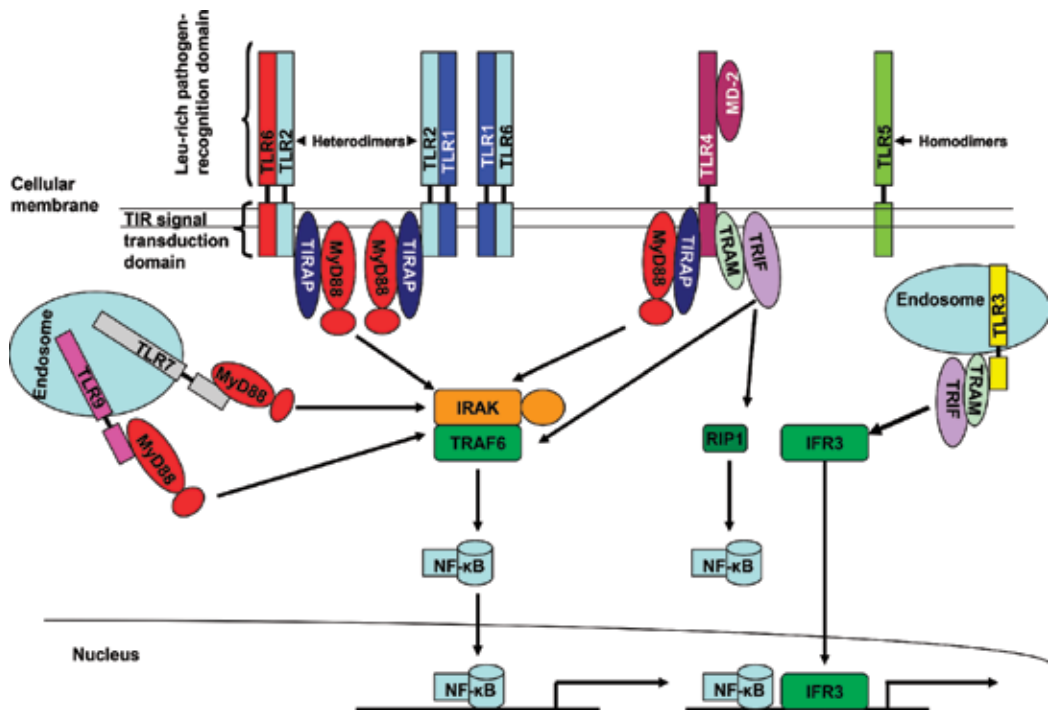
Toll-like receptors (TLRs) play an important role in immune surveillance and responses to pathogenic and nonpathogenic microorganisms. They act as innate immune sensors against

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endogenous and exogenous danger signals by using pattern recognition molecules (DAMPs and PAMPs) and drive an adaptive immune response through their signaling pathways, which leads to NF- $\kappa$ B and IRF3 transactivation and induces different inflammatory cytokine genes. Over the past several years, different studies have indicated that TLR polymorphisms modify the cellular immune response and that some of these polymorphisms are associated with susceptibility to infectious and inflammatory diseases as well as cancer incidence and severity [1–3]. However, previous studies have reported that the polymorphisms on the TLR1-TLR10 cluster have been associated with increased risk of cancer. A meta-analysis also confirmed the association of TLRs with increased risk of cancer development. Our previous studies have demonstrated a strong link between TLR polymorphisms and colon as well as breast cancer in the Saudi Arabian population. Similar studies have been conducted with Korean patients with papillary thyroid cancer and their clinicopathologic features in age-matched controls using direct sequencing. The general objective of this chapter is to investigate the role of different TLR (i.e., TLR2, TLR4, and TLR6) polymorphisms and their association with cancer development.

## 2. Toll-like receptors signaling pathways and their activation

Toll-like receptors (TLRs) are transmembrane glycoprotein receptors that play a key role in the innate immune system. Usually, they are present on the cell surface, except for TLR3, TLR7, TLR8, and TLR9. These are located in the endosomal membranes of sentinel cells such as macrophages and dendritic cells that recognize structurally conserved molecules derived from microbes. TLRs are the first pattern recognition receptors (PRRs) identified and characterized in mammals [4]. They initiate key inflammatory responses, shape adaptive immunity against microbial infection, repair and regenerate tissues [5, 6]. Through their role in the immune system, TLRs are a possible tool for curing and preventing cancer. TLRs belong to type I transmembrane glycoproteins characterized by an extracellular leucine-rich domain and a cytoplasmic tail, which are primarily responsible for mediating ligand recognition and a single transmembrane helix. TLRs also contain a cytoplasmic tail domain that is homologous to the interleukin-1 receptor and is responsible for initiating various intracellular signaling cascades. These signaling cascades include activation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B), which is considered a key transcription factor that promotes expression of genes involved in immune response such as cytokines and chemokines, as well as co-stimulatory and adhesion molecules [7, 8] (**Figure 1**). To date, 10 types of toll-like receptor families have been identified in humans, and each of them recognizes a specific PAMP [9–12]. Moreover, TLRs can form heterodimers such as TLR2/TLR1 and TR2/TLR6 to recognize the tri- or diacyl lipopeptides of bacteria, respectively [13, 14]. TLR2/6, along with CD36, has been found to have a role in recognizing the lipoteichoic acid (which is diacylated) of Gram-positive bacteria [15]. TLR2 can recognize the peptidoglycan of most bacterial species and fungi, while TLR4 along with CD14 recognizes the lipopolysaccharide (LPS) of Gram-negative bacteria. TLR3 can recognize the double-stranded RNA (dsRNA) that is found during the replication cycle of most viruses [15]. TLR5 recognizes bacterial flagellin [13, 14]. Furthermore, TLR7 and TLR8 can recognize the



**Figure 1.** Toll-like receptors: localization and signaling pathways.

single-stranded RNA (ssRNA) found in certain viruses and also the imidazoquinoline compounds, imiquimod and resiquimod (R-848). Correspondingly, TLR9 recognizes hypomethylated CpG motifs of bacterial double-stranded DNA (dsDNA) and DNA generated during the replication process of dsDNA viruses such as the herpes simplex virus [13, 14]. On the other hand, the PAMP recognized by TLR10 is unknown. However, TLRs can be classified into two groups based upon cellular location [16]. TLRs 1, 2, 4, 5, and 6 are found on the cell plasma membrane and can be activated by extracellular PAMPs. In contrast, TLRs 3, 7, 8, and 9 are principally found in membranes of intracellular compartments, such as endosomes and lysosomes [17]. The intracellular location of TLRs 3, 7, 8, and 9 enables them to detect nucleic acids (i.e., DNA or RNA) that have been released from viruses or bacteria and degraded within endosomes and lysosomes inside the cell [15, 16]. Thus, the inactivation of TLRs will certainly hamper immune function, leading to significant side effects for human health and well-being. The TLR signaling cascade involves (or not) the activation of the adapter molecule MyD88. Both cascades lead to the activation of NF-κB to promote transcription of pro-inflammatory cytokines, chemokines, and cationic peptides. These mediators are involved in innate and adaptive immune responses.

TLRs present in various immune cells are used to sense multiple pathogens [18, 19]. In this sense, TLR2 activation allows the activation of NF-κB and subsequent production of IL-8 and iNOS [19]. Also, it was demonstrated that the activation of TLR3 induced the production of IL-8,

TNF $\alpha$ , IL-18, and type I interferon (IFN $\alpha/\beta$ ) and promoted Th-1 type immune responses [20, 21]. Furthermore, other studies have demonstrated that the activation of TLR5 in human keratinocytes by its ligand, flagellin, also resulted in the production of TNF $\alpha$ , IL-8, and the antimicrobial peptides human  $\beta$ -defensins 2 and 3 (hBD2 and hBD3) [22, 23]. TLR9 activation leads to the selective production of the chemokines CXCL9 and CXCL10, which promote memory T-cell responses and the production of type I interferon [20, 21]. Miller et al. showed that TLR5 and TLR9 promote the wound healing process by production of the differentiation factor TGF $\alpha$  [24]. Recently, Semlali et al. reported that gingival epithelial cells in engineered human oral mucosa sense *C. albicans* infection by activating TLRs and producing antimicrobial peptides, such as HBD-2 and HBD-3 [25]. Also, Rouabhia et al. demonstrated that the mutation of the *Candida* genes *IPT1* or *ECM33* prevents TLR activation and cationic peptide (beta-defensin) expression [26]. Together, these available studies demonstrated that TLRs are expressed in the immune cells, and the deregulation of TLR expression by immune cells may promote cancer initiation and development.

### 3. Deregulation of TLR expression may promote cancer initiation and development

The expression of TLRs, their function in cancer cells and the association with tumor progression have become a very exciting field of investigation. However, it is well reported that functionally active TLRs are expressed by multiple immune cells such as human cancer cells (lung, gastric, laryngeal, cervical, prostate, etc.) [2, 27]. The general pattern of TLR expression in tumor cells suggests that TLR-mediated signaling plays a crucial role in cancer tumor development. It is possible that tumor cells express multiple TLRs to recognize various danger-associated molecular patterns (DAMPs) in their microenvironment. This may enhance the biological process mediated by TLR activation to produce favorable conditions for growth and survival. However, the significance of the expression of several TLRs in various cancer cells is not fully understood. Semlali et al. reported that different TLRs, specifically TLR 2, 6, and 9, are expressed in normal colon epithelial tissues, and their expression has been reported to be decreased in most colorectal cancer tissues compared to normal matching tissues [8]. Conversely, TLR4 expression increases in colon and breast cancer tissues compared to normal tissues [28, 29]. To date, TLRs have been found to have the opposite effect on tumor progression. On the one hand, TLR ligands can suppress tumor growth. On the other hand, TLR agonists can promote the survival of malignant cells and increase their resistance to chemotherapy [30]. It is possible that tumor cells express multiple TLRs to recognize various DAMPs in their microenvironment. This may enhance the biological process mediated by TLR activation to produce favorable conditions for growth and survival. Furthermore, the ligation of TLRs in tumor cells increases the production of immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)- $\beta$  [2], suggesting that tumor cells also utilize TLR activation to escape from tumor immune surveillance. However, several available studies support the idea that TLRs are cancer inhibitors. *Thus, further investigation is mandatory to decipher the role and the genetic variation of TLRs in cancer.*



## 4. TLR polymorphisms and their role in cancer development

Multiple single nucleotide polymorphisms (SNPs) have been identified in TLRs with potential functional consequences for infectious disease or cancers.

- TLR1 polymorphisms and cancer susceptibility

Several studies have linked TLR1 polymorphism to different types of cancer, including breast, colon, and gastric. Two SNPs related to TLR1 and breast cancer were previously investigated. Rs7696175 was found to be associated with increased risk of breast cancer in two populations: Chinese and of European ancestry (OR > 1) [31, 32]. Moreover, Chen et al. investigated the association of rs4833095 SNP and breast cancer in a Chinese population, and they found no association [33]. In colon cancer, rs5743618 (T1805G) on exon 4 results in I602S amino acid substitution in the junction of cytoplasmic and transmembrane TLR1 domain, affecting ligand binding [34]. However, several studies have demonstrated a significant decrease in cytokine response in rs5743618 as compared with wild type [34]. In patients with metastatic colorectal cancer (mCRC), TLR1 rs5743618 SNP was associated with a significant response to FOLFIRI plus bevacizumab chemotherapy. Based on these findings, TLR1 rs5743618 might be a predictive biomarker for the advantage of FOLFIRI plus bevacizumab response in mCRC patients [35]. According to Castano-Rodriguez and colleagues' review, rs5743618 polymorphism has also been associated with decreased risk of gastric cancer among the German population (OR < 1) [36]. Moreover, Ravishankar et al. investigated the association of rs4833095 polymorphism in TLR1, and they found that this SNP has conferred susceptibility of *Helicobacter pylori* patients to the development of gastroduodenal diseases, especially gastric cancer [37].

- Association between TLR2 and cancer

The link between TLR2 polymorphisms and cancer was specifically investigated in the context of chronic inflammation, which likely increases the risk of cancers. An association between TLR2-174 to -196 del polymorphism, 22 base-pair deletions in the promoter, has been found with breast, colon, gastric, and cervical cancer; therefore, the presence of this polymorphism might be used as a biomarker for these cancers. Theodoropoulos et al. demonstrated the association between -174 and -196 del of TLR2 and breast cancer in Caucasian patients, and they found that this polymorphism may confer increased susceptibility to breast cancer [38]. Furthermore, Proença et al. found that -174 to -196 del are related to an elevated risk of colorectal cancer [39]. According to a review by Castano-Rodriguez and colleagues, TLR2-174 to -196 del have been associated with gastric cancer among different populations: Chinese, Brazilian, and Japanese (OR > 1). On the other hand, -174 to -196 del (Ins/Ins genotype) have been associated with cervical cancer as a protective factor among Tunisian women [40]. Among the Korean population, two TLR2 polymorphisms have been studied to investigate their association with papillary thyroid cancer (PTC)—rs3804099 and rs3804100—and it was found that the two SNPs are associated with PTC [41]. Additionally, rs3804100 and rs3804099 have been associated with increased gastric cancer risk in Brazilian and Chinese populations, respectively [36]. Slattery et al. have investigated some TLR2 SNPs for their impact on

colon cancer risk and survival under cigarette smoking and NSAIDs usage. They found that rs7656411 (T > G) variant allele in normal colon of aspirin/NSAIDs consumers has been associated with a lower risk of colon cancer development, but not for those who did not consume aspirin/NSAIDs recently. Also, rs3804099 (T > C) and the variant allele (CC) have been linked to reduced colon cancer risk in cigarette smokers, but not for nonsmokers. While rs5743704 (C > A) CA/AA genotypes and rs5743708 (G > A) were associated with decreased survival at developed colon cancer stages III and IV, there was no effect among diagnosed stages I and II [42]. Related to hepatocellular cancer (HCC) susceptibility, Junjie et al. investigated the association of two synonymous SNPs in the coding region of TLR2 among the Chinese population. They found that rs3804099 C/T and rs3804100 C/T polymorphisms are associated with decreased risk of HCC susceptibility (OR = 0.493, 0.509, respectively) [43]. Another study was done among the Chinese population that investigated the association of rs3804099 C/T and rs7656411 G/T polymorphisms in TLR2. Huo et al. found that these two SNPs have a significant association with increased risk of HCC [44].

- Association between TLR3 polymorphisms and risk of cancer

In general, there are few reports investigating the correlation between TLR3 polymorphism and different types of cancer. Yeyeodu et al. detected that rs10025405 G allele SNP, which is located at 3'-near gene in TLR3, is associated with breast cancer. Among 100 cases of African American women, this SNP with OR < 1 was associated with a fivefold reduced risk of breast cancer [45]. Another study conducted among the Chinese population ( $n = 715$ ) investigated the role of missense rs3775291 SNP, which is located in exon 4 of TLR3, and found that rs3775291 with A allele is associated with an increased risk of relapse in breast cancer [33]. However, the same rs3775291 polymorphism has been associated with increased survival when diagnosed at stage II among German colorectal cancer patients [46]. Furthermore, a study was done among 900 sample cases from Asian ethnic backgrounds to investigate the correlation between rs5743312 SNP and breast cancer. Rs5743312 with T allele, located in intron 3 of TLR3, was found to increase the risk of breast cancer (OR > 1) [47]. Slattery et al. investigated the association of two TLR3 polymorphisms (rs11721827 and rs3775292) and colon cancer and found that rs11721827 variant allele in the normal colon of aspirin/NSAIDs consumers is associated with a lower risk of colon cancer development, but not for those who did not consume aspirin/NSAIDs recently. The rs3775292 CG/GG genotypes in colon cancer with dietary carbohydrate intake have been associated with no significant increased risk at high intake levels and decreased risk at low intake levels. On the other hand, CG/GG genotypes have somewhat influenced survival when diagnosed at advanced colon cancer stages, with no impact among earlier stages [42]. Related to susceptibility to HCC, Li and Zheng investigated the association of two polymorphisms in TLR3: -976 T/A and +1234C/T. They found that -976 T/A polymorphism is not associated with HCC. On the other hand, the prevalence of +1234CT and +1234TT genotypes was found to be significantly increased in HCC cases compared to normal so +1234C/T polymorphism could be a risk factor for HCC [48]. Otherwise, investigation of TLR3 rs5743305 and rs3775291 polymorphisms has shown no significant correlation between these SNPs and the risk of cervical cancer, among a Swedish population [49]. Among North Indian population, Pandey et al. found no association between rs3775290 TLR3 polymorphism and

cervical cancer development [50], while Zidi et al. found that rs3775290 TLR3 polymorphism (+1377 C > T genotype) increased the risk of cervical cancer in advantage stages [40].

- Potential association between TLR4 polymorphisms and cancer risk

In Saudi Arabian population, Semlali et al. investigated the association between breast cancer and four TLR4 polymorphisms: rs2770150, rs10759931, rs10759932 in the promoter region, and rs4986790 in the exon region. They found that three of them—rs2770150, rs4986790 (Asp299Gly), and rs10759932 are not associated with breast cancer, while rs10759931 is strongly associated with increased susceptibility to breast cancer [28]. In Caucasian patients, Theodoropoulos et al. demonstrated an association between Asp299Gly SNP of TLR4 and breast cancer, and they found that this polymorphism may confer increased susceptibility to breast cancer [38]. Apetoh et al. also found that TLR4 rs4986790 (Asp299Gly) SNP is associated with increased risk of breast cancer [51]. Regarding lung cancer, Kurt et al. investigated two SNPs (rs4986790 and rs4986791) on the TLR4 gene, rs4986791 cytosine/thymine substitution at nucleotide 1196, and rs4986790 adenine/guanine substitution at nucleotide 896. They found no relation between rs4986790 polymorphism and lung cancer. In contrast, an rs4986791 polymorphism associated with lung cancer compared with CC genotype presences of CT genotype was 3.857 higher risk of lung cancer [52]. Additionally, Vacchelli et al. investigated the impact of TLR4 rs4986790 in response to chemotherapy in non-small-cell lung cancer. They reported that loss of function of TLR4 alleles did not affect overall survival in non-small-cell lung cancer (NSCLC) patients [53]. Another study investigated the association between the +3725 G/C polymorphism in TLR4 and breast cancer among 665 Chinese patient samples. They found that the +3725 G/C polymorphism increased the suitability to breast cancer and decreased the survival time (OR = 2.34) [54]. However, rs10759931 has been associated with colon cancer development risks among Saudi population, regardless of gender or age, while rs2770150 has been associated with colon cancer in Saudi women over 50 years old, and it was closely linked to decreased levels of female sex hormones during the postmenopausal period [55]. Another study investigated the association between rs10759932 C allele polymorphism on CRC development risks and found that there was no influence on TLR4 gene expression in CRC tumor tissue [39]. Furthermore, rs1554973 (T > C) has been associated with improved survival in colon cancer in earlier and advanced stages [42]. In exon 3, rs4986791 + 119 C/T (C > T), which has a Thr399Ile amino acid substitution, was associated with cancer development risk for Caucasians, but not for Asians [56]. A synergistic relationship has been found between rs1927911 (C/T) TT genotype and InterLukin17 (rs6973569) polymorphism with high spicy food intake, which ultimately increased the risk of CRC development [57]. Wang et al. investigated the association of TLR4 SNPs Asp299Gly (rs4986790) and Thr399Ile (rs4986791) in the Chinese population. They found that in ovarian cancer patients, rs4986790 and rs4986791 presented at a lower incidence [58]. Related to gastric cancer, a meta-analysis done by Zhou et al. demonstrated that TLR4 + 896AA/G and + 1196C/T polymorphisms may be associated with significantly increased gastric cancer risk among the Caucasian population [59]. Rs4986790, +896 (A > G) SNP causes an amino acid substitution (Asp299Gly), which leads to an altered TLR4 extracellular domain structure. Also, G allele has been reported with a diminished response to the ligands and thus reduced pro-inflammatory cytokine production

[60]. This observation led [39] to confirm the lack of association between colorectal cancer development risk and rs4986790 polymorphism. A slight frequency of rs4986790 SNP and absence of G/G homozygous have been associated with CRC in different populations, including in Croatia [61], China [62], Spain [63], Japan [64], Greece [38], Brazil [65], Saudi Arabia [55], and Kashmir [66].

- TLR5 polymorphisms and cancer risk

Recently, Shuang et al. investigated the association between the TLR5 polymorphism and breast cancer using two SNPs conducted a study among a Chinese population: the nonsense SNP rs5744168 and the missense SNP rs2072493. They found that rs5744168 T allele encodes a stop at codon 392, affecting TLR5 signaling, and it is associated with sporadic breast cancer occurrence (OR > 1). On the other hand, they found no association between rs2072493 G allele and breast cancer risk [67]. Klimosch et al. found that rs2072493 (A > G), nonsynonymous SNP coding for N592S is associated with colorectal cancer-specific and overall survival. Rs5744174 (T > C), non-synonymous SNP results in the amino acid substitution F616 L. Having a CC genotype was associated only with colorectal cancer-specific survival. These two SNPs, rs2072493 and rs5744174, were both associated exclusively with colon cancer patients [68].

- TLR6 polymorphisms and risk to cancer development

The same SNP in TLR1 (rs7696175) that was found to be associated with breast cancer was also found in TLR6, and it was found to be associated with increased risk of breast cancer in two populations: Chinese and of European ancestry (OR > 1) [31, 32].

- TLR7 and TLR8 polymorphisms and their association with cancer

Generally, TLR7 and TLR8 polymorphisms have not been associated with cancer, except rs3853839 (G/C) polymorphism in TLR7, which might be a biomarker for the benefit of cetuximab-based chemotherapy for *KRAS*-wild type metastatic colon cancer patients. They found that having the GG genotype is related to a longer progression-free survival benefit than having the CC genotype [69].

- Association between TLR9 polymorphisms and cancer risk

Resler et al. have examined two single nucleotide polymorphisms (SNP, rs352140 and rs187084) in TLR9 and their association with breast cancer. In over 800 Caucasian case samples, they found that rs352140 does not alter the protein amino acid sequence but might alter protein function or stability. In addition, this SNP was found to be associated with breast cancer risk (OR = 0.85, protective effect) while rs187084 has not been found to have an association with breast cancer [70]. In a comparison study of African American (AA) and European American (EA) breast cancer patients, the association of rs352140 was investigated. It was found that rs352140 SNP, located at the CpG site, has a protective effect that is 1.6X more common in EA compared to AA [71]. In contrast, Etokebe et al. found no association of rs352140 with breast cancer among 130 case samples of Croatian patients [72]. Rs187084 (C/T) was found to be significantly associated with colon cancer development risk in female patients, which might be linked to sex hormones, including estrogen and progesterone. It has been suggested by previous studies that female sex hormones might have a role in protecting against colon cancer [73, 74]. Additionally, reduced TLR-9 expression was observed in colon

cancer tissues compared with normal tissues. Previous studies demonstrated that reduced TLR-9 transcription activity is relatively associated with C genotype frequencies as compared to T genotype. However, female T allele frequency is lower than controls. The authors suggest that some introns might have some regulatory functions including alternative splice influence, which finally affects both mRNA and protein products [8]. Moreover, rs352139 (A/G) and rs352144 (A/C), in the promoter region, were found to be significantly associated with colorectal cancer development and localization. Reduced TLR-9 expression has been observed in colon cancer, and that might be linked to the fact that promoter mutations seem to affect the stability of regulation processes [8]. On the other hand, Lee et al. investigated the rs5743845 polymorphism in the TLR9 gene. They found that the 2588 G/A SNP in TLR9 did not correlate with increased lung disease in the European population [75]. The relation between cervical cancer susceptibility and TLR3 (rs3775290) and TLR9 (rs352140) polymorphisms has been studied by Pandey et al. in a North Indian population. They found no association between rs3775290 and rs352140 polymorphisms and cervical cancer development. In terms of the effect, TLR 3 (c.1377C/T) and TLR 9 (G2848A) SNPs with clinical stages of cervical cancer, the AA genotype of TLR 9 presents a marginally increased risk for advanced cancer stages (OR = 2.63). In contrast, the TLR3 SNP did not present any significant correlation with cervical cancer clinical stages [50]. Another study investigated the polymorphisms -1486 T/C (rs187084) [76] in TLR9 and cervical cancer susceptibility in the Chinese population and found that rs187084 is associated with cervical cancer development. Moreover, genotype TC was significantly correlated with increased cervical cancer risk in the Polish population [76]. In a meta-analysis, Mu et al. investigated whether (TLR9) -1486 T/C and 2848G/A polymorphisms have a role in cervical carcinogenesis. They demonstrated that rs187084 was associated with an increased risk of cervical cancer while rs352140 did not affect cervical cancer risk [77]. Among a Swedish population, TLR-9 (rs5743836, rs352139, and rs352140) polymorphisms showed no significant correlation with the development of cervical cancer risks [49].

## 5. Conclusion

TLRs SNPs could serve as a predictive biomarker for different cancer patient treatment. These available studies demonstrate that TLR polymorphism and its functional consequences could be a significant step forward in preventing and curing colon cancer.

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# Developmental Genetics and Preimplantation Genetic Diagnosis

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Additional information is available at the end of the chapter

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## Abstract

The genetic code information programs the embryogenesis. In this chapter the three phases of prenatal development the pre-embryonic, the embryonic and the fetal are elaborated. The role played by transforming growth factor beta related genes in the growth and differentiation of early embryo will be described. The implications of Fibroblast growth factor gene in binding heparin its effect on cell survival and mitogenic pathways is discussed. The orchestrated symphony of Sonic Hedgehog genes in early human embryo development is elaborated. The Homeobox genes (Hox gene) and their role in early human embryogenesis are described. The different tests available for prenatal genetic testing are briefly described. Preimplantation genetic diagnostic procedures are indispensable in clinical embryology. The microsurgical techniques of the Polar body biopsy, Blastomere biopsy and Blastocyst biopsy are discussed at the end of the chapter. A diagrammatic representation of individuals and relationships in clinical genetics is described. A brief description of procedures of invasive fetal tests for collecting the fetal tissue is also discussed.

**Keywords:** preimplantation genetic diagnosis, clinical genetics, developmental genetics

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## 1. Introduction

A diploid embryo is formed by the fusion of two haploid gametes. All cells are totipotent till the embryo is 17 days. After 17 days there is specification that is followed by determination. Specification is the first stage of initial commitment which is labile the cells are adaptable. During specification if a few cells are moved to another location, they can acquire a different fate and develop according to the area to which they are shifted. Following specification the commitment is irreversible. The cells are not adaptable and changing the location of cells cannot change their fate. Congenital birth defects are often caused by errors in embryogenesis.

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This is important because an embryonic injury when the cells are pluripotent may be lethal or may not have any effect at all. But when the cells cross the phase of specification and determination an injury invariably results in a structural anomaly. For example, exposure to rubella virus causes loss of cells in fetal lens and results in congenital cataract and microphthalmia.

## 2. Phases of prenatal life

Prenatal life can be divided into three main stages, i.e., pre-embryonic, embryonic and fetal. The pre-embryonic phase is the period during which the small collection of cells gets differentiated to form three germ layers, i.e., ectoderm, mesoderm and endoderm by the process of gastrulation. The body axes, i.e., anteroposterior, dorsoventral, left and right also established during gastrulation. The embryonic phase lasts from 5 to 8 weeks.

The pre-embryonic and embryonic phases are the times during which a single cell progresses to form the organ primordia which is the first 8 weeks of human development.

The final fetal stage after 8 weeks leads to rapid overall growth and maturation of the embryo into a viable human fetus. The integration of complex phenomena, which leads to the formation of a viable infant, has generated interest in understanding the molecular and structural aspect of this process [1].

Various comparative and evolutionary studies have been done to understand this fascinating developmental phenomenon. Many experiments were done to trace cells during their development. Transparent embryos were observed and even living cells stained with vital dyes were used to observe their fate. Later radiographic labels and autoradiography techniques were used.

Grafting experiments using quail cells into chick embryos at early stages of development were some of the pioneering efforts in monitoring cell fates that lead to origin of different organs and tissues. Other grafting experiments were grafting the primitive node from its normal position on the body axis to another site could induce formation of a second body axis.

It was also concluded that a piece of tissue from posterior axial border of one limb if grafted to the anterior border of another limb then digits on the host limb are duplicated. This posterior signaling was called zone of polarizing activity and now the signaling molecule is identified as Sonic Hedgehog. Thus the advent and progression in the field of molecular biology has led to better understanding of embryology of normal and abnormal developmental processes.

## 3. Regulation of gene expression

The cell fates can be mapped by identifying cells using reporter genes, fluorescent probes and other markers. The gene expression can be regulated at several levels

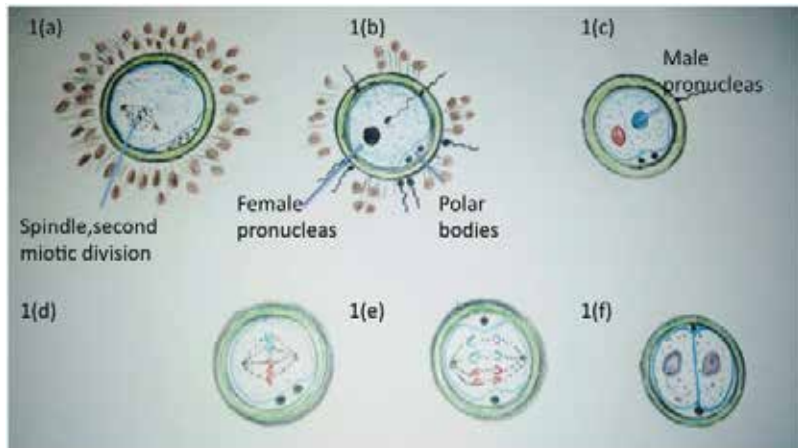
1. How different genes may be transcribed?
2. How DNA transcribed from a single gene may be selectively processed to regulate which RNA s will reach the cytoplasm to become mRNAs?
3. How mRNAs are selectively translated?
4. How the proteins from mRNA can be modified?

Fertilization is the process of union of male and female gametes and it occurs in the ampullary part of the uterine tube. Out of 100–200 million spermatozoa deposited in the female genital tract only a single spermatozoon penetrates through the corona radiates and zona pellucida. Sperm cell membrane fuses with the oocyte membrane.

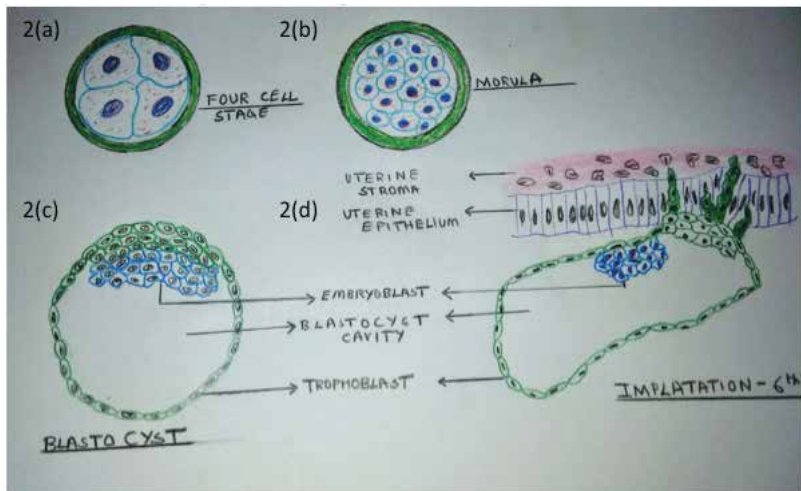
The penetration of sperm induces cortical and zona reactions, which prevents further sperm binding and penetration. This also leads to the resumption and completion of second meiotic activation of oocyte. The results of fertilization lead to restoration of diploid number of chromosomes, determination of gender of the zygote and initiation of cleavage (**Figure 1**).

The zygote undergoes repeated mitotic divisions through the process of cleavage. As it attains an eight-cell stage it undergoes compaction and segregates into an inner cell mass and outer cell mass. Inner cell mass, which is the embryoblast, forms the embryo proper and outer cells mass forms the trophoblast. With accumulation of fluid in the embryo the embryoblast and trophoblast separate from each other and a blastocyst is formed (cyst = fluid filled cavity). The zona pellucida disintegrates and the embryo is implanted.

By Day 8 of fertilization the blastocyst gets partially embedded into the endometrial stroma (**Figure 2**). The inner cell mass differentiates into hypoblast layer and a layer of tall columnar



**Figure 1.** (a) Oocyte after ovulation. (b) Spermatozoa penetrate the oolemma. (c) Syngamy with male and female pronuclei seen. (d) Chromosomes are arranged in the equatorial plane. (e) Chromosomes are migrating towards poles. (f) Two-cell stage.

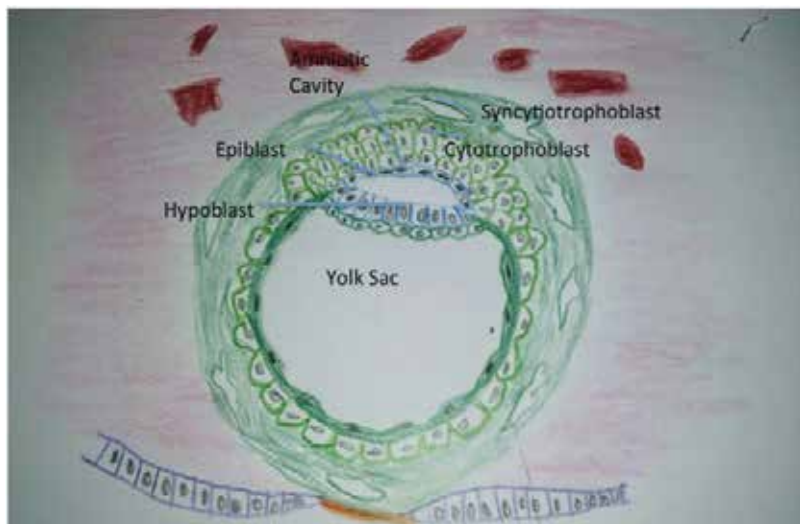


**Figure 2.** (a) Blastocyst stage. (b) Morula formation. (c) Blastocyst formation. (d) Blastocyst on 6th day of development showing trophoblast cells of blastocyst penetrating the uterine mucosa.

cell which is epiblast. This also leads to formation of two fluid filled cavities in the blastocyst, the primitive yolk sac and the amniotic cavity (**Figures 3 and 4**).

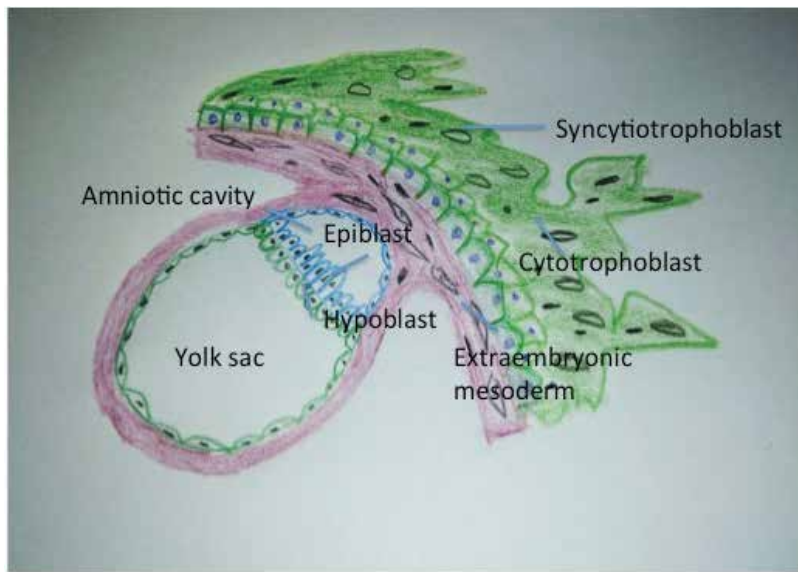
The most important event during the third week of gestation is gastrulation by which all three germ layers are formed, i.e., ectoderm, mesoderm, and endoderm. Gastrulation starts with the formation of primitive streak on the epiblast (**Figure 5**).

At the cephalic end of the streak a small primitive pit develops which the primitive node surrounds. The epiblast cells of the primitive streak undergo proliferation and migration by



**Figure 3.** Blastocyst in the endometrial cavity at 9 days.

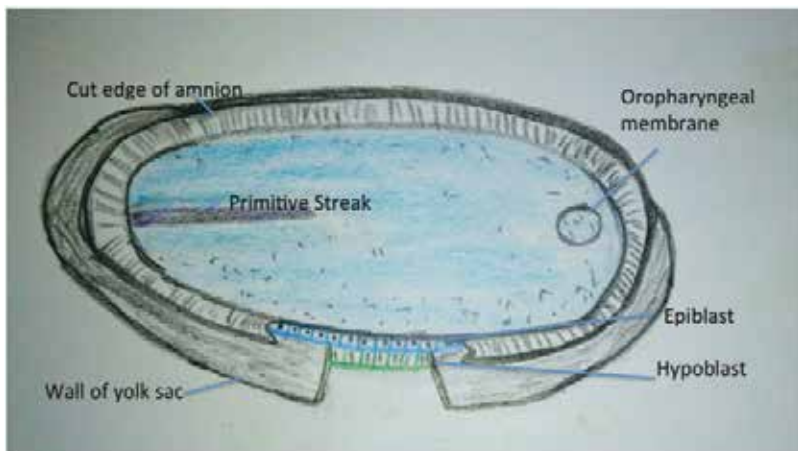




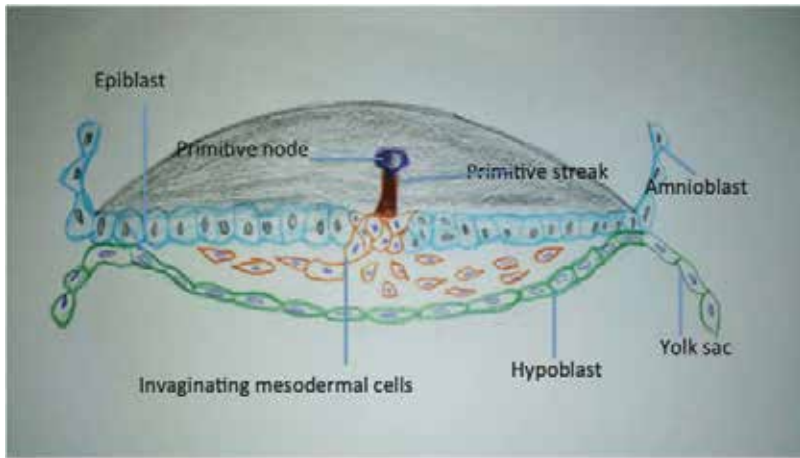
**Figure 4.** Bilaminar embryonic disc at 14 days in the implantation site in endometrium.

inward movement called invagination. This process is controlled by Fibroblast growth factor 8 (FGF 8) synthesized by the cells of primitive streak.

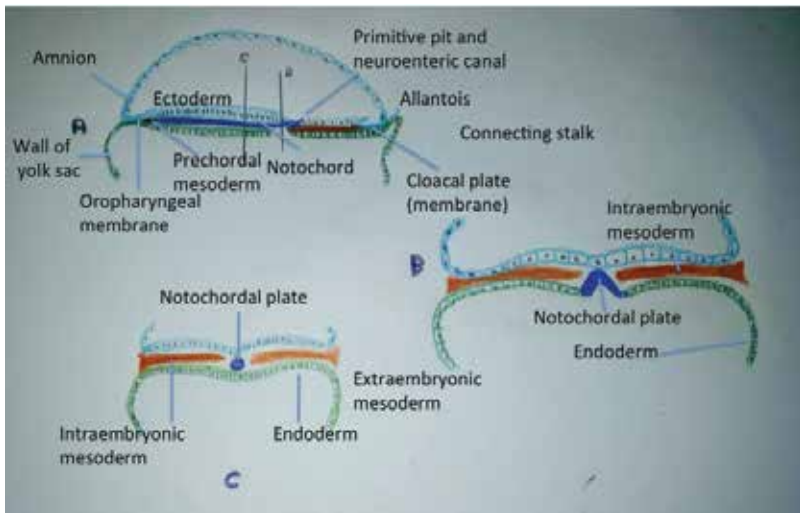
Fibroblast Growth Factor 8 down regulates E cadherin, which is the binding protein of epiblast cells thus helping in cell migration and invasion into underlying layer. Cells that are specified by regulation of Brachyury T expression form the mesoderm layer. FGF 8 also controls this expression. Some of the epiblast cells will even replace the hypoblast Layer and proliferate to form the embryonic endoderm (**Figures 6 and 7**). Cells, which continue to remain in epiblast,



**Figure 5.** Dorsal view of germ disc at 14 days showing epiblast and hypoblast. A primitive node is formed at the cephalic end and a primitive streak is seen at the caudal end.



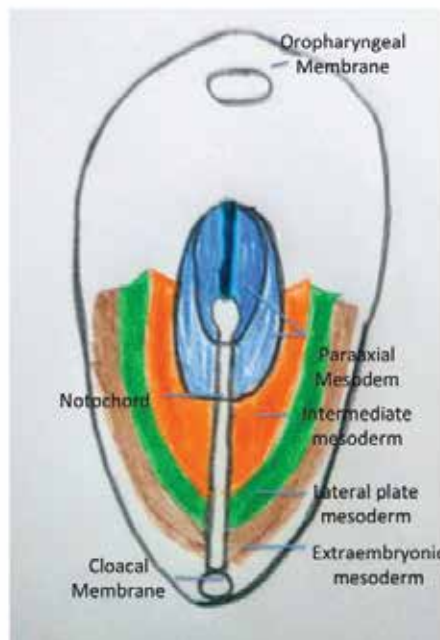
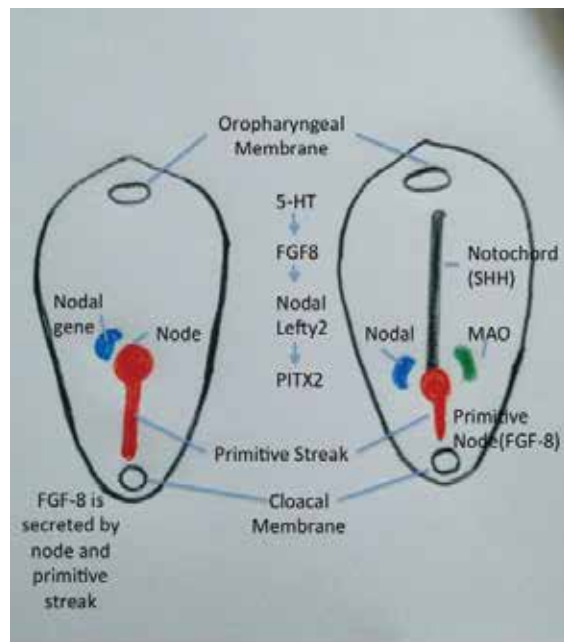
**Figure 6.** Cross section through the cranial region of primitive streak at 15 days showing invagination of epiblast cells.



**Figure 7.** (a) Sagittal section of a day 17 embryo showing most cranial part of notochord. (b) Schematic cross section through the region of notochord plate. (c) Schematic view showing definitive notochord.

give rise to the embryonic ectoderm. Thus all the three germ layers are gradually formed in the epiblast and hypoblast and the process of gastrulation is complete [2].

During and before gastrulation another important event, which occurs, is the establishment of body axes—anteroposterior, dorsoventral and left—right (**Figure 8**). As already discussed the primitive streak with the primitive node at its head end determines the anteroposterior axis of the developing human embryo [3]. The anterior visceral endodermal cells at the anterior end of embryonic disc expresses genes and transcription factors like OTX2, LIM1 and HESX1 and also signaling proteins like Cerberus and Lefty which decreases the primitive node activity at head



**Figure 8.** Dorsal view of the embryonic disc showing gene expression patterns responsible the laterality in the embryo (om: oropharyngeal membrane, cm: cloacal membrane, N: notochord, pm: paraaxial mesoderm, im: intermediate mesoderm, lpm: lateral plate mesoderm, eem: extraembryonic mesoderm). (a) FGF8 secreted by the node and primitive streak establishes expression of nodal gene which leads to TGF $\alpha$  family on the left side. (b) Expression of nodal and LEFTY on the left side of the ventral aspect of neural tube and this is mediated by serotonin monoamine oxidase (MAO) on right side breaks down 5-hydroxy traptamine (5HT). (c) Dorsal view of the primitive streak and fate map of epiblast cells.

end of the embryo. Nodal which is a protein of transforming growth factor beta family establishes and maintains the primitive streak.

Another important protein of Transforming growth factor beta is Bone Morphogenic Protein 4, which is secreted throughout the embryonic disc. This is important for ventralising mesoderm for kidney, blood and body wall development through the formation of lateral plate and intermediate mesoderm. The primitive node acts as an organizer and inhibits action of Bone Morphogenic Protein 4 by promoting other genes. Chordin, noggin and follistatin antagonize the action of BMP4. This results in dorsalisation of cranial mesoderm to form notochord, somites and somatomeres.

Brachyury T gene regulates the dorsal mesoderm formation in middle and caudal regions of the embryo. Laterality, i.e., left -right -sidedness is development by the interaction of signal molecules and genes. Genes regulating left side development are well known like LEFTY-2, Nodal that up regulates PITX2, which is a transcription factor. A neurotransmitter serotonin (5HT) also plays an important role in establishing left sidedness in the embryo. Defects of Lefty-2 and PITX2 and 5 HT can lead to defects like dextrocardia, situs inversus and cardiac abnormalities.

The cells of the epiblast, the regions of streak and node from they migrate determines the fate and the type of mesoderm they will eventually form. The cells which migrate from cranial end of node form the notochord and prochordal plate. The cells that proliferate and migrate from lateral edges of the node and cranial part of primitive streak form the paraxial mesoderm. The cells that proliferate and migrate from middle of primitive streak form the intermediate mesoderm. The cells migrating from caudal part of the streak form the lateral plate mesoderm. The cells from the caudal most part of the streak contribute to extra embryonic mesoderm. Thus the fate map of development of various organ systems is established at the time of gastrulation itself [2].

#### **4. Genes as carriers of information**

Genes are not only carriers of inherited information but also they are extremely important instructors for embryological development. In the last decade there is a tremendous progress in identifying specific genes, which are responsible for control of development in human embryo. The earlier studies were initially performed in *Drosophila* and other laboratory animals have immensely contributed in understanding the pathways and genes involved in human development.

There are genes, which are involved in development of code for proteins, which include signaling molecules and receptors, DNA transcription factors, enzymes, transport systems and components of extracellular matrix. Thus mutations and defects in all of the mentioned factors above can lead to birth defects [3].

The genetic factors which initiate, maintain and establish embryonic development is not clearly determined yet. There are some key developmental pathways, which are identified

after exhaustive experiments in genetic expression studies, but still the processes are not clear. The developmental gene families identified in vertebrates and mammals show sequence homology with developmental regulators of fruit fly *Drosophila*. The developmental genes produce proteins called transcription factors, which regulate RNA transcription from DNA template, by binding to regulatory DNA sequences to form complexes, which induces transcription by RNA polymerase. Thus gene expressions can be controlled by transcription factors thus in other words they can switch genes on and off. The variety of processes taking part during development include induction during which extracellular signals change fate of one type of cells to another, segmentation, migration, differentiation and apoptosis or programmed cell death. The protein signaling molecules identified to be involved in the above processes during development across specific belong to Transforming growth factor family, (TGF-beta), Wingless (Wnt) family and the Hedgehog (HH) family.

## 5. Cell signaling

Cell signaling plays a very important role in in normal embryogenesis. The receptor tyrosine kinase (RTK) signaling phosphorylates tyrosine residues and is activated by ligands and thus brings about diverse functions during development. Certain ligands, which activate RTK, are fibroblast growth factor (FGF), epidermal growth factor, TGF, Platelet derived growth factor, etc. The intracellular cascade of reactions that follows once binding of ligands activates the receptors has been studied in vitro experiments and is believed to bring about multiple changes though not fully understood [4].

The TGF-beta family of signaling molecules constitutes at least 33 members of this cytokine family. They are involved in cross talk between cells thus establish cellular communication. These extracellular signaling polypeptides are transduced to regulate other gene expression. These signaling molecules bind to the receptors on the cell surface brings about a cascade of reactions and activation of receptor kinases which leads to translocation of complexes into nucleus of cells and thus either activate or suppress the target gene expression.

The TGF-beta family can be grouped into (1) the BMPs and (2) TGF-beta s, activin, nodal, and myostatin. These groups of signaling molecules are actively involved in various cellular and developmental processes like cell cycle, cell migration, cell size, gastrulation and axis specification and various metabolic processes. BMP signaling is also involved in dendritogenesis and axonal transport.

## 6. Segmentation

The end of third week also marks the beginning of segmentation of the paraxial presomitic mesoderm into somatomes proceeding rostrocaudally. By the end of fifth week there are about 42–44 pairs of somites divided into occipital, cervical, thoracic lumbar and coccygeal pairs. The Wnt, Notch and FGF signal molecules play a vital role in the formation of somites.

The notch delta pathway is responsible for the somite formation and it is expressed in oscillating pattern. The Notch accumulates in that paraxial mesoderm which is destined to form the somite and then once the somite is formed it reduces in concentration [1].

The Sonic Hedgehog -Patched Gli pathway promotes cell proliferation in the notochord, floor plate of neural tube, brain and developing limbs and gut. This Sonic Hedgehog Gli pathway malfunctioning can lead to a variety of birth defects and cancers which includes holoprosencephaly to a basal cell carcinoma, medulloblastoma, etc. The Sonic Hedgehog is first broken down to form an active N-terminal form to which a cholesterol moiety gets added which binds to the Patched 1 and Patched 2 trans membrane proteins in the target cells.

The normal action of Patched is to inhibit a transmembrane protein Smoothed but Sonic Hedgehog-Patch combination the inhibition is released. Smoothed thus activated. Further activates a cascade within the cell involving the GLI family of transcription factors, i.e., activation of GLI 1 and GLI 2 and Inhibition of GLI 3. Disruption of hedgehog signaling during development either due to mutation or teratogens can lead to developmental anomalies. Excessive activation of Hedgehog pathway does promote increase in angiogenic factors and lead to development of cancers of brain, lungs, mammary gland, prostate and skin [5].

Sonic Hedgehog functioning is essential for human development and its absence or mal expression is associated with certain specific defects as holoprosencephaly, limb defects and ventral defects of neural tube. This sonic hedgehog which is a morphogen and its signaling is dependent on its concentration and duration. The fundamental cellular processes like proliferation, survival, cell fate determination, migration, apoptosis during development are controlled by key developmental signaling pathways like Receptor tyrosine Kinase, Hedgehog, BMP, WNT, Notch, Retinoic acid signaling pathways [4].

## 7. Transcription factors

The transcription factors are the genes whose products will activate or suppress other genes and thus mutations in the transcription factors can lead to a variety of birth defects. These include the Homeobox (HOX) genes, Paired box Genes (PAX), T Box (TBX), SRY type HMG Box (SOX) genes, Zinc Finger Genes. The transcription factors as mentioned above leads to an orderly distribution of already differentiated cells to form specific tissues and organs by spatial arrangement and this process are called pattern formation. Studies in *Drosophila* the homeotic or HOX genes determine segment identity and faulty expression of these genes result in major structural abnormalities.

*Drosophila* has eight HOX genes arranged in single cluster whereas in humans there are 39 HOX genes arranged in four homeobox cluster genes on chromosomes 7p, 17q, 12q, 7q as HOX A, HOX B, HOX C, HOX D respectively numbered as 1–13. These genes regulate the cell fate and thus establish a pattern along the cephalocaudal axis and also limb bud axis and genital axis. Thus these genes play important role in development of CNS, axial skeletons, limbs gastrointestinal and urogenital systems.

The Hox genes are numbered and always expressed in cephalocaudal manner and there is a direct linear relation between the position of a particular gene and its spatial expression so playing a major role in early morphogenesis. Thus it results in a coordinated patterning of the derivatives of all the three germ layers. There are 39 HOX genes in humans but it is very astonishing that very few syndromes and malformation is attributed to HOX gene mutations. This can be explained by assuming that mutation in HOX genes can be so devastating that the embryo cannot survive. There can also be another explanation that the paralogous group of HoX genes like HOXA4, HOXB4, HOXC4 and HOXD4 though situated in different chromosome segments can compensate for loss of function or mutation in paralogous genes.

The paired box genes (PAX-genes) are a highly conserved DNA sequence and 9 PAX genes have been identified in humans. Loss of function of 5 PAX genes has been associated with developmental abnormalities in humans. PAX2 mutations causes renal coloboma syndrome in which renal malformations occur along with defects in eye. PAX 6 mutations also lead to eye defects as absence of iris. The SRY is a Y linked gene and plays a major role in male sex determination. This SRY genes show homology with the SOX genes and in humans it is seen that mutations in SOX2 have shown to cause anophthalmia or microphthalmia, esophageal atresia, and genital hypoplasia in males.

The T-box genes or TBX genes are dispersed throughout the human genome. Loss of function or mutation of TBX3 causes ulnar-mammary syndrome, which includes developmental abnormality of upper limb and mammary gland hypoplasia. Mutations in TBX-5 cause Holt Oram syndrome, which is an autosomal dominant disorder, characterized by congenital heart abnormalities and upper limb abnormalities.

The zinc finger genes are the genes, which have a zinc finger motif, and it acts as a transcription factor, which binds to DNA, and thus they can result in single gene developmental disorders. GLI3 is a zinc finger motif containing gene and large deletion or translocations involving GLI3 results in Grieg cephalopolydactyly and on the other hand frame shift mutation of the same gene result in Pallister-Hall syndrome which is characterized with polydactyl imperforate anus hypothalamic hamartomata. Mutations in WT1 another zinc finger motif gene causes Wilms tumor. Mutations in other zinc finger genes such as ZIC2 and ZIC3 results in holoprosencephaly and lateral polarity defects, which are essential for development of left-right axis.

The testis determining factor or the SRY gene, which is now determined to be located in the sex-determining region of the Y chromosome and evidence, shows that this is the primary factor, which determines maleness. Expression of SRY gene leads to activation of other genes like SOX9 which leads to the differentiation of the medulla of the undifferentiated gonad to develop into a testis in which the Leydig cells are formed which starts producing testosterone which leads to Wolffian duct stimulation and formation of male external genitalia.

The Sertoli cells on the contrary start producing the Mullerian Inhibitory hormone, which regresses the Mullerian duct. On the absence of SRY gene the medulla of the undifferentiated gonads develop into ovary the Mullerian duct forms the internal genitalia and the external

genitalia does not fuse and develop into normal female genitals. Normal sexual differentiation is completed by 14 weeks though the testis descends at a much later part of pregnancy [1].

## 8. Gene expression during development

Different cells in an embryo express different sets of genes at different times. A common set of genes is present in all cells at all times and is called as the “House-keeping genes.” These genes carry out the normal functions of a cell. Speciality genes are the special genes that enable a cell to carry out special functions. The genes responsible for embryonic body plan Homeobox genes, proto-oncogenes and PAX genes.

The gene expression is regulated by three sets of proteins called as the promoter, silencer and enhancer proteins. These proteins can modify the gene expression and define a particular cell type. There are also signaling proteins that facilitate the development of adjacent areas in a particular organ.

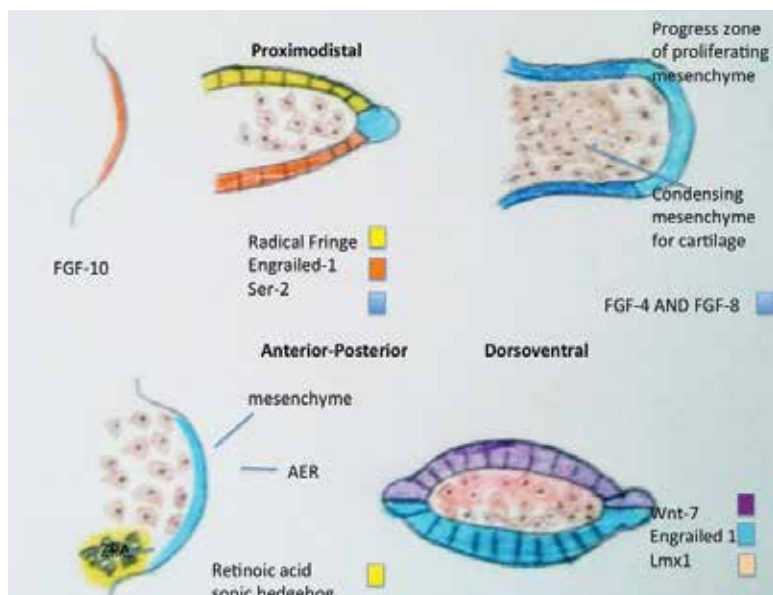
The SRY gene on the Y chromosome produces testis determining factor, which in turn activate the SOX9, and steroidogenesis factor (SF1) and it stimulates development of Sertoli and Leydig cells. WNT4 is the master gene for ovarian development. It inhibits SOX9 and up regulates DAX1. WNT4 along with other genes promotes formation of cortical cords and causes regression of medullary cords and prevents the tunica albuginea to develop [2].

The extracellular growth factors regulate cell division and differentiation by the process of signal transduction and thus mutations in genes, which produce these factors and receptors, have been implicated in cancer development and also in developmental anomalies. The Fibroblast growth factor receptors (FGR) play an important role in embryogenesis. Mutations in FGR have been associated with two groups of disorders of skeletal system, which broadly include the craniosynostosis syndromes and achondroplasias. Genes such as Fibrillin-I and Elastin code for proteins that are needed for arranging microfibrils in the matrix and thus the mutations are associated with Marfans’ syndrome [3].

## 9. Limb development

The process of development of limbs is well understood as the genes controlling the growth, patterning and signaling pathways for its development is well conserved from *Drosophila* to mammals. The developing limb is divided into a proximal stylopod, middle zeugopod and distal autopod. In humans the stylopod becomes the arm or thigh, the zeugopod becomes the forearm or leg and the autopod changes into the hand or foot. The limb buds appear on Day 26 for upper limb and 1 or 2 days later for lower limb. The limb bud to begin with consists of a mesenchyme core as an extension from the parietal layer of lateral plate mesoderm which leads to the formation of bones and connective tissues and it is covered with a layer of cubical ectoderm. The positioning of the limb buds depend on the correct functioning of HOX genes, which control the expression of FGF8, and the latter determines the limb type, i.e., fore or hind limb. The apical





**Figure 9.** Molecular regulation of limb development. Fibroblast growth factor 10 and AER initiate limb growth. These are induced by bone morphogenetic protein S (RA is retinoic acid, ZPA is zone of polarizing activity, AER is apical endodermal ridge).

ectodermal ridge (AER), which is a localized area of thickening on the surface ectoderm covering the limb bud, produces growth signals such as FGF4 and FGF8, which result in growth in proximal-distal axis. A collection of cells at the posterior margin of limb bud near body wall also undergoes proliferation to form the zone of polarizing activity which produce morphogen retinoic acid which in turn expresses SHH (Sonic Hedge Hog) which plays a huge role in determining the anteroposterior axis for limb development (**Figure 9**). Bones of the limb are derived by endochondral ossification of the parietal layer of the lateral plate mesoderm which is determined by HOX genes (HOXA and HOXD), which regulate the type, and shape of the bones. Digits are formed due to organized apoptosis of the AER into separate ridges and ultimately by interdigital apoptosis. HOXD13 mutations result in synpolydactyly, which is a dominantly inherited condition. Hand foot genital syndromes results from mutations of HOXA13 and presents hypospadias in males and bilateral thumb and great toe hypoplasia [6]. Dorsalization of the limb bud is under the control of genes like radical fringe and Wnt7a. Holt Oram syndrome an autosomal dominant inheritance is due to mutation of T-box 5 gene and is characterized by forelimb abnormalities and cardiac defects thus T-box genes determine the limb identity by activating FGFs. Another gene is the PitX, which contribute in forming hind limb characteristics [6].

## 10. Head and neck formation

The most important feature in development of head and neck is the formation of pharyngeal arches resembling the gills of the fish. These arches are bars of mesenchyme tissue appearing at fourth or fifth week of development with an outer covering of ectoderm and inner layer of

endoderm at the cranial most part of the primitive gut. The arches are separated from each other by ectodermal clefts and form out pouching in the lateral wall of developing pharynx as endodermal pouches. The mesoderm which lies in the center of the arches form the arch arteries and musculature and the skeletal elements are formed from the neural crest cells, which migrate early from hindbrain region. The FGF signaling plays an important role in determining the migration and fate of neural crest cells in the arches, endodermal pouch formation and differentiation [7]. Though it was previously thought that the neural crest cells are important for pharyngeal arch development but nowadays it is shown that the endoderm of the pharyngeal apparatus acts as the principal organizer and thus it is proved that neural crest cells are not necessary for pharyngeal arch development. The out pocketing of the endoderm is significant for pharyngeal arch development and regulated by TBX1 and RA signaling [8]. The most well-known condition due to faulty development of third and fourth pouches is the DiGeorge syndrome which results due to submicroscopic chromosome deletion 22q11 and loss of approximately 30 genes. Treacher Collins is hypoplasia of the maxilla and mandible with coloboma of the lower eyelid, cleft palate, hearing impairment has an autosomal dominant inheritance affecting the first arch and results due to mutation in TCOF1 gene [1].

## 11. Ciliary development

The cilia on the apical surface of the cells have an important role in human embryological development deciding cell specification, axis determination and pathology of human congenital disorders. A variety of congenital defects like polycystic kidney, laterality defects, nervous system defects, and retinal degeneration are associated with ciliopathies thus proving that cilia can serve as signaling factor in embryonic development. Cilia plays an important role in Hedgehog patch smoothed Gli pathway and thus it regulates the Gli protein activity. The cilia transition zone a short segment just above the basal body acts as an essential gatekeeper for movement of protein in and out of the cilia. Cilia also play an important role in movement of fluid in various confined embryonic spaces, which may act as a developmental shear force [9]. The congenital polycystic kidney disease, which results in numerous cyst formations, can be inherited as autosomal dominant or recessive. In autosomal recessive type the cysts are formed in the collecting tubules and it is a progressive disorder though less common but highly progressive and leads to kidney failure early in infancy and childhood. The autosomal dominant type is more common (1/500 to 1/1000) results in cyst formation in all segments of the nephron is less progressive and does not result in renal failure till adulthood, The above two types of conditions are related to the mutation of genes that code ciliary protein and thus fall under the group of disorders the ciliopathies which are creating a lot of interest in recent times. Bardet-Biedl syndrome and Meckel-Gruber syndrome are characterized with renal cysts and other features are also some of the ciliopathies recently identified [2].

## 12. Reciprocal interactions

Organogenesis results due to reciprocal interaction between the differentiated epithelial cells and the underlying mesenchyme, which is brought about by signaling molecules. The

transcription factor NKX2 which is a homolog of the gene *tinman* that regulates heart development in *Drosophila*, is induced in the endoderm overlying the splanchnopleuric mesoderm for human heart development. NKX2 is considered as the master gene for human heart development and its expression is brought about by BMP activity and WNT inhibition. The venous portion of the heart is specified and formed under influence of retinoic acid and cardiac looping is due to expression of PITX2 in lateral plate mesoderm.

The cephalocaudal and lateral folding of the embryo gives rise to formation of primitive gut tube which is endoderm lined yolk sac cavity incorporated into the embryo by fourth week of development. The retinoic acid (RA) concentration varies in this primitive gut and it is the highest in colon region and least in that part of this gut tube which give rise to the pharynx. This variation of RA concentrations causes expression of certain transcription factors, which leads to the specification of the gut tube to develop the parts. SOX2 is transcription factor for development of esophagus and stomach, PDX1 is for duodenum development, CDXC is for small intestine, and CDXA is for large intestine and rectum. The interaction of the epithelial and underlying mesenchyme components of the gut follows the initial patterning and brought about by sonic hedgehog expression throughout the gut. The transition of the foregut to midgut and thus hind gut derivatives and the change in the epithelial characteristics are brought about by nested expressions of HOX genes which are activated by sonic hedgehog to bring about an orchestrated development of the gut. The secretion of FGFs from cardiac mesoderm and BMPs from the septum transversum leads to the hepatic development. The FGFs inhibits the activity of inhibitors and thus specifies the development of hepatic endoderm by a negative feedback mechanism as suppressing the inhibitors brings about the induction. The sonic hedgehog expression in gut endoderm, which is destined to form dorsal pancreatic bud, is suppressed in the gut endoderm by FGF2 and activin. The ventral bud is induced by visceral mesoderm, which up regulates expression of pancreatic and duodenal homeobox gene (PDX). Two types of PAX genes specify the endocrine cells. PAX4 and PAX6 genes specify the insulin, somatostatin, and pancreatic polypeptide secreting cells whereas PAX 6 genes specify the glucagon secreting cells [5].

### 13. Urogenital system

The urogenital system develops from a common mesodermal ridge, i.e., the intermediate mesoderm. The intermediate mesoderm gives rise to three overlapping kidney systems the pronephros, the mesonephros and the metanephros. The metanephros, which appears by fifth week, gives rise to the definitive kidney in humans. The mesonephric duct of the intermediate mesoderm gives rise to an ureteric bud which forms the collecting part of the kidneys the renal pelvis, calyces, 1–3 million collecting tubules. The interaction of this ureteric bud from the mesonephric duct with the mesenchyme of the metanephric blastema is the key factor in determining the initiation of kidney development. WT1 is the transcription factor expressed by the mesenchyme of metanephros, which increases the sensitivity of the metanephros to interact with the ureteric bud and respond to it. The branching and growth of ureteric bud is under control of production of Glial derived neurotrophic factor (GDNF) and Hepatocyte growth factor produced by the mesenchyme of metanephros which bind to the receptor on

ureteric bud. The ureteric bud too produces FGF and BMP7, which induce the metanephric mesenchyme to undergo proliferation, and also blocks apoptosis. Epithelial conversion of the mesenchyme of metanephros occurs under the influence of WNT9B and WNT6 from the ureteric buds, which in turn activate PAX2 and WNT4 in the metanephric mesenchyme. All these interactions lead to the modifications in extracellular matrix protein to form the characteristic epithelial basal lamina rich in laminin and type IV collagen. Wilms tumor, a malignant neoplasm of the kidney usually affecting children of age group 5–10 years, is due to mutations in WT1 gene on chromosome 11p13 and can be associated with other abnormalities. Mutations in GDNF gene, which causes branching of the ureteric bud and interaction of the ureteric bud and metanephros, can result in renal agenesis [2].

## 14. Molar pregnancy

Hydatiform mole can be either partial or complete in which the placenta may proliferate abnormally. This can be partial with 69 chromosomes or triploid in which 46 chromosomes are derived from father and 23 from mother, which can be either due to dispermy or endoduplication of haploid sperm. In complete hydatiform molar pregnancy there are only 46 chromosomes and solely paternal in origin. It's due to fertilization of an empty ovum by two sperms or duplication of single sperm. The complete mole can undergo malignant change into invasive choriocarcinoma and outcome can be fatal if untreated [1, 3].

## 15. Multifetal gestation

Multiple births result from simultaneous nurturing two or more embryos in the uterus and can be of monozygotic or dizygotic types. In monozygotic twins a very early division of the zygote before separation of the cells which form chorion result in dichorionic twins, twinning at blastocyst stage results in monochorionic diamniotic twins which constitutes about 70% of monozygotic twins. Twining after first week leads to monoamniotic twins. There is 2–5 times increase in monozygotic twins in babies born by IVF. Very late divisions after 14 days can lead to conjoined twins which is common in females about 75% and thus late twinning and X inactivation can be interrelated. Dizygotic twins result from fertilization of two ova by two sperms thus they are also called fraternal twins. Dizygotic twins are diamniotic and dichorionic. Increased maternal age, family history and intake of ovulation inducing drugs can increase risks for dizygotic twins [1, 3].

In 1961 Dr. Mary Lyon experiments on mice led to the development of Lyon's hypothesis regarding X chromosome deactivation. Later this was recognized and the term lyonization is used for the process of X chromosome, which occurs around 15th, or 16th day of development in female embryos. Either the maternal or paternal X chromosomes can be deactivated in any particular cell and thereafter the same X chromosome is inactivated in daughter cells. Barr bodies are the inactivated darkly stained mass of chromatin seen during interphase. During

cell division this inactive X chromosome is late replicating. But not all of the X chromosome is inactivated. Genes at tip of Xp (short arm) and other genes at other loci of Xp also escape deactivation. There are more genes which get deactivated in Xq thus resulting in less severe phenotypic effects in people with Xq deletions compared to Xp deletions. In men and women with more than one X chromosomes the number of Barr bodies seen during interphase is one less than the total number of X chromosomes. Dosage compensation mechanisms are evolved which lead to a balance in X linked gene products between sexes. This can be achieved by two fold increase in expression of X linked genes in males, a two fold down regulation of X linked genes in females and lastly complete inactivation of one of the two X chromosomes in females. A complex of above mentioned strategies are adapted in mammals [10].

## **16. Pathogenetic mechanisms of birth defects**

The mechanism of birth defects can be broadly grouped as deformation, disruption, dysplasia and malformation. Major congenital anomalies are those that are life threatening but minor abnormalities are those that do not pose a threat to extra uterine survival. Examples of structural anomalies are cleft lip and palate, diaphragmatic hernia, hydrops, congenital heart diseases, choroid plexus cysts and short femur.

## **17. Markers of structural anomalies**

In the first trimester, nuchal translucency is a marker of chromosomal anomalies. In the second trimester there may be echogenic bowel, echogenic chordae or absent nasal bone. Presence of these biophysical markers in early ultrasound becomes an indication to test for chromosomal anomalies in the fetus. Chromosomal anomalies can be detected by performing direct tests (fetal blood sampling, amniocentesis, cordocentesis) on the fetus or chorionic villi (chorionic villus sampling). Indirect screening tests can be done in mother (fetal DNA in maternal serum) to obtain indirect evidence.

## **18. Genetic testing**

There are about 25–30 trillion cells (1 trillion = 1,000,000,000 000) in the human body. They are of two types: somatic cells and gametes. The somatic cells are diploid with 23 pairs of chromosome. The gametes are haploid with 23 chromosomes [11].

Each chromosome has 100–1000 genes. The genetic information contained in Chromosomes is in the form of base pairs. The total base pairs in a haploid set of 23 chromosomes are around 3000 million. Direct testing can be karyotype, FISH or QF PCR, Micro deletion detection, microarray, mutation specific testing or exon sequencing. Single nucleotide polymorphism testing is also important [12–14].

## 19. Karyotyping

Karyotyping detects the number of chromosomes (Aneuploidies or the gross structure of chromosomes). A karyotype detects any chromosomal anomaly greater than 5 Million base pairs. This could be monosomy, trisomy, tri and tetra ploidy, deletions, duplications, etc. Karyotyping is done by culture that may take 3–4 weeks. An example is detection of Down's syndrome by karyotyping of fetal cells obtained after amniocentesis). Any abnormality lesser than 5000 million base pairs cannot be detected by karyotyping. For example a small micro deletion or a mutation responsible for a genetic disease like CAH (Congenital genital hyperplasia) or spinal muscular atrophy.

## 20. FISH

FISH will detect a specific chromosomal anomaly that has been previously suspected. In prenatal diagnosis typically abnormality of chromosome 21, 13, 18, X, Y. It gives results between 72 h. For example if the triple markers in maternal serum and first trimester are suggesting Down's syndrome, a specific FISH for chromosome 21 can be performed. But if there is no specific suspicion it is better to do karyotyping.

A FISH may detect micro deletions also but it has to be planned which micro deletion we are looking for. For example 22q micro deletions responsible for Di George Syndrome in a cardiac anomaly like tetralogy of Fallot. Laboratories usually have a panel for common micro deletions. 22q micro deletion is the second most common chromosomal anomaly after Trisomy 21.

## 21. Microarray

Almost all mutations are very small with only 100–1000 pairs. For these we have to depend on cytogenetic tests like Mutation testing (Sanger Sequence), microarray, Exome sequencing and genome sequencing. If we do not know what to test for than we have to order a microarray. It will scan the entire genome if some few hundreds of base pairs are missing or not. For example, microarray gene testing of uterine endometrium in cases of recurrent implantation failure following in vitro fertilization and embryo transfer.

## 22. Exom and genome sequencing

These are the most precise tests telling us abnormalities in the minute base pairs. These are expensive with last resorts.

An analogy is a library with 23 bookshelves. Each shelf has 10 rows and each row has 25 books. A karyotype is to find if a bookshelf is in excess or missing. Also it can also tell if a major part of bookshelf is broken. A micro deletion is like a missing row in a bookshelf.

A microarray can identify if a book or two is missing in the library but then sometimes it tells a book which is not important. A mutation specific test is like checking if a particular book is missing. For example a book on Indian cooking in a row of cookery books.

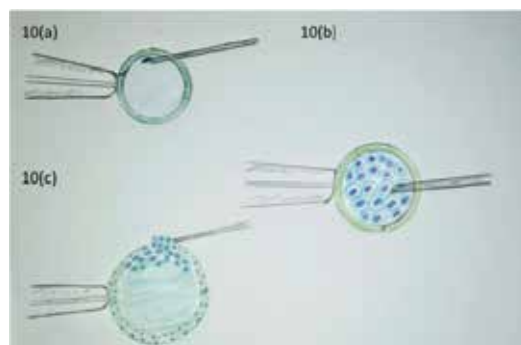
An Exom sequencing is like checking if a few pages are missing in any book in any shelf of the whole library. Genome sequencing is like checking if any paragraph or sentence is missing in any book in any shelf in the whole library. Majority of the fetal birth defects are due to single gene/multiple gene mutations and cannot be identified in karyotyping.

### 23. Preimplantation genetic testing

Preimplantation genetic testing is defined as a procedure to remove one or more nuclei from oocytes (polar bodies) or embryos for genetic testing before transfer [15, 16]. Preimplantation genetic diagnosis PGD is a term used to determine whether a certain mutation or an unbalanced chromosomal complement has been transmitted to the oocyte or embryo when one or both genetic parents carry a genetic mutation or a balanced chromosomal rearrangement. Preimplantation genetic diagnosis is done to avoid transfer of embryos with mutation and identify healthy embryos for transfer [17].

Preimplantation genetic screening is term used when both genetic parents are chromosomally normal and their embryos are screened for any genetic defects before implantation to improve the success rate of embryo transfer [18, 19].

In preimplantation genetic screening and 24 chromosome copy number analysis (CGH, array comparative genomic hybridization, real-time quantitative PCR, SNP microarray) the aim is



**Figure 10.** (a) Diagrammatic illustration of the method for polar body biopsy. (b) Blastomere biopsy. (c) Multiple cell biopsies from blastocyst.

only to improve the IVF rates. So the requirements of accuracy are less strict and high false positive results may be acceptable. The test should more importantly be less costly, rapid and non-invasive.

In preimplantation genetic diagnosis diagnostic accuracy is most important. The test should be highly sensitive and specific with very low false negative results. There are three procedures polar body biopsy, blastomere biopsy and blastocyst biopsy [20] (**Figure 10**).

## 24. Counseling for genetic testing of embryos

The risks associated with ovarian stimulation should be empathically communicated. The couple should be given the option of not proceeding with IVF and PGD/PGS and the choice should be voluntary. The risks associated with embryo biopsy and extended cultures while waiting for the result of biopsy should be explained. The possibility that in case of a false positive result a healthy embryo will be discarded should be explained. The possibility that in case of a false negative result an abnormal embryo may be transferred should be explained. The couple should also be explained about the options of prenatal testing like maternal serum markers, fetal ultrasound, amniocentesis, chorionic villous sampling and fetal blood sampling. The couple should also be explained about the possibility that if all embryos are found affected embryo transfer will not be carried out. Finally the issue of embryos not transferred (discard, cryopreservation, donation, research) should be discussed.

The couple should also be made aware of alternative methods of avoiding genetic defects (like use of donor gametes).

## 25. Polar body biopsy

Laser or mechanical dissection can be done for opening the zona pellucida and retrieval of the polar body [21, 22]. Laser assisted biopsy are less time consuming. Acid Tyrode solution used for chemical denudation of blastocysts is not tolerated by ovum and is not used for ovum biopsy.

The removal of both polar bodies is done in the time window of 8–14 h. After fertilization. Simultaneous removal of both polar bodies is preferred over sequential removal of polar bodies as there are fewer traumas to the oocyte.

Even with the biopsy of both polar bodies, around 26% of errors are missed. Even in the presence of an abnormal polar body screening 90% of embryos will still be euploid [23]. This is because copy number analysis only determines a relative loss or gain of genetic material-not an absolute copy number. Nondisjunction represents only 10% of errors; others are due to premature segregation of sister chromatins. When reciprocal errors occur for a given chromosome in first and second polar body, 90%of embryos will be euploid. So, with 55% of abnormal



polar bodies 10% will be due to nondisjunction and the remaining 45% from half of the premature separation of sister chromatids.

## 26. Embryo biopsy

Blastomere biopsy can be obtained at day 3 embryos. Blastocyst biopsy can be done on Day 5 or Day 6 embryos.

Blastomere biopsy is done by zona drilling with acid Tyrode solution or mechanical aspiration. Alternatively laser denudation can be done. If removal of two cells is considered it should be done only after an embryo has six or more cells. Blastomere biopsy is associated with low implantation rates [24].

Blastocyst biopsy on Day 5 or Day 6 embryos is done with noncontact infrared lasers. Laser is used to create an opening in the zona pellucida and the herniating trophoctoderm is excised. Around 10 trophoctoderm cells are removed and studied. Blastocyst biopsy of trophoctoderm does not lead to reduced implantation and delivery rates. It should be remembered that the RCTs that demonstrated a beneficial effect of embryo biopsy were only done on blastocyst stage with trophoctoderm biopsy.

## 27. Blastocyst comprehensive screening and single-embryo transfer

After a comprehensive chromosome testing a single best embryo can be transferred in women. The initial data collected has revealed that frozen embryo transfer after comprehensive gene testing leads to a better on going pregnancy rates as compared to a fresh blastocyst transfer that was screened only morphologically [25, 26]. With the development of newer modalities like comprehensive chromosome screening and single embryo transfer older women will benefit and risks of multiple embryo transfer will be minimized.

## 28. Direct testing

Direct testing on the fetus is offered when there is an ultrasound marker of anomaly; serum screening (triple markers, quadruple markers) is positive or when one of the parents or siblings is a carrier of chromosomal abnormality. Individuals and relationships are described as a diagram when clinical history is obtained (**Figures 11 and 12**).

### 28.1. Clinical indications for prenatal diagnosis

Diagnostic interventions in obstetrics are mainly directed at some form of fetal tissue sampling for genetic, biochemical, hematological and histological processing. Samples of fetal tissues like amniotic fluid, chorionic villi and blood can be obtained by a variety of tissue sampling

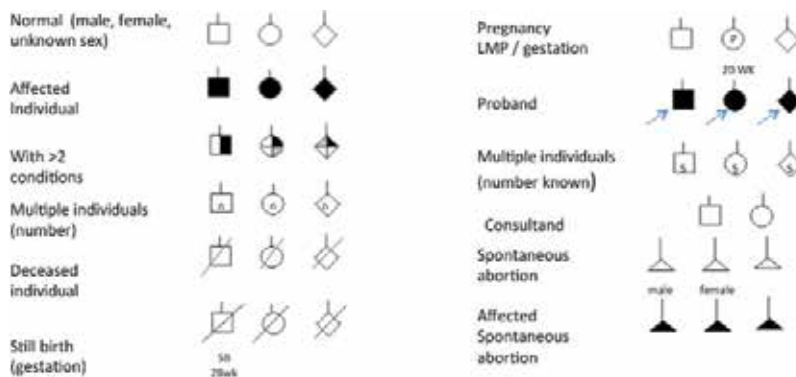


Figure 11. Diagrammatic representation of individuals in clinical genetics.

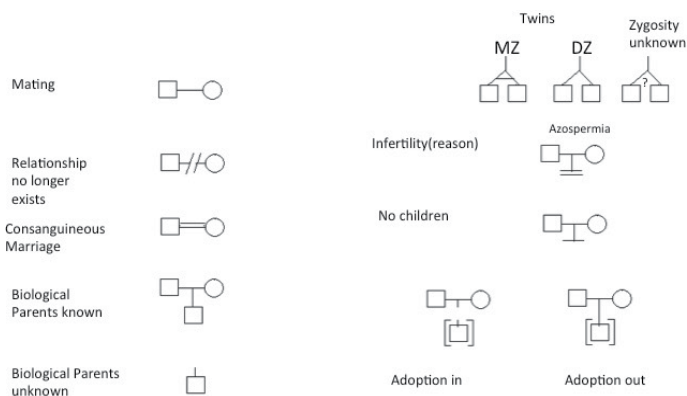


Figure 12. Diagrammatic representation of relationships in clinical genetics.

methods under ultrasound guidance. Other fetal tissues that can be sampled for prenatal diagnosis are fetal urine, skin, liver and brain.

## 28.2. Indications of prenatal diagnosis

1. Advanced maternal age
2. History of chromosomal abnormality or single gene disorder in a sibling
3. Chromosomal translocation in one parent
4. Rh isoimmunization
5. Congenital infections
6. Fetal growth disorders
7. Increased nuchal translucency in first trimester ultrasound scan

8. Multiple soft tissue markers seen on ultrasound in the second trimester
9. Positive maternal serum biochemistry

The fetal tissue sample obtained by these invasive procedures can further be processed.

1. Karyotyping
2. DNA analysis
3. Hematological parameters
4. Biochemical analysis
5. Enzyme assays
6. Microbial assays
7. Histological studies by light and electron microscopy.

## **29. Amniocentesis**

Amniocentesis is the oldest procedure performed for prenatal diagnosis [27]. As early as 1952, it was found that cells in the amniotic fluid represented fetal tissue and could be used for gender determination. This procedure was performed initially without any guidance. Now, with the help of real time ultrasound and color flow mapping, amniocentesis is performed easily with accuracy. There are minimal complications and the procedure is safe.

Amniocentesis is performed in the second trimester when the uterus has become an abdominal organ and the amniotic fluid is about 200 ml. At any gestation, 10% of amniotic fluid volume can be aspirated for diagnostic procedures. A prerequisite is that there should be an adequate amniotic fluid volume for that gestational age.

### **29.1. Early amniocentesis**

Improved laboratory techniques have made it possible to culture amniotic fluid cells from as little as 10 ml of amniotic fluid at 12 weeks. However, the volume of 10 ml will account for almost a third of the amniotic fluid volume at this gestational period [28]. This may lead to problems in the fetus like lung hypoplasia and limb deformities like congenital talipes equinovarus. To overcome the loss of fluid volume, a technique of entrapping the cells in a filter and returning the amniotic fluid into the cavity has been tried. Randomized controlled trials are required to establish the safety of this procedure.

### **29.2. Technique of amniocentesis**

Under continuous ultrasound guidance and strict aseptic precautions, a 20–22 gauge spinal needle is introduced in a pool of amniotic fluid, which is devoid of fetal parts. It is preferable to avoid the placenta. If the placenta is anterior, the needle is introduced laterally or above the

placental margin. The amnion is pierced with a sudden controlled force, a jab of needle to prevent tenting of the membranes. The first few ml of fluid is discarded to minimize the contamination with maternal blood. About 20 ml of fluid is withdrawn for analysis. Not more than two insertions should be performed at one sitting.

### **29.3. Amniocentesis in twins**

Individual sampling of both sacs using two different needles under direct ultrasound guidance can perform amniocentesis in twins. A single insertion technique in which needle is advanced to the second sac after aspirating the first sac has been advocated by Jeanty.

### **29.4. Complications**

A blood stained aspirate can occur in 1–2% of the procedures. If the color is red, it indicates fresh blood possibly due to maternal blood contamination. Dark red or dark brown color of the fluid indicates prior intraamniotic bleed that is associated with poor fetal outcome. A brown color amniotic fluid can also indicate fetal aneuploidy. The overall pregnancy loss following amniocentesis is estimated as 0.5%.

The incidence of fetomaternal hemorrhage is 63% in anterior placentas wherein the needle has traversed through the placenta. It reduces to 18% in posterior placentas. Hence, it is essential to determine Rh type of the mother prior to the procedure. Anti D immunoglobulin must always be given in rhesus negative women after any prenatal procedure. Fetal trauma is unlikely if the procedure is done continuous ultrasound monitoring.

## **30. Chorionic villous sampling**

Chorionic villous sampling was initiated in clinical practice in 1980s [29]. It is performed between 10 and 14 weeks. Placental biopsies are also done in second and third trimesters for rapid karyotyping.

### **30.1. Technique**

Prior to the procedure the crown rump length is measured to confirm the gestational age. Fetal heart activity is documented and a sample of villi can be obtained by the transcervical or transabdominal route [30].

### **30.2. Trans cervical route**

A special transcervical cannula developed by Rodeck with a malleable obturator is used. With a partially filled bladder, the gravid uterus is imaged transabdominally and the chorionic frondosum is identified. With the patient in lithotomy position a cannula is introduced through the cervical canal. The cannula guided into the chorionic frondosum under continuous ultrasound guidance. A 10 ml syringe with 2 ml of culture media is then attached to the

proximal end of cannula. Suction is applied and a gentle to and fro motion of the cannula will ensure aspiration of villi into the medium in the syringe. The cannula is then withdrawn with continuous suction being maintained. The sample is then examined under a microscope to ensure the presence of branching villi. A minimum of 15 mg of tissue will be required for culture.

### **30.3. Trans abdominal approach**

Rodeck transabdominal chorionic villous biopsy forceps can perform transabdominal collection of fetal villi. A 20 Gauge spinal needle can also be used for sampling the villi transabdominally. The needle is advanced under ultrasound guidance and suction is applied as via the transcervical technique. A double lumen technique can also be used. In this, a large lumen outer needle is introduced into the uterus and a smaller gauge needle is passed through the outer needle to sample the villi. The advantage of this technique is that resampling can be done easily if the sample is insufficient in the first passage of needle. Transabdominal approach has been found safer with few complications than trans cervical route.

### **30.4. Complications of chorionic villous sampling**

Fetal loss can occur in 0.6–2% of cases. Loss rates are greater than 10% if more than two needle insertions are made to collect the chorionic villi. A sub chorionic hematoma may form in 4% cases, which usually resolves spontaneously. Chorioamnionitis is a rare complication occurring in less than 0.3% cases. A delayed rupture of membranes can happen weeks to days after chorionic villous sampling in about 0.3% of cases.

The risk of fetomaternal hemorrhage is dependent on the amount of tissue aspirated and is detected by a rise in maternal serum alpha fetoprotein [31]. All Rh Negative non-sensitized mothers should receive a prophylactic Anti D immunoglobulin [32]. Perinatal complications like premature rupture of membranes, small for gestational age or intrauterine growth restriction have not been noticed after chorionic villous sampling. Chorionic villous sampling performed prior to 9 weeks of gestation is known to be associated with specific fetal malformations. Oromandibular limb hypoplasia syndrome and terminal transverse limb reduction anomalies have been documented when chorionic villous sampling was performed prior to 9 weeks of gestation. Chorionic villous sampling should not be performed prior to 9 weeks of gestation [33, 34].

## **31. Fetal blood sampling**

Fetal blood can be sampled after 18 weeks of gestation. Fetal blood sampling rules out the possibilities of pseudomosaicism that are more common in chorionic villi and amniotic fluid cultures. Fetal blood can also be used for hematological estimations and enzyme and hormone assays. Direct access to fetal circulation is also used to give intra uterine exchange transfusions in cases of Rh isoimmunization.

### **31.1. Technique of fetal blood sampling**

Fetal blood can be obtained from the umbilical vein or artery in the umbilical cord near its insertion into placenta. Fetal blood can also be obtained from the intrahepatic umbilical vein or a free loop of umbilical cord. A 22 Gauge spinal needle is used for the procedure. Ultrasound with color Doppler is used to image the site of cord insertion in the placenta. The needle is introduced under ultrasound guidance about 1 cm from the insertion site of the umbilical cord into the placenta. If the cord is punctured close to the placental insertion there are increased chances of contamination with maternal blood. After obtaining the fetal blood sample it is tested for contamination with maternal blood. Kleihauer-Betke test of acid elution with hydrochloric acid can distinguish between fetal and maternal cells. Fetal red blood cells do not get eluted with hydrochloric acid due to presence of fetal hemoglobin. Fetal Red blood cells have a larger Mean Corpuscular Volume compared to maternal red blood cells.

Intrahepatic portion of the umbilical vein is an alternative site, which yields pure blood samples. Direct fetal cardiac sampling has been resorted to in few centers. When blood is obtained directly from the fetal heart or intrahepatic portion of umbilical vein testing for contamination with maternal blood is not necessary.

### **31.2. Complications**

When a needle is inserted into the umbilical vein the most common complication is fetal bradycardia. However it should be monitored and it quickly reverts back to normal after the needle is withdrawn. Sometimes a brisk spurt of blood from the puncture site into the amniotic fluid is seen lasting for 2 min. However this bleeding invariably stops and the fetus is unaffected. In cases of continued hemorrhage a bleeding disorder in the fetus should be considered. The overall fetal loss rate following umbilical blood sampling is 1–2%. There is no increased risk of preterm labor, intrauterine growth restriction or fetal congenital malformations.

## **32. Fetal skin biopsy**

Fetal skin biopsy is done between 18 and 20 weeks of gestation [35, 36]. This is indicated for the detection of genodermatoses like epidermolysis bullosa, epidermolytic hyperkeratosis, harlequin ichthyosis and Sjogren-Larsson syndrome [37, 38]. Another indication is oculo cutaneous albinism.

### **32.1. Technique of fetal skin biopsy**

Fetal skin sampling is done using a special biopsy forceps, which is introduced through a trocar needle. The site of sampling is chosen according to the indication. The site of sampling is different in different indications. In suspected genodermatosis the site of biopsy is gluteal region. When oculo cutaneous albinism is suspected the skin biopsy is taken from the eye-brows and scalp. An electron microscope study of the fetal skin sample is required to confirm

the diagnosis. The procedure is safe and in a study of 52-skin biopsy by Rodeck no complications were recorded [39].

### 33. Fetal liver biopsy

Fetal liver biopsy has been used for the prenatal detection of urea cycle disorders and G6PD deficiency. The procedure is performed with a double lumen fetal liver biopsy needle [40].

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# Gene Polymorphisms of Immunosuppressants in Solid Organ Transplantation

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Yingzi Ming and Meng Yu

Additional information is available at the end of the chapter

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## Abstract

The rapid development of immunosuppressants (IS) has already improved the prognosis of recipients post solid organ transplantation (SOT) in the past decades. However, the individual difference in IS metabolism may lead to either rejection or drug toxicity, thus suggesting the importance of personalized therapy. Gene polymorphisms (GP) regarding metabolic enzymes and medication transporters of the IS consist the footstone for personalized therapy, which are the hotspots in recent years. However, although a great of efforts have been put into researching the association between GP and pharmacokinetics (PK) or pharmacodynamics (PD), controversial results still remained. The only consensus that has been reached is the use of GP-based IS therapy could help the recipients to reach target concentration faster with less dose adjustment. Whether the GP-based IS therapy could improve the clinical long-term outcome needs further confirmation. In our chapter, we would summarize the information associated with GP of IS, and discuss the potential impact of GP on clinical practice to provide new insights into guiding tailored therapy of IS in SOT.

**Keywords:** gene polymorphisms, immunosuppressants, solid organ transplantation, pharmacokinetics, pharmacogenetics

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## 1. Introduction

SOT has prominently reduced mortality and morbidity when facing conditions of end-stage organ failure [1]. To prevent rejection of SOT, the use of IS is essential. And with the continuous development of medical technology, individualized treatment has been paid much more attention. With the advent of new drugs, more choices of IS combination are available in present clinical practice, and the most widely used of which is a calcineurin inhibitor (CNI) + an

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antiproliferative agent + corticosteroid. Tacrolimus (Tac) and ciclosporin (CsA) are both CNI, and Tac has held most of the market in recent years. Mycophenolate (MMF) is the most widely used antiproliferative agent, which is a prodrug of mycophenolic acid (MPA) that has immunosuppressive activity [2]. There is also a monosodium salt of MPA that was developed to overcome the gastrointestinal side effects of MMF. Ahead of the “triple immunosuppressive therapy”, many maintenance regimens also have induction therapy, which consists of antibodies to lymphocytes. The mammalian target of rapamycin (mTOR) is a more recent IS, which includes sirolimus (SRL) and everolimus (EVE). They were first envisaged as a primary IS in regimens without CNI [1, 3, 4], while now, most of the time they are used at low doses in CNI-sparing regimens [5, 6].

To achieve the target blood concentration fast with less dose adjustment is clinically important, for the therapeutic window of these IS is narrow, which may lead to either acute rejection or over-immunosuppression [7–9]. There are also numerous drug-specific adverse effects, CNI has a closer relation to high blood pressure hypertension, nephrotoxicity, and new-onset diabetes after transplantation (NODAT) [10], steroids may also lead to NODAT [11], and the mTOR inhibitors are often associated with delayed wound healing and hyperlipidemia [8]. In the majority of cases, these adverse effects are associated with high drug concentrations in the blood, but this is by no means universal [12].

In consideration of inter-individual variation in PK of IS, monitoring the blood concentration has been essential. Therapeutic drug monitoring (TDM) has been widely used to ensure the blood concentration of CNI and SRL [13]. In daily clinical practice, the blood sample is taken and measured at a single time-point immediately before the next dose (called  $C_0$  or trough concentration). Although recent studies have suggested that, for measurement of CsA, 2 h after dosing ( $C_2$ ) correlates better with the area under the concentration-time curve (AUC) compared to  $C_0$  [9]. This may also apply for Tac, but the practice has not been adopted widely [12, 14, 15].

TDM is no doubt powerful and indispensable in the current management of IS, while many patients may still experience a significant delay in achieving target blood-concentrations, and drug dosage need to be adjusted repeatedly, which could significantly increase the risk of acute graft rejection [9, 16]. The narrow therapeutic window of the IS makes it impossible to use a higher initial dose for all patients, and here comes an unmet need for a strategy, which can lead to the fast achievement of IS target blood concentration in the period immediately following transplantation for all the patients. Fortunately, the investigation of GP combined with critical patient data and concomitant medications may help and thus potentially reduce the adverse events related to over- or under-exposure. In addition, studies may also possibly reveal the association between GP and IS PD, which may help clinicians to choose a better combination of IS for the specific group. A strategy based on GP is most likely to be effective when a single gene has a major influence on the absorption, disposition, elimination or tissue compartmentalization of a drug [12].

This chapter discusses the published genetic associations with IS PK and PD, and their potential use in clinical practice to guide drug dosing in SOT.

## 2. GP and Tac

Tac is a 23-membered macrolide lactone isolated from *Streptomyces tsukubaensis* in 1987 for the first time [17], and in 1994, the US Food and Drug Administration firstly approved Tac for liver transplantation. Due to its excellent efficacy, Tac has been extended as a first-line regimen for kidney, heart, lung, intestinal and bone marrow transplantation. Genetic factors including CYP3A5\*3, CYP3A4\*1B, CYP3A4\*22, ABCB1, and POR\*28 have been reported frequently for their influence on Tac dose requirement, which reveals the importance of GP of Tac.

### 2.1. CYP3A5 and Tac

Reportedly, polymorphisms in the CYP3A5 gene may explain 40–50% of the variability in Tac dose requirement [18, 19]. The hottest SNP studied in CYP3A5 is CYP3A5\*3, which is an A to G transition at position 6986 within intron 3 (rs776746) [20]. This mutation leads to alternative splicing, and truncation of the protein, which decreases the function of the CYP3A5 enzyme [21]. As a result, CYP3A5 expressers (CYP3A5\*1/\*1 or CYP3A5\*1/\*3 genotype) have significantly lower dose-adjusted  $C_0$  compared to CYP3A5 non-expressers (CYP3A5\*3/\*3 genotype), and the requirement of Tac dose is CYP3A5\*1/\*1 > \*1/\*3 > \*3/\*3 [20]. A large number of retrospective studies have shown that kidney graft recipients who are CYP3A5 expressers require an approximately 2-fold higher Tac dose compared with non-expressers [22–24]. In addition, no matter in adult or pediatric heart recipients [25, 26], in lung transplantation recipients [27], as well as in liver transplantation recipients [28], the same relationship has also been observed.

Other CYP3A5 SNPs include CYP3A5\*6 (rs10264272) and CYP3A5\*7 (rs41303343). CYP3A5\*6 encodes a G to A transition at position 14,690, causing a splice variant mRNA and deletion of exon 7, resulting in nonfunctional CYP3A5 protein [21, 29]. CYP3A5\*7 denotes a single base insertion at codon 346, causing a frame shift and resulting in a truncated mRNA and nonfunctional CYP3A5 [30].

As for the association between CYP3A5 and the early prediction of the risk of acute rejection, the results are quite inconsistent. Some studies involving Tac therapy did not find any significant association [23, 31–34], while some other studies reported that CYP3A5 expressers had a higher risk of experiencing biopsy-proven acute rejection (BPAR) [35, 36].

As for other kidney transplantation outcomes involving Tac therapy, such as chronic allograft nephropathy or delayed graft function (DGF). Some studies reported that CYP3A5 expressers had a higher risk of experiencing biopsy-proven Tac-related nephrotoxicity [37, 38] and reduced renal function during the first year after transplantation [36], and CYP3A5 non-expressers were more likely to develop DGF [39] and early renal graft injury as assessed by the urine test [40].

### 2.2. CYP3A4 and Tac

As for CYP3A4 gene, two SNPs in relation to Tac PK have been investigated extensively: CYP3A4\*1B SNP (rs2740574) and CYP3A4\*22 SNP (rs35599367).

The CYP3A4\*1B SNP involves an A to G transition at position -392 in the promoter region of CYP3A4 and is associated with an increase of CYP3A4 activity [41]. It showed that the  $C_0/D$  ratio of Tac in patients with the \*1B mutation was reduced by 35% compared with that of wild-type homozygotes [42]. However, there is a linkage disequilibrium (LD) between CYP3A4\*1B and rs776746 of the CYP3A5 gene. It is possible that the effect of CYP3A4\*1B on Tac PK and PD is caused by rs776746, which has been shown in several published studies [43, 44]. Therefore, the exact effect of CYP3A4\*1B alone on Tac is still unclear.

The CYP3A4\*22 SNP (rs35599367) contains a transition of C to T in intron 6 and is associated with reduced CYP3A4 mRNA expression and CYP3A4 enzyme activity in vitro [45]. In clinic observation of kidney transplantation, the CYP3A4\*22 required less Tac dose to achieve the target exposure. What's more, it was not influenced by the CYP3A5 genotype [46]. However, it should be noted that the frequency of CYP3A4\*22 is relatively low. About 5% of the Caucasian population, 3% in the American population, and not found in Asians or Africans [47].

Other CYP3A4 SNPs such as CYP3A4\*18 (rs28371759) may also have an impact on Tac PK. This SNP is located in intron 10, with a transition of T to C at position 878. This mutation may increase the activity of the CYP3A4 enzyme and thereby increase the Tac clearance rate and plasma drug concentration [48].

There is also a new and rare CYP3A4 variant, which is now designated as CYP3A4\*26. This variant is an 802C>T transition and results in a premature stop codon at position 268 in exon 9 (R268\*) [49]. The truncated CYP3A4 protein is non-functional.

When combining CYP3A4 and CYP3A5 genotypes, Elens et al. [50] were able to predict Tac dose requirements better compared with the CYP3A4 or CYP3A5 genotype alone. Based on these observations, it has been proposed to prescribe different Tac doses for ultrarapid (CYP3A5 expressers and CYP3A4 \*1/\*1), intermediate (CYP3A5 non-expressers and CYP3A4\*1/\*1) and poor (CYP3A5 non-expressers and CYP3A4\*22 carriers) CYP3A metabolizers, respectively [51].

### 2.3. ABCB1 gene and Tac

P-gp, also known as ABCB1 or MDR1 is a glycoprotein encoded by the human ABCB1 gene, which serves as drug transporter of Tac, and plays an important role in Tac PK. Recently, P-gp has been found to contain more than 50 SNPs. Among them, the ABCB1 3435C>T (rs1045642), 1236C>T (rs1128503) and 2677G>T/A (rs2032582; Ala893Ser/Thr) SNPs have drawn the most attention after intensive investigation [52–54].

The ABCB1 3435C>T (rs1045642) might be the hottest locus among all the ABCB1 gene SNPs. Reportedly, the frequency of this mutation in orientals is 37–49% [55]. The variation of rs1045642 locus might reduce the expression and function of P-gp in the duodenum, and thus potentially affect the bioavailability of Tac [56].

As for ABCB1 2677G>T/A SNP, wild-type patients required 40% higher Tac dose compared with homozygous carriers of 2677G>T/A SNP ( $P \leq 0.05$ ), while the concentration/dose ratio was 36% lower in the wild-type patients ( $P \leq 0.02$ ). The haplotype analysis further confirmed the

results and suggested that 3435C>T and 2677G>T/A SNPs were associated with daily Tac dose requirements. In addition, the study of these three SNP haplotypes (1236C>T, 2677G>T/A and 3435C>T, which are in linkage disequilibrium) found that C-G-C (haplotype 1) and T-T/A-T (haplotype 2) accounted for 45.4 and 36.2% of the haplotypes, respectively; individuals with haplotype 1 required significantly higher daily doses of Tac than those with haplotype 2 [57].

Although there are many studies on the association of ABCB1 GP with Tac PK or PD, the results remain inconsistent. To further confirm the association, large-scale genotype-phenotype correlation trials are encouraged.

#### **2.4. POR and Tac**

POR is essential for CYP-mediated drug oxidation as an electron donor [58]. POR\*28 (rs1057868; A503V) is a coding variant in POR gene, which is believed to be effective in increasing the activity of POR and thus leads to the increasing activities of CYP3A4 and CYP3A5 [59, 60].

It has also been reported frequently that POR\*28 carriers have lower adjusted Tac  $C_0$  and Tac  $C_0$ /Tac dose in heart or kidney transplantation, no matter for the adult or the pediatric [61–64]. Although the strength of this association seems weak and has a limited clinical impact on Tac dose requirements (15–20%), POR\*28 may explain a part of Tac variability, and POR\*28 carriers may experience faster Tac metabolism [61, 63, 65].

As for the association between POR\*28 allele and BPAR after Tac or CsA therapy, no significant evidence was found [63]. As for other graft clinical outcomes, the POR\*28 allele was not found to be associated with the higher risk of DGF after Tac therapy in patients with renal transplantation [63]. While it should be noted that one study in recipients with kidney transplantation demonstrated a higher risk of post-transplantation diabetes mellitus (PTDM) in patients carrying the POR\*28 allele [60].

#### **2.5. PXR and Tac**

As a nuclear transcription, the human pregnane X receptor (PXR), which is encoded by NR1I2, regulates the expression of CYP3A and ABCB1. Polymorphisms of NR1I2 have been reported, but the results regarding their association with Tac dose requirement are conflicting [18, 66, 67].

#### **2.6. PPAR- $\alpha$ and Tac**

The expression and activity of CYP3A are also related to the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ). Two sequence variants in the PPAR- $\alpha$  gene (PPARA), PPARA c.209-1003G>A and c.208+3819A>G, can reduce the PPAR- $\alpha$  expression and contribute to the intra- and inter-individual variability of CYP3A [68]. At present, PPARA c.208+3819A>G appears to have the strongest influence on Tac PK, though it still needs confirmation.

As for graft clinical outcome, one study in kidney transplant recipients demonstrated a higher risk of PTDM who carry the rs4253728 SNP [59].

## 2.7. Other SNPs and Tac

The multidrug resistance-associated protein 2 (MRP2), which is encoded by the *ABCC2*, may also be associated with Tac metabolism [69].

The CYP2C8 enzyme, which is highly expressed in the liver, can also be found in extrahepatic tissues like kidney [70]. Reported by Suarez-Kurtz et al. [71], the CYP2C8\*3 was associated with higher Tac  $C_0/D$ , but only in CYP3A5 non-expressers. Furthermore, CYP2C8\*3 and CYP2J2 -76G>T SNPs were reported to influence the renal function of the patients and the occurrence of adverse events during treatment with Tac and mycophenolate sodium [72].

Genetic polymorphisms in IL-18 (e.g., rs5744247) and IL-10 (e.g., -819 C/T and -592 C/A) can also affect Tac dose requirements [50, 62]. However, the exact mechanism by which they affect Tac dose requirements is unknown [73, 74].

Recently, it was also reported that IL-3 rs181781 and CTLA4 rs4553808 genetic polymorphisms probably influence the Tac dose requirements in Chinese kidney transplant recipients [75].

## 3. GP and CsA

Both CsA and Tac are CNI. The GP that closely related to Tac as mentioned above could have more or less the effect on CsA, and as it should be, the effect is not exactly the same. In recent years, Tac gradually replaces the use of CsA, and here we will not make an in-depth introduction.

Reportedly, the influence of the CYP3A5\*3 and CYP3A4\*22 variants on the PK [19, 42, 50, 76–80] or PD [33, 81–84] of CsA are controversial. The effect of CYP3A5\*3 on the CsA PK appears to be weaker than observed for TAC, while the CYP3A4\*22 allele appears to be stronger, which would decrease the clearance of CsA by 15%. As stated in [47], for the time being, no precise dose adjustment has been proposed based on these variants.

## 4. GP and SRL

Both SRL and EVE are the mammalian target of rapamycin inhibitors (mTORi). SRL was not used as IS until the late 1990s. SRL can form a complex with the tacrolimus-binding protein (FKBP), which binds to mTOR. It inhibits the entry of cells from G1 phase to S phase and inhibits T lymphocyte activation (proliferation) [85, 86].

Despite the potential advantages of sirolimus, such as antitumor activity [87–89] and antiviral activity [90–92], there are also a number of side-effects that result in a limit use of mTORi in clinical trials and practice, including PTDM, hyperlipidemia, anemia, proteinuria, oral ulcers, diarrhea, impaired wound healing, interstitial pneumonitis and edema [93].

### 4.1. CYP3A5 and SRL

At least 2 studies found no association between the CYP3A5\*3 allele and SRL trough levels ( $C_0$ ), dose requirement,  $C_0$ /dose [94, 95]. On the contrary, several other studies described



significant associations between SRL exposure and the CYP3A5\*3 genotype [96–99]. The difference between the two kinds of results may be caused by the co-treated use of CNI. The effect of CYP3A5\*3 genotype on SRL may only be notable in the patients taking no CNI [100].

#### 4.2. CYP3A4 and SRL

The CYP3A4\*22 allele was found to be associated with a moderately lower SRL hepatic metabolism *in vitro*, which, is inconsistent with another study [101]. A 113 stable recipients post renal transplantation switched from a CNI to SRL and found no significant association between this allele and SRL PK.

As for CYP3A4\*1B allele and SRL PK, a study including 149 recipients with renal transplantation confirmed a significant association between this allele and SRL  $C_0$ /dose in the subgroup of 69 patients taking no CNI [96, 97], as the same case in CYP3A5\*3. However, another study reported differently [97].

#### 4.3. ABCB1 and SRL

As for ABCB1 gene, two studies showed no significant association between the ABCB1 c.3435C>T SNP and the SRL  $C_0$ /dose [96, 98]. In another study, no association was found between SRL  $C_0$ /dose and any of the ABCB1 exon 12, exon 21, and exon 26 SNPs, nor with their haplotype [94].

On the other hand, some reported that ABCB1 haplotype combination has a significant influence on SRL PK [44]. According to the report, the mean SRL  $C_0$ /dose was approximately 30% lower in Chinese renal transplantation recipients carrying ABCB1 CGC/CGC as than those carrying the CGC/TTT or TTT/TTT combinations (no effect of ABCB1 individual SNPs was found).

#### 4.4. Other SNPs and SRL

As for the POR\*28 allele, the PPARA rs4253728 SNP, and CYP2C8\*3, no significant association was found between these SNPs and SRL PK [60, 68, 102–104].

Reported data about the influence of P450 or ABCB1 gene variants on the PK/PD of SRL are inconsistent [96, 97, 101, 105, 106]. CYP3A5\*3 genotyping might be potentially useful in kidney transplant recipients with no CNI because of the possible competition for CYP3A5 metabolism [100]. As was stated in [47], at the present time, data are insufficient to recommend any genotype test for this immunosuppressant.

### 5. GP and EVE

EVE is the hydroxyethyl derivative of SRL, with a similar mechanism of action but much more predictable PK. Clinical trials using EVE followed, first in combination with CNI then in CNI-sparing regimens [100].

Again, the reported data about the association between SNP polymorphisms (including CYP3A5\*3, CYP3A4\*22, ABCB1 c.3435C>T, CYP2C8 and PXR) and the PK/PD of EVE are

inconsistent [26, 104, 107, 108]. For more details, readers may refer to [100, 109], one of which was specifically devoted to EVE.

## 6. GP and mycophenolic acid (MPA)

MMF is the most widely used antiproliferative agent [2], after administration, MMF is hydrolyzed to form MPA, which is in turn glucuronidated by several members of the uridine diphosphate-glucuronosyl transferase (UGT) family to form the main metabolite 7-O-MPA-glucuronide (MPAG). MPAG is excreted into bile by ABCC2 (or MRP2) and undergoes enterohepatic circulation [100]. Organic anion transporting polypeptides (OATPs, encoded by the *SLCO* genes), ABCB1 (P-glycoprotein, encoded by the *ABCB1* gene), and cytochrome P450 (CYP) 2C8 and CYP3A4/5 are also involved in the PK of MPA [110].

### 6.1. UGT1A9 and MPA

As mentioned above, the UGT family plays an important role in the metabolic process MMF, of which, UGT1A9 is the most important family member that may affect the PK of MPA [110]. Reportedly, there is a significant influence of the UGT1A9-2152C>T (rs17868320) and -275T>A (rs6714486) SNPs on MPA PK, while this conclusion seems to depend on the MPA dose, type of concomitant CNI (CsA or Tac), and time after transplantation [111–114].

Another UGT1A9 SNP, -98T>C (or UGT1A9\*3) has also been found to be associated with higher MPA exposure in healthy volunteers and kidney transplantation recipients [111, 113–115].

As for the association between MPA PK and genetic variants in UGT1A8 or UGT2B7, reported results remain conflicting. Further investigation is needed to reveal the associations between MPA PK and UGT genotype [110, 116].

### 6.2. ABCC2 and MPA

ABCC2 is responsible for the biliary and renal excretion of MPAG and is inhibited by CsA [117]. According to the reports, the ABCC2 -24C>T has been studied most extensively among the SNPs that have been identified in the *ABCC2* gene [110]. Some studies did find a significant relationship between various ABCC2 SNPs and MPA PK [118, 119], while many other studies have reported differently [110, 112, 113].

### 6.3. *SLCO1B1* gene, *SLCO1B3* gene, and MPA

The OATPs 1B1 (*SLCO1B1* gene) and 1B3 (*SLCO1B3* gene), 2 uptake transporters located on the sinusoidal side of the hepatocytes, are involved in the uptake of circulating MPAG in hepatocytes [112], which contributes to MPA enterohepatic circulation.

Among the SNPs that have been described in *SLCO1B1*, the nonsynonymous 521T>C (Val174Ala) and 388A>G (Asn130Asp) SNPs are associated with altered transport activity. These 2 SNPs are in LD and form haplotypes designated as *SLCO1B1*\*1A (388A-521T),

SLCO1B1\*1B (388G-521T), SLCO1B1\*5 (388A-521C), and SLCO1B1\*15 (388G-521C) [120]. In the studies of [112, 121], no significant association was found between the SLCO1B1 SNPs or haplotypes and MPA PK in renal transplantation recipients. In another study, SLCO1B1\*15 allele carriers are found to be related to a lower level of MPAG than in noncarriers [122], suggesting a decreased hepatic uptake of the metabolite.

As for the various of SNPs in SLCO1B3, the most frequent are a T>G substitution at position 334 and a G>A substitution at position 699 (in complete LD), which result in 2 amino acid changes (Ser112Ala and Met233Ile). In a study of renal transplant recipients receiving MMF with no CsA immunosuppressive regimen, the SLCO1B3 334G allele was found to be associated with a significantly lower MPA dose-normalized exposure, Whereas in the group of MMF + CsA, no significant effect was observed [112].

#### **6.4. Inosine monophosphate dehydrogenase (IMPDH) and MPA**

The mechanism of action of MPA is the inhibition of the rate-limiting enzyme in de novo purine synthesis, inosine monophosphate dehydrogenase (IMPDH). Followed by the characterization of 2 isoforms in humans, IMPDH1 and IMPDH2 [123], many other genetic variants of both isoforms have been identified. Although a few of them seem to have an effect on the expression or the enzyme activity directly, most of the genetic variants are either rare or ineffective on enzyme activity [124, 125].

As for the potential influence of IMPDH variants on IMPDH activity, a study in a group of renal transplantation recipients on MPA demonstrated that the enzyme activity over 12 h was 49% higher in patients with the IMPDH2 variant rs11706052 than in patients with the wild-type. While in the group with no MPA, no difference was found [126]. In addition, for the IMPDH2 variant rs121434586, which has only been reported at a very low frequency, the enzyme activity is reduced to approximately 21% of wild-type activity, probably because of accelerated protein degradation. For the IMPDH1 variant only found in the Han Chinese-American group (rs72624960), the enzyme activity is as low as 10% compared with the wild type, also explained by accelerated degradation [124].

## **7. Conclusions**

Tremendous efforts have been made in order to better understand the individual differences of IS. The genetics polymorphisms mentioned above are more or less related to the variability of IS PK/PD. To date, our knowledge of GP associated with IS in SOT is insufficient. It is still not sure whether genotype testing of these alleles would improve clinical outcome of SOT, this technique is definitely effective in depicting the PK parameters of IS, and has the potential to increase the chance of determining the best drug and the correct initial dose. A benefit of genotyping as a predictive test is that this is a fixed characteristic that will not change with pharmacological and physiological status [12]. Algorithms based on multiple genotypes may have a better performance in predicting the required dose, which helps recipients achieve target IS concentration faster with fewer dose adjustments. The Clinical Pharmacogenetics Implementation Consortium (CPIC) has also published Tac dosing guidelines based on

CYP3A5 genotype expression [127], which is inspiring that the transplant community devotes such great efforts to the PG research.

Whatever, based on the present literature, a study of initial tacrolimus dosing based on the CYP3A5 genotype would be logical. And according to [47], the French National Network of Pharmacogenetics (Réseau national de pharmacogénétique [RNPGx]) considered CYP3A4 (CYP3A4\*22) genotyping would be potentially useful.

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## Conflict of interest

No conflict of interest.

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# Pharmacogenomics

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# Pharmacogenetics of Cardiovascular Disease: Genetic Variation and Statin Intolerance

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Additional information is available at the end of the chapter

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## Abstract

Statins are very effective for lowering low-density lipoprotein cholesterol for primary and secondary cardiovascular disease prevention. While statins are usually well tolerated, individual response to statin therapy varies and intolerance, predominantly muscle symptoms, may appear in a significant proportion of patients. Besides clinical factors, variation in genes coding for proteins with drug transporting, immune or enzymatic function have been implicated in the pathogenesis of statin intolerance. In this review, we will characterise the candidate gene variants for development of statin intolerance, describe their population distribution and summarise current knowledge on their biological plausibility. Clinical relevance and current guidelines/recommendations will be also discussed.

**Keywords:** genetic variation, pharmacogenetics, *SLCO1B1*, statin, statin-induced myopathy

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## 1. Introduction

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase) inhibitors are highly effective drugs lowering plasmatic concentration of LDL-C cholesterol by 30–50% [1]. Despite the fact that they are usually considered safe and very well tolerated, a significant proportion of the treated patients does not tolerate the drug: they suffer from side effects, which may result in non-compliance of patients, drug dose-lowering and even discontinuation of therapy [2–4]. Undesirable effects of statins restrict their administration or reaching LDL-C cholesterol target values and limits effective treatment of patients at risk. Non-adherence or discontinuation of therapy is associated with an increased risk of cardiovascular events [5–7].

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By analogy to individual nature of patients' response to treatment [8–10] there are also inter-individual differences in occurrence and extent of statin intolerance and its symptoms. The knowledge of the risk factors predisposing for intolerance development including characteristic genetic background is crucial for its understanding and prevention. In this chapter we will review the polymorphic gene variants implicated in development of statin intolerance, briefly describe their biological plausibility and characterise clinical relevance.

## **2. Statin intolerance**

Statin intolerance is the inability to tolerate sufficient dose of statin needed to reduce cardiovascular risk due to side effects or intolerance to treatment [11]. The most frequent are muscle symptoms characterised below.

### **2.1. Statin-associated muscle symptoms**

Statin-induced muscle symptoms range from myalgia to mild or severe myopathy and even to rare rhabdomyolysis [12]. The symptoms appear in about 75% in the first 10–12 weeks and in 90% of cases in the first 6 months after treatment initiation or dose up titration [13]. The true frequency muscle related side effects has been widely debated: while an observational study reported as much as about 20% of patients on statins [14], clinical trial data suggests frequencies to be equal or lower than 5% [15], however there was a study reporting that clinical trials did not use a standard definition for statin myalgia [16], which may result in underestimated occurrence of statin-induced muscle symptoms. In any case, given very high usage of statins (the third most frequently prescribed drug), even lower relative frequency numbers would mean substantial absolute number of symptomatic patients.

### **2.2. Clinical-related risk factors**

The available data shows that the side effects of statin therapy are group-dependent, time-dependent and dose-dependent; their frequency is greater at a higher statin dose [17].

Endogenous factors known to increase occurrence of side effects are as follows: another lipid-lowering therapy, alcohol abuse, surgery, heavy exercise. Importantly, interactions with medication may be serious [18]; particularly drug interactions likely contribute the susceptibility to statin related muscle symptoms [19].

Further factors predisposing to statin intolerance are: advanced age (>70 year), female sex, race/ethnicity, family history of muscle disorders, vitamin D deficiency, history of creatine elevation, hepatic and renal impairment, hypothyroidism, low body mass index [20].

### **2.3. Genetic factors**

Besides the above characterised factors, genetic “make-up” of a given patient is important component in susceptibility to statin intolerance. Indeed, genetic variation represents the major factor responsible for inter-individual differences in patient responsiveness and their inclination towards undesirable side effects of statins.

### 3. Genes responsible for statin intolerance

The following section characterises the gene variants that have been implicated in mechanisms of statin intolerance represented by statin-induced myopathy. These are listed in the **Table 1**.

#### 3.1. *SLCO1B1* gene

*SLCO1B1* gene encodes the OATP1B1 (organic anion transporting polypeptide), which has been reported to regulate the hepatic uptakes of statins [27, 28]. Strong support for its nomination as a risk factor for statin intolerance came from the GWAS study which investigated genetic variation in 85 subject with myopathy and 90 controls, all taking 80 mg of simvastatin [21]: strong association was identified between statin-induced myopathy and single nucleotide polymorphism (SNP) rs4363657 located within the *SLCO1B1* gene. This noncoding SNP was in nearly complete linkage disequilibrium with the nonsynonymous rs4149056 SNP variant, which has been linked to statin metabolism: the odds ratio for myopathy was 4.5% per one copy of the C allele and 16.9% in CC homozygotes compared with homozygotes for standard allele (TT). More than 60% of observed myopathy cases could be attributed to this particular genetic variation, [21], which is also due to its relatively high population prevalence - rs4149056 C allele frequency is 15%.

#### 3.2. *LILRB5* gene

A potential role for immune system genetic variation in development of statin-induced myopathy has been recently reported for a variant in leukocyte immunoglobulin-like receptor subfamily-B, *LILRB5* gene (rs12975366:T > C:Asp247Gly) [25]. The missense variant Asp247Gly has been associated with serum creatine kinase (CK) levels; the mean levels of this enzyme were elevated in Asp247 homozygotes (TT). The *LILRB5* Asp247 homozygous genotype has, therefore, been associated with increased risk of statin intolerance [25]. No independent replication data on this plausible new variant has been available so far.

Gene	Chromosome	Allele	rs number	Coding variation	Study
<i>SLCO1B1</i>	12p12.2	*5 ≠	rs4149056	521 T > C	[21]
<i>CYP2D6</i>	22q13.1	*3	rs35742686	2549delA	[22]
		*4	rs3892097	splicing defect, G > A	
		*5		gene deletion	
<i>CYP3A4</i>	7q21.1	*1B	rs2740574	-392A > G transition	[23]
<i>GATM</i>	15q15.3	—	rs9806699	G > A, cis-e QTL	[24]
<i>LILRB5</i>	19q13.4	—	rs12975366	T > C: Asp247Gly	[25]
<i>COQ2</i>	4q21.22-q21.23	—	rs6335454	synonymous	[26]
		—	rs4693075	non-coding	

Note: rs, reference sequence; ≠ denotes haplotype (not allele) designation, see Section 4, second paragraph.

**Table 1.** Gene and their variants implicated in development of statin intolerance presented as statin myopathy.

### 3.3. *GATM* gene

Glycine amidinotransferase, *GATM* gene encodes a mitochondrial enzyme, which is involved in creatine biosynthesis. SNP rs9806699 within the *GATM* gene has been associated with statin induced myopathy, specifically minor allele A conferring a protective effect and reduced risk of myopathy [24]. However, as this result was not replicated [29], further investigations are required before a possible role for this variation in statin tolerance is clarified.

### 3.4. Family of cytochrome P450 genes

The cytochrome P450 family is a group of isoenzymes important for catalysing oxidation of xenobiotics. There is a wide spectrum of polymorphic variants affecting various pharmacogenetics aspects. Regarding cardiovascular setting, CYP gene variation plays role in warfarin and clopidogrel metabolism with clear clinical relevance (e.g. [30]). In context of statin adverse drug reaction, Mulder et al. [22] reported higher incidence of statin intolerance in the group of patients who carried two of the less effective *CYP2D6* \*3,\*4,\*5 alleles. Regarding another gene within cytochrome P450 system, namely *CYP3A5*, an association was observed between nonfunctional *CYP3A5*\*3 allele and the magnitude of CK elevation in case of patients experiencing myalgia during atorvastatin treatment [23]. Importantly, patients who develop myalgia while taking atorvastatin were more likely to experience a greater degree of muscle damage if they express two copies of *CYP3A5*\*3.

### 3.5. Other plausible gene variants

*COQ2* gene encodes Coenzyme Q2, involved in synthesis of ubiquinon (Coenzyme Q10, CoQ10), a redox carrier in the mitochondrial respiratory chain and a lipid-soluble antioxidant. Two variants within the *COQ2* gene (**Table 1**) have been associated with increased odds of statin intolerance, defined primarily through muscle symptomatology [26]. This observation has been subsequently replicated [31].

From other molecules functioning as drug transporters, *ABCB1* gene variation may also participate in development of statin muscle symptoms. This gene encodes the P-glycoprotein, an independent efflux pump. From its variants, the 1236 T, 2677non-G, and 3435 T alleles were less frequent in cases undergoing statin therapy than in the control group [32]. The authors also demonstrated a reduced T-non-G-T haplotype frequency (20.0%) in patients in whom myalgia developed during simvastatin treatment, as compared with the control, non-myalgia group (41.4%).

Most recently, a variant of a *UGT1* gene coding for uridine diphosphate glucuronosyltransferase, specifically *UGT1A1*\*28 variant allele (rs8175347), was reported to possess plausible protective effect in development of statin intolerance [33], however again this finding must be replicated.

In the following text we will concentrate on the *SLCO1B1* gene variation and describe its population distribution and clinical relevance. The reason for our focus is that to date, the rs4149056 *SLCO1B1* variant has been repeatedly evidenced to possess the strongest effect in response to statin therapy.

#### 4. Genetic variability and population distribution of *SLCO1B1*

More than 45 nonsynonymous variants in *SLCO1B1* gene have been identified [34]. Some of the variants have altered function [35]. Genotypic frequencies of *SLCO1B1* variants depend on ethnicity, and genetic difference between populations correlated with the geographical distances [34, 36, 37]. In particular, single nucleotide polymorphism the 521 T > C (rs4149056) appeared more commonly in European-Americans while it was less frequent in African-Americans. In opposite, single nucleotide polymorphism the 388A > G (rs2306283) was detected predominantly in African-Americans. Pasanen et al. [38] investigated the frequencies of 12 SNPs in *SLCO1B1* in 941 persons from 52 populations across Europe, Asia, Africa, Middle East, Oceania and the Americas (Amerindians).

*SLCO1B1* single nucleotide polymorphisms 521 T > C and 388A > G form four haplotypes: \*1A (388A/521 T), \*1B (388G/521 T), \*5 (388A/521 T) and \*15 (388G/521C) [38, 39]. The low activity haplotypes-\*5 (388A/521C) and \*15 (388G/521C) occur with combined haplotype frequency of approximately 15–20% in Europeans, 10–15% in Asians, 2% in sub-Saharan Africans. The \*1B (388G/521 T) haplotype occurs in approximately 26% Europeans, in 39–63% Asians and in 77% sub-Saharan Africans. The haplotypes \*5 and \*15 are associated with significant reductions of statin hepatic uptake [40], resulting in increase of systemic substrates exposure.

#### 5. Clinical relevance of the variation in the *SCLO1B1* gene

Clinical relevance of the *SCLO1B* variation is based on biological role of its gene products in hepatic transport of statins. Statins are mainly delivered within hepatocytes to their site of actions by uptake transporters and eliminated into the bile by efflux transporters [41]. Many statins are substrates of hepatic uptake transporters including OATP1B1, OATP2B1 and OATP1B3 [28] with OATB1B1 as the main one. The loss of function the *SLCO1B1*\*5 (Val174Ala, 521 T > C, rs4149056), located in exon 5, downregulates OATB1A1 transporter cell membrane and protein expression [42] which leads to decreased hepatic uptake, greater systemic statin plasma concentrations, and therefore greater muscle statin exposure, all these resulting in adverse effects [20, 36, 43–45].

Importantly, the impact of the rs4149056 variant on statin metabolism appears to differ between distinct statins. The effect of rs4149056 genotypes was much greater for simvastatin, less for atorvastatin and rosuvastatin in healthy volunteers [46, 47]: For simvastatin the area under curve, AUC (0-infinity) was increased by 221% in genotype CC individuals in compared with wild-type TT individuals. For atorvastatin this parameter was increased by 145% and for rosuvastatin by 62%. Individuals carrying C allele also reached maximum concentration (C<sub>max</sub>) earlier, and its value was 200% higher compared with TT individuals of rs4149056 [47]. Further, the rs4149056 polymorphism was significantly associated with simvastatin treatment cases of severe statin induced myopathy, which did not occur after atorvastatin [45] or pravastatin [48] treatment. Similar conclusions regarding simvastatin

Study	Simvastatin dose	Outcome	Reference
SEARCH	80 mg	OR 4.5 per C allele OR 16.9 for CC homozygotes	[21]
Heart protection study	40 mg	OR 2.6 per C allele	[21]
Brunham	10-80 mg	OR 2.3 per C allele	[45]

Note: OR, odds ratio.

**Table 2.** Basic studies that have reported a strong association between the rs4149056 single nucleotide polymorphism and simvastatin induced myopathy.

were obtained in animal model, again for simvastatin however not for pravastatin or atorvastatin [49]. **Table 2** lists main studies investigating effect of simvastatin on statin intolerance/myopathy in humans.

It is crucial that in agreement with the rules for performing association studies [50] the data obtained in the SEARCH study has been independently replicated within the Heart Protection Study, in 10,000 patients who received 40 mg Simvastatin [21]. The meaningful data obtained in this way provided starting point for reflection of the observations from genetic and pharmacokinetic studies into clinical practice, including formulation of treatment recommendations which will be subject of the next section of our chapter.

## 6. Testing for statin intolerance in clinic—current status and treatment recommendations

The spectrum of evidence supporting the association between the lead SNP rs4149056 and statin, namely simvastatin-induced myopathy prompted application of the *SLCO1B1* genotyping for clinical usage. This translation to diagnostics aims mainly at reducing risk of simvastatin induced muscle toxicity and at increased adherence to therapy [43, 51, 52]. Another possible outcome of genotyping is the option to use alternate agents of the statin class.

In clinical practice, the adverse effect of *SLCO1B1* polymorphism depends on the genotype (being highest in homozygotes), statin dose and statin type. This has been reflected in the guidance for prescribers provided primarily by the Clinical Pharmacogenetics Implementation Consortium (CPIC); the working group produces guidelines for simvastatin use in individual carriers risk allele in *SLCO1B1* gene [43]; the guidelines have been recently updated [53]. In patients with one or two copies of *SLCO1B1* rs4149056 C allele, simvastatin should be avoided or reduced dosage should be considered, pravastatin or rosuvastatin are preferred alternatives according to the CPIC guidelines, however other clinical and patients specific factors should be taken into account [43, 53].

Apart from this general, non-compulsory guidance, there have been more systematic efforts to apply *SLCO1B1* genotyping into practice. This direction is represented e.g. by pre-emptive programs performed from the initiative by U.S. Pharmacogenetics Research Network at eight sites [54]. Similarly, aiming of introducing genotype-guided prescribing, pre-emptive genotyping has



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KEY MESSAGE

- Statins are the basis of dyslipidemia treatment in patients with increased cardiovascular risk.
  - Genetic factors may predispose to statin intolerance.
  - *SLCO1B1* gene variation has strong association with statin-induced myopathy.
  - Guidance for prescribers reflects individual risk for *SLCO1B1* rs4149056 C allele carriers.
  - Investigations of clinical relevance of other plausible gene variants are ongoing.
- 

also been investigated in Europe by the EU Horizon 2020-funded Ubiquitous Pharmacogenomics (U-PGx) Consortium (<http://upgx.eu>) in seven European countries [55]. Last but not least, recommendations were formulated also on a national level - in France: the French National Network of Pharmacogenetics (RNPGx) [56] is in favour of rs4149056 testing before starting therapy or early after treatment onset in patients with one or more risk factors. If the genotype is not known early, the RNPGx considers that a polymorphism test is potentially useful also in the event of already occurring muscle toxicity in patients treated with statins, in order to rule out or confirm a genetic cause. From the above examples it is clear that pharmacogenetic genotyping for prediction/confirmation of statin intolerance undergoes ongoing development and progress; further updates of the recommendations are expected. It should be noted that there have been opinions as well that the current status of knowledge has not been yet sufficient to allow clinical application of genotyping for risk of statin intolerance [52]. There have been several arguments, however especially those economical (“the tests are too costly”) are not substantiated; some “con” opinions have been also “traditionalistic”, from conservative point of view on doubting any new test or medical management measure including pharmacogenetics. However, this reluctant or at least “sceptical” attitude about pharmacogenetic contribution to routine statin usage, well known also from other applications of pharmacogenetics, has been gradually changing—it only takes time, systematic information on the evidence and particularly education to overcome it [57].

## 7. Future perspectives including economic aspects of genetic test for statin intolerance

Implementation of a genetic test for statin intolerance into routine practice definitely requires analysing its benefits not only for patients but also for health care providers. In this context, pharmaco-economic data on genetic testing statin intolerance have been scarce. The existing literature on cost-effectiveness of pharmacogenetic testing has been either general [58] or described economic savings solely due to hypolipidemic effect of statins [59]. The first specific data for statin intolerance and its genetic testing appeared only very recently [60]—the authors estimated 356 Canadian dollars as the cost limit for economic feasibility and at the same time dominant health effect for cardiovascular prevention. In extension of this very first report [60], this topic should be, therefore, addressed more intensively and also from other angles in the future. This has been the case with other pharmacogenetic applications (e.g. [61]), it will be also innovative to use new approaches which utilise alternative parameters for assessing effectiveness (e.g. [62–64]).

Though important, inclusion of economic criteria is the only one part of the future priorities in the field of application of genetic variation for testing statin intolerance. Other avenues for future may address (1) further search for and verification of other genetic markers than *SLCO1B1* including providing pharmacokinetic data [65, 66], (2) reflection of ethnic differences in distribution of genetic markers between populations [64, 67], (3) inclusion of the results of genetic test into electronic medical records [68], (4) performing meta-analyses of studies reported so far, and last but not least, (5) performance of well-designed clinical studies implementing also other non-genetic criteria in order to propose a risk-score or clinically applicable algorithm. The existing examples from other pharmacogenetic applications (e.g. [69]) and above described initiatives such as U-PGX [55], RNPGX [56], or the recent idea to provide patients with their DNA (pharmacogenetic) passport [70], allow us to expect further developments targeted at patient benefit and innovation of medical care.

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## Conflict of interest

The authors do not report any conflict of interest.

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# Immunopharmacogenomics in Cancer Management

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## Abstract

With the unavoidable progress of genomics technologies, “one size fits all” strategy has switched to individual-specific treatment approaches. Hence pharmacogenomics-based personalized cancer medicine has emerged. Promising treatment option immunotherapy includes either “take the brakes off immune system (i.e., checkpoint blockade therapy) or the use of immune cells expanded in an in vitro tumor-free environment”. Both options have been varied and included unpredictable results. Combination of cancer immunotherapy and pharmacogenomics applications may contribute to solve the complexity of outcome prediction and variations between individuals receiving the same immunotherapeutic treatment. To enhance the tumor immunity and determine cancer patients who response to immunotherapy, classification based on gene polymorphisms in key immunoregulatory molecules including antigen-presenting molecules, immunoglobulins and their receptors, cytokine/chemokines and their receptors, adhesion and costimulatory molecules, toll-like receptors, and intracellular signaling molecules plays a vital role in redirecting or modulating the function of immune cells. Therefore, polymorphisms in immunoregulatory molecules and their impact on immunotherapeutic outcome should be considered in cancer management.

**Keywords:** cancer, immunopharmacogenomics, personalized immunotherapy, polymorphisms

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## 1. Introduction

The host immune system has a crucial role on the pathogenesis of many diseases, including cancer. Tumorigenesis depends not only on cellular biology but also on immune responses. The tumor development and progression are associated with the interaction between the immune system and malignant cells. The effect of immune responses has been counted on for both cancer immunotherapies and a broad range of cytotoxic agents as well as molecular

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targeted drugs. With the accumulation of information about the immune responses in tumor immune surveillance, the fame of cancer immunotherapy is increasing. Some cancer immunotherapies, such as monoclonal antibodies, cytokines, cancer vaccines, and cell-based therapies, have been developed and incorporated into clinical practice for the activation of the host immune response to eliminate cancer cells [1, 2].

## 2. Importance of personalized cancer therapy on immunotherapy

With characterization of alterations on immunity-related molecules through deep sequencing tools in the genomic era, immunotherapies are revolutionizing cancer treatment. Natural variations in nucleic acid sequence named as gene polymorphisms exist in general population with a high frequency and include no side effect for the people. Single nucleotide polymorphisms (SNPs) are the most common polymorphism in population [3]. SNPs can be also recognized as biological markers to identify genes that are associated with diseases. It has been displayed that some SNPs have effects on individual's response to some drugs. Therefore research groups are focused on the investigation of how SNPs in the human genome correlate with drug response to get more successful therapy regimes in personalized medicine era [4]. At this point, immunopharmacogenomics that incorporates immunogenomics and pharmacogenomics aims to improve a better approach for how the immune system impacts to the immunotherapy response.

### 2.1. PD-1/PD-L1 polymorphisms

The blockade of immune checkpoints modulating immune responses has recently been emerged as an immune therapy against cancer. Different immune checkpoint molecules such as programmed cell death 1 receptor (PD-1), an immune checkpoint receptor on immune cells (especially on T lymphocytes); and programmed death-1 ligand-1 (PD-L1) on tumor cells or tumor-infiltrating immune cells, have been associated with tumor immune evasion [5]. Interaction of PD-1 with PD-L1 starts antitumor immune response suppression; therefore blockade of PD-1/PD-L1 binding has been recently applied for antitumor immune therapy [6]. PD-1 is expressed on some immune cells, including T cells, natural killer T (NKT) cells, mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells, some dendritic cells, B cells, lymph node, and bone marrow cells [4]. PD-1 is commonly expressed immunoinhibitory receptor that belongs to CD28/B7 family, and it is expressed on some immune cells, including T cells, natural killer T (NKT) cells, mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells, some dendritic cells, B cells, lymph node, and bone marrow cells. PD-1 has two opposite roles such as downregulation of ineffective immune responses and expansion of malignant cells by preventing of protective antitumor immune responses [7]. PD-1 has function on the inhibition of T-cell activation, production, and survival [4]. PD-L1, ligand of PD-1, is a member of the B7 family of immune-regulatory ligands. It is expressed on functioning active T cells, B cells, dendritic cells, and macrophages (antigen-presenting cells) and activates various tissue groups by inflammatory cytokines [7]. PD-L1 has two different forms as membrane bound and soluble form. Both of these forms have been found on CD28/B7 family such as CTLA-4, CD28, and B7-H4 [8].

PD-L1 has role on the negative regulation of immunological response. PD-L1 overexpression can prevent to form antitumor immune responses against cancer cells; hence increased expression of PD-L1 on tumor cells may be predictive for a blockade of the PD-L1/PD-1 binding [9]. Some studies have shown that PD-L1 is also expressed on many types of cancer cells (including melanoma, lung cancer), and it has been reported that expression of PD-L1 ligands by cancer cells results in the evasion of the immune system. They stop the production of tumor-specific T cells by propagation of inhibitory signals which cause damaged antitumor immunity [7, 10]. Based on their function on antitumor immune response suppression, PD-1 and PD-L1 can be considered as powerful biomarkers for new tumor formation or progression of cancer. In literature some studies are focused on their role on genetic susceptibility, and some of them are focused on their prognostic or predictive significance. In literature some polymorphisms on PD-1 which is encoded by *PDCD-1* gene (Gene bank ID: 5153) on chromosome 2q37 location are associated with tumor susceptibility. *PD-1.1* polymorphism (dbSNP reference cluster ID rs36084323, c.-606G > A), located in the promoter region, has been accepted as a risk factor for non-small cell lung cancer and breast cancer [11, 12]. *PD-1.3* (rs11568821, c.627 + 189G > C,) located in intron 4 has been associated with colon, thyroid, and breast cancer [13–15]. *PD-1.5* (rs2227981, c.804 T > C), located in exon 5, is associated with increased cancer risks in non-small cell lung, cervical, colon, and ovarian cancer [4, 16]. A recent study showed that *PD-1.9* (rs2227982, c.644C > T), located in exon 5, has significance on the gastric cancers [17]. *PD-L1* gene (Gene bank ID: 29126) polymorphisms (rs2890658, c.683-369C > A, and rs10815225, c.-114C > A) might serve as risk markers for esophageal squamous cell carcinoma and gastric cancer [18, 19]. Blockade of PD-1/PD-L1 pathway with specific anti-PD-1 treatments such as nivolumab, lambrolizumab, pidilizumab, BMS-93659 (MDX-1105), RG7446/MPDL3280, and MEDI4736 (B7-H1) has been tested in different cancers including ovarian, colorectal, bladder, and non-small cell lung cancer and melanoma. Even though responses vary in different tumor types, anti-PD-1 treatments are supported. Responses not only vary in different tumor types; some patients show clear responses to blockade of PD-1/PD-L1 pathway, whereas they do not respond [4]. Currently, it is difficult to predict treatment responses to anti-PD-1 treatments. Expression of PD-L1 is not an optimal biomarker, because determination of PD-L1 expression by immunohistochemistry is not always proper due to the different antibody selection and cutoff levels. The PD-L1 expression may not anticipate the therapeutic responses because the PD-1/PD-L1 interactions on B cells have role on adaptive and innate immune systems. Nowadays, researchers are focused on the potential impact of single nucleotide polymorphisms as predictive therapeutic markers and their improvement capacity of the expression markers [20]. In literature, there are some studies about the effect of *PD-1/PD-L1* SNPs on non-small-cell lung cancer (NSCLC) treatment [20–22]. Nomizo et al. evaluated the association between *PD-1/PD-L1* SNPs and response to nivolumab, PD-1 immune checkpoint inhibitor, and survival in NSCLC. Five SNPs in *PD-L1* (rs1411262, c.394 + 1999C > A; rs2282055; c.-14–368 > G; rs4143815; c.\*395G > C; rs2890658; c.683-369C > A; rs822339, c.-15 + 2576A > G) and two SNPs in *PD-1* (rs2227981; c.804 T > C; rs2227982, c.644C > T) were investigated by using real-time polymerase chain reaction (RT-PCR). The G allele of rs2282055 and the C allele of rs4143815 in *PD-L1* were significantly associated with better clinical response than T or G allele, respectively [20]. In a study conducted by Do et al., SNPs involved in immune checkpoints were used to predict the clinical outcomes of patients with advanced-stage NSCLC after paclitaxel-cisplatin chemotherapy. The SNP rs2297136, c.\*93G > A

in PD-L1 gene, was significantly associated with better chemotherapy response and better overall survival, and rs4143815 in PD-L1 was also found as associated with better response to paclitaxel-cisplatin chemotherapy [21]. *PD-L1* polymorphisms, especially rs4143815, may be promising markers for the prediction of clinical outcome of chemotherapy. However further studies with larger patient groups are needed to validate the results from different research groups, and also more studies should be designed to figure out PD-L1 in chemotherapy responses of different cancers.

## 2.2. CTLA4 polymorphisms

After the determination and characterization of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) as a key negative regulator in immune response (broadly named as checkpoint molecule), studies have increased to develop cancer immunotherapy targeting this co-inhibitory molecule. CTLA-4 (CD152) is a member of immunoglobulin (Ig) superfamily and functions for downregulation of T-cell activation. CTLA-4 expressed by activated T cells binds to B7.1 (CD80) and B7.2 (CD86) on antigen-presenting cells and transmits an inhibitory signal to T cells, so it can restrict the density and extent of the immune responses. The complex formed from CTLA-4 and B7 proteins (B7.1 and B7.2) can switch activated T cells into inhibitory T cells. This change promotes tumor escape from immunosurveillance [23]. Although lymphoproliferative disorders and severe autoimmune diseases have been shown in CTLA4-knockout mice; CTLA-4 blockade can boost immune responses in tumor-transplanted mice, as well as extending antitumor immune responses and rejection of tumors [24, 25]. It has been shown that blocking of CTLA-4, namely, downregulation of T-cell activation, can lead the cancer regression in patients with different cancers due to the enhancing immune responses as well as antitumor activity [23, 26]. However, not all patients can benefit from the treatment of CTLA-4 blockade, and some of them developed severe autoimmune reactions. The mechanisms of this interindividual variability in response to immunotherapy are not well understood.

Some *CTLA-4* polymorphisms have been investigated with the determination of genetic factors that may change the response and toxicity of CTLA-4 antibody therapy [27]. Human *CTLA-4* gene with four exons is located on chromosome 2q33, and different functional subunits of CTLA-4 protein such as a leader sequence, an extracellular, a transmembrane, and a cytoplasmic domain are encoding [28]. Many SNPs have been identified in the CTLA-4 gene that contains three exons and two introns. To date, at least five SNPs that were well studied in immune diseases have been investigated in cancer association studies. Among them, the rs5742909, c. - 318C > T located in the promoter region; rs231775, c.49A > G located in exon 1; and rs3087243, c.6230A > G (CT60) located in 3'-untranslated region (3'UTR) have attracted more attention. There are more than 100 SNPs in *CTLA-4* gene, and most of them are spotted on three exons and two introns of CTLA-4 gene. Until now, at least five attracted SNPs have been associated in cancer-related studies, such as rs11571316, c. - 1577G > A in the 5'-untranslated region (5'UTR); rs5742909, c. - 318C > T in the promoter region; rs231775, c.49A > G in exon 1; and rs3087243, c.6230A > G (CT60) in 3'-untranslated region (3'UTR). These alterations change the expression or functional activity of CTLA-4 protein [29]. Some common SNPs have been reported that they alter either expression or function of the *CTLA-4* gene product, as well as responses to immunotherapy with anti-CTLA-4 antibodies. A study conducted in melanoma

by Breunis et al. has shown that G allele of rs4553808, c.-1660A > G; T allele of rs11571317, c.-657C > T; and A allele rs231775, c.49A > G, are significantly associated with response to anti-CTLA-4 antibody (MDX-010). SNPs rs4553808 and rs11571317 are spotted on the promoter region; and rs231775 is on the exon 1 and causes p.Thr17Ala substitution [27]. In another study performed by Queirolo P. et al. in metastatic melanoma patients, rs11571316, c. - 1577G > A in the 5'UTR, and rs3087243, c.6230A > G (CT60) in the 3'UTR of the gene, were associated with best overall response to ipilimumab which is a CTLA-4 immune checkpoint blocker [30]. In lung cancer-based study, which is performed by Song B et al., patients with the A allele had a significantly shorter survival time than those with the G allele (p value <0.001; survival times: AA genotype 9.8 months, GA genotype 12.0 months, GG genotype 12.5 months) [31]. With the presented studies above, it can be said that several SNPs in *CTLA-4* are biologically functional and might be predictive factors for CTLA-4 antibody therapy used for cancers.

### 2.3. CCR5 polymorphisms

Pro-inflammatory cytokines, chemokines and other protein-structured molecules are secreted from cancerous cells and their stromal environment. Chemokine family belongs to chemotactic cytokines and has 44 members. Chemokines have role in homeostasis and immunity by controlling the leukocyte trafficking and induction, so they have role in organogenesis, cell growth, and differentiation. Tumor-related chemokines have important role in cancer biology, in terms of leukocyte infiltration, angiogenesis, and immune evasion. Based on the number and spacing of conserved cysteine residues on their N-terminus, chemokines consist of four subgroups named as CX3C, CXC, CC, and C [32, 33]. Chemokines act through their interactions with specific chemokine receptors located on the cell surface. Most of the cancerous cells overexpress these specific chemokine receptors which have role together with chemokines in cellular survival, proliferation, migration, invasion, and metastasis. In humans, the chemokine receptor family includes 24 members, and they are divided into four subfamilies (CX3CR, CXCR, CCR, and CR) based on the class of chemokines that they interact. Specific ligand-receptor binding induces GDP to GTP exchange, and this activates the downstream signaling effectors for the propagation of cellular pathways [34, 35].

It is considered that chemokine receptors are promising targets for new immunotherapies since the detection of overexpressed chemokine receptors on the surface of cancer cells [36]. Systemic administration of interleukin-2 and checkpoint inhibitors increases the secretion of chemoattractants such as ligands of CCR5 and CXCR. Therefore polymorphisms on CCR5 and CXCR chemokine receptors affect the migration capability of tumor-infiltrating lymphocytes to the tumor location [37]. So response rates to immunotherapy vary among patients. Ugural et al. showed that genotypic status of CCR5 chemokine receptor has an impact on the immunotherapy (interferons or interleukin-2) responses in patients with metastatic melanoma. A strong association was determined between the *CCR5*Δ32 genotype and overall survival as poor prognostic factor. *CCR5*Δ32 polymorphism (rs333, c.554-585del32) consists of 32 bp deletion. Heterozygosity causes decreased levels of receptor expression, and homozygosity causes the absence of expression. Immunotherapy outcome was worse in metastatic melanoma patients who had *CCR5*Δ32 genotype as homozygotes or heterozygotes [38]. Above observation of Ugurel et al. clashes with findings of study performed by Bedognetti et al.

The presence of *CCR5*Δ32 polymorphism was not correlated with worse response in metastatic melanoma patients undergoing adoptive therapy [39]. Conflicting studies exist on the effect of *CCR5*Δ32 polymorphism on immunotherapy or immunochemotherapy. Hamid et al. did not report any significant relationship between the existence of *CCR5*Δ32 genotype or rs1799987, c.-301 + 246A > G, polymorphism and responsiveness to ipilimumab [40].

#### 2.4. KIR, HLA, and Fcγ polymorphisms

Natural killer (NK) cells, large granular lymphocytes, have role on the early innate immune response. In contrast to T cells, which remember foreign antigens through T-cell receptors in the context of major histocompatibility complex (MHC) molecules, NK cells are programmed to eliminate infected or transformed cells. Activation of NK cells, known as “missing-self” model due to the absence of MHC molecules depends on the numerous signals through their respective activating or inhibitory receptors [41]. The *killer immunoglobulin-like receptor (KIR)* gene encodes both activating and inhibitory NK-cell surface receptors. *HLA class I* gene encodes ligands of inhibitory KIR (HLA-C1 for KIR2DL2/3, HLA-C2 for KIR2DL1, HLABw4 for KIR3DL1), and the interaction between KIR and HLA class I ligands leads to NK inhibition. NK cells also express Fc receptors [42]. The interplay between KIR and HLA is crucial for positive outcomes of immune-related therapies. KIR/KIR-ligand mismatch is associated with improved outcome to immune-related therapies as well as autologous stem cell transplantation [42].

Delgado et al. reported that KIR receptor-ligand mismatch was related with response or improvement of relapsed or refractory neuroblastoma patients receiving interleukin-2-based treatment, consistent with a role for NK cells in this clinical response [43].

There are three fragment c gamma receptor (FcγR) classes such as (i) FcγRI, capable of high-affinity binding monomeric IgG, (ii) FcγRII with low-affinity binding, and (iii) FcγRIII interaction with complexed IgG. FcγRII and FcγRIII have variants with different binding affinity immune complexes such as FcγRIIa (131H/R) and FcγRIIIa (Val158Phe) [44].

Several groups have investigated the role of *FcγR* polymorphisms in the response of monoclonal antibodies (mAbs) such as rituximab, cetuximab, and trastuzumab. A correlation was reported between FcγRIIa polymorphisms and complete response in rituximab-based regimen received by non-Hodgkin lymphoma patients [44]. In another study performed to understand the influence of the *FCGR3A* gene polymorphism on rituximab response of non-Hodgkin lymphoma patients, again a correlation was found between *FCGR3A* genotype and clinical response [45]. In a preliminary study, *FCGR2A* and *FCGR3A* polymorphisms were shown as useful markers to predict clinical outcome in metastatic colorectal cancer patients treated with cetuximab, a chimeric immunoglobulin G1 (IgG1) monoclonal antibody (mAb) against epidermal growth factor receptor (EGFR) [46]. Musolino et al. reported a correlation between *FCGR3A* and objective response rate and progression-free survival in patients with HER-2/neu-amplified breast cancer receiving trastuzumab. Also an association was determined between the combination of *FCGR2A* and *FCGR3A* and better objective response rate and progression-free survival [47]. Cheung et al. reported an association between *FcγR2A* polymorphism and progression-free survival in the response of neuroblastoma patients to the anti-GD2 antibody [48]. As a result, KIR-HLA immunogenetics can give useful information



about the activation of innate immunity, and combining KIR-HLA genotyping with other molecular markers such as Fc $\gamma$  receptor polymorphisms that can give insight about immune responsiveness may allow clinicians to determine the most effective and least toxic personalized immunotherapy.

### 3. Conclusion and future aspects

Immunogenomics uses advance genomic analysis tools to distinguish the limitations of the immune system, and pharmacogenomics identifies the variability of pharmacologic responses based on individual's genetic/germline variations. Integration of both immunogenomics and pharmacogenomics forms immunopharmacogenomics to revolutionize the immunotherapy applications through the identification of genetic status of immunoregulatory molecules. This approach could be used to develop a better understanding for immunologic reactions, select patients for immunotherapy, and predict the side effects and response to anticancer treatment (not only immunotherapy but also chemo/radiation therapy). Although the immunopharmacogenetic applications are limited in clinical practice, it is clear that immunopharmacogenomics will become an important approach of cancer management in immunotherapy era.

### Conflict of interest

Authors declare no conflict of interest.

### Author details

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Polymorphism or variation in DNA sequence can affect individual phenotypes such as color of skin or eyes, susceptibility to diseases, and response to drugs, vaccines, chemicals, and pathogens. Especially, the interfaces between genetics, disease susceptibility, and pharmacogenomics have recently been the subject of intense research activity. This book is a self-contained collection of valuable scholarly papers related to genetic diversity and disease susceptibility, pharmacogenomics, ongoing advances in technology, and analytic methods in this field. The book contains nine chapters that cover the three main topics of genetic polymorphism, genetic diversity, and disease susceptibility and pharmacogenomics. Hence, this book is particularly useful to academics, scientists, physicians, pharmacists, practicing researchers, and postgraduate students whose work relates to genetic polymorphisms.

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