Genotyping of Single Nucleotide Polymorphisms

Tian Ye, Ran Tong, and Zhiqiang Gao

Contents

Abstract Single nucleotide polymorphisms (SNPs) are the most abundant DNA variations in human genome. Numerous reports have indicated that SNPs are closely associated with diseases such as cardiovascular diseases, different types of cancer, and other genetic diseases. As such, SNPs are currently considered as potentially important cancer biomarkers that may significantly improve cancer diagnosis and prognosis, aid drug development, and offer personalized treatments for cancer patients. The importance of SNPs, especially on the genetic diseases, has urged researchers to develop SNP detection methods for their sensitive and accurate identification in the presence of excessive wild-type (WT) genes. Unlike other DNA assays, the intrinsically subtle difference between WT and mutant genes—a single-base variation—makes it a challenging task to specifically detect low abundant SNPs out of large amounts of coexisting WT genes. To date, many methods have been proposed for SNP detection, and they can be classified into two categories—allele-specific hybridization methods and allele-specific enzymatic methods. A thorough review of the state-of-the-art detection technologies for SNPs will be helpful to researchers in the development of more efficient SNP genotyping technologies. In this article, recent advances in the detection and genotyping of SNPs are summarized, and emerging techniques for SNP genotyping are assessed.

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1 Introduction

Human Genome Project successfully sequenced the whole human genome of some volunteers (Collins et al. 2003; Venter et al. 2001). During sequencing, a large number of single nucleotide polymorphisms (SNPs) were discovered together with other types of variations (Sachidanandam et al. 2001). Among all kinds of DNA variations, SNPs have the largest amount (Durbin et al. 2010). SNPs are defined as single base pair positions where variable sequences are available (Brookes 1999). The frequency of the variation was estimated to be at least 1 % of the whole allele frequency. The existence of SNPs inspired further projects on the determination of SNPs throughout the whole genome, of which the HapMap and 1,000 Genomes Project are representatives (Frazer et al. 2007; Siva 2008). The 1,000 Genomes Project successfully detected 1.5 million SNPs within populations of human beings, and the phase II of the HapMap project characterized 3.1 million SNPs. By the construction of databases, the data are accessible through the Internet, of which the representative databases are National Center for Biotechnology Information (Pruitt et al. 2005), HapMap (Thorisson et al. 2005), and The Human Genome Variation Database (HGVbase) (Fredman et al. 2004).

The importance of SNPs has many aspects, especially on the pathogenic genetic diseases. It is proved that SNPs are related to diseases such as cardiovascular diseases, different kinds of cancer, and other genetic diseases (Pecoits-Filho et al. 2003; Paynter et al. 2009; Wallace et al. 2008; Onay et al. 2006; Dumur et al. 2003; Tuupanen et al. 2009; Zacharova et al. 2005). The discovery of the relationship between typical SNPs and diseases triggered the improvement of SNP genotyping method. Ideal SNP detection and genotyping should have the following features: rapid and robust sequencing, high automation, promising accuracy, low cost, and so on (Kwok 2001). Based on the knowledge of genotyping, mainly two categories of SNP assays are developed, which are allele-specific hybridization method and allele-specific enzymatic method (Syvanen 2001). Allele-specific enzymatic method is further divided into three branches, namely, the technology based on ligation, primer extension, and enzymatic cleavage (Myakishev et al. 2001; Chen et al. 1998; Nollau and Wagener 1997). In this article, the two categories of SNP genotyping methods are reviewed with typical examples listed in each method, followed by a brief summary.

2 Genotyping of Single Nucleotide Polymorphisms

2.1 Allele-Specific Enzymatic Ligation-Based Genotyping of Single Nucleotide Polymorphisms

The application of ligase to SNP genotyping was first introduced more than 25 years ago (Landegren et al. 1988), after which the importance of enzymatic reaction to SNP detection was gradually noticed by other researchers. The principle of the assay lies in the ligation of two adjacent oligonucleotides which are connected at or near the site of SNPs. After a 30-year development, the ligation-based SNP discrimination has well characterized, and many methods are developed based on different amplification methods, such as polymerase chain reaction (PCR), ligase chain reaction (LCR), and rolling circle amplification (RCA), all of which can significantly increase the sensitivity and accuracy of the results (Lizardi et al. 1998; Wee et al. 2012; Duan et al. 2009). Some ligation-based methods are introduced in the following two sections.

2.1.1 Magnetic Bead-Based Genotyping of Single Nucleotide Polymorphisms

In allele-specific ligation detection, the application of magnetic beads can effectively minimize the noise generated by unreacted reagents, which can further increase the detection power of the assay (Fig. 1) (Shin et al. 2014). By applying such an idea, a multiplexed SNP genotyping method utilizing universal amplification of separated ligation-dependent probes was developed—amplified sequence length polymorphism (ASLP) assay (Shin et al. 2014). Ligation of allele-specific probes, wild-type allele-specific oligonucleotides (ASOW) and mutant allele-specific oligonucleotides (ASOM) with locus-specific probe (LSO) was executed first by DNA ligase, the separation probes, functionalized with biotin, were extended using the ligation product as templates. The double-stranded DNA formed in the previous steps was separated by streptavidin-coated magnetic beads, which were further amplified by PCR, introduced to microarray, and detected. By such an assay, 15 SNPs were discriminated simultaneously.

Another method of separating reacted and unreacted probes by magnetic beads can be achieved by the incorporation of ultrasmall magnetic nanoparticles into oligonucleotides, leveraged on the size-dependent magnetic properties of ferrofluidic nanoparticle probes (FNPs) in ligation reaction to detect SNPs

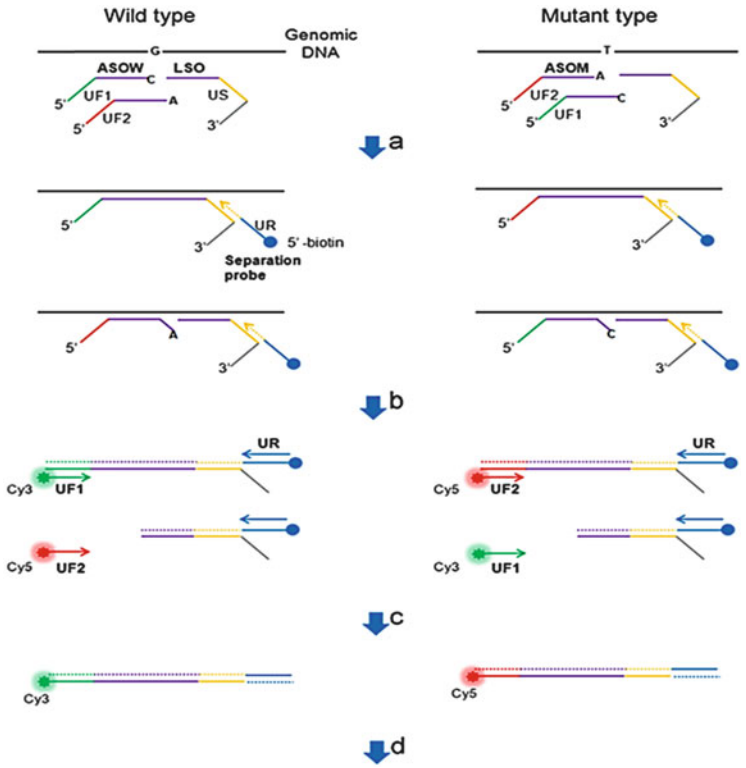

Fig. 1 ASLP technology for multiplexed SNP genotyping (reproduced with permission from Shin et al. 2014)

(Fig. 2) (Shen et al. 2013c). Ligation of two FNP-modified oligonucleotides (FNP-1 and FNP-2) by LCR with perfectly matched target was carried out, after which the aggregated product facilitated by FNP made the reacted nanoparticles easily separated by an external magnetic field. Colorimetric detection, thereafter, could discriminate the SNP by reflecting the concentration of remaining FNPs. In another study, the catalytic properties of horseradish peroxidase and the magnetic separation of magnetic beads were utilized to discriminate SNPs with 1,000-fold excess of wild-type genes (Fig. 3) (Chen et al. 2012). The limit of detection was as low as 1 fM after LCR amplification.

2.1.2 Gold Nanoparticle-Based Genotyping of Single Nucleotide Polymorphisms

Gold nanoparticles (AuNPs) have attracted special attention in SNP detection technology because of their unique optical properties, of which not only the fluorescence but also the quenching of the fluorescence of gold nanoparticles is

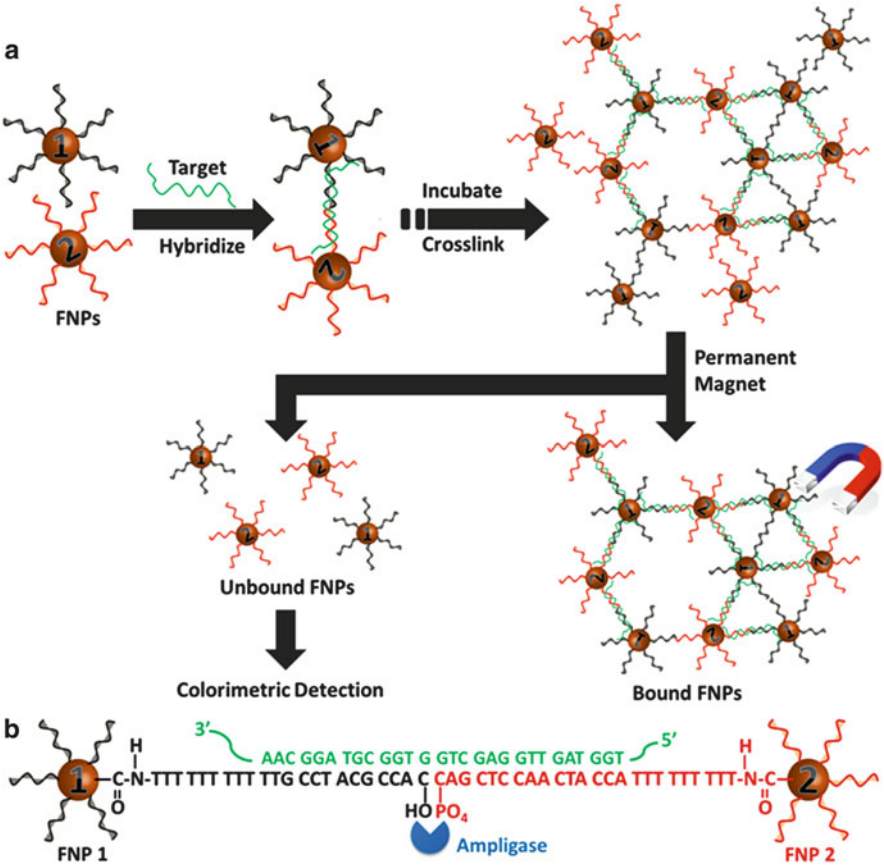

Fig. 2 Principle of the SNP detection assay by FNPs (reproduced with permission from Shen et al. 2013c)

interesting (Wang et al. 2010; Zu et al. 2011). The first report on the colorimetric detection of DNA sequences using nonfunctionalized AuNPs appeared in 2004 (Li and Rothberg 2004). Some selected methods based on the cooperation of AuNPs and allele-specific ligation SNP genotyping are elaborated.

An SNP discrimination chip-based assay applying the optical properties of AuNPs, which is enhanced by silver and the ligation reaction, has been developed (Fig. 4) (Xue et al. 2009). When the perfectly matched target was introduced, capture strand on the chip and the probe on the AuNPs were ligated. Discrimination by the naked eye or flatbed scanner can be executed after a silver attachment and non-stringency wash. One interesting feature of the method is that not only can it detect the SNP at the designed site, but also the discrimination of SNP on the close to the ligation position is still practical.

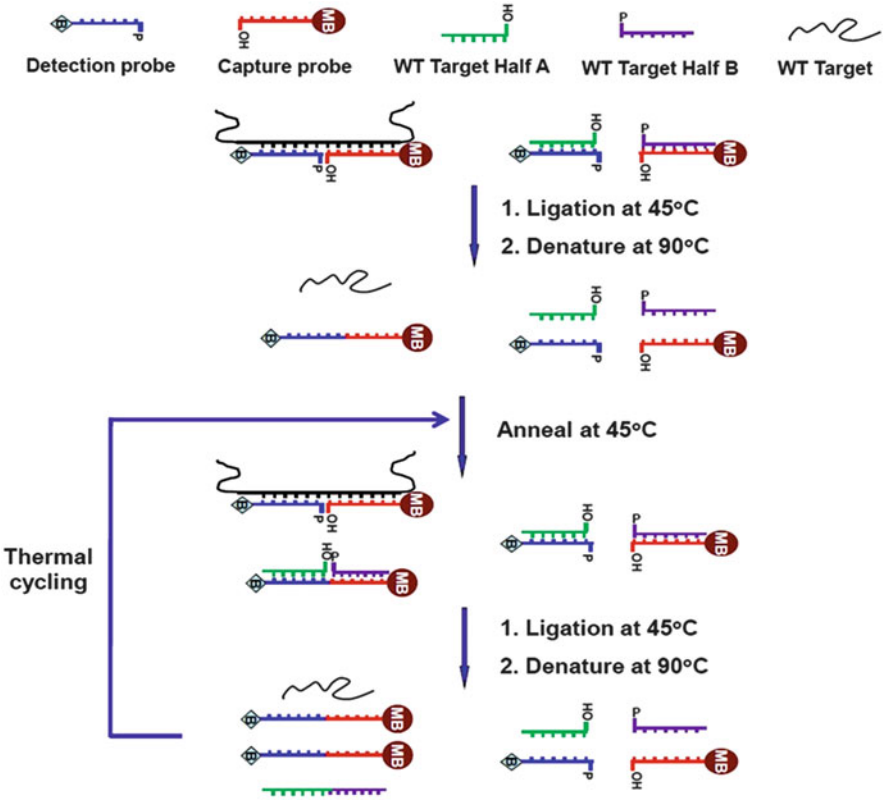

Fig. 3 SNP genotyping assay using magnetic beads (reproduced with permission from Chen et al. 2012)

The detection of SNP with real-time PCR sensitivity utilizing LCR and AuNPs has been developed (Fig. 5) (Shen et al. 2012a, b). The method was controlled by thermal cycle. The hybridization and ligation of the target with two types of AuNP-modified probes happened before raising the denaturing temperature to separate the target and the ligated strand, of which both were templates for the next round LCR reaction. Exponential amplification of ligation product was achieved after the first round of LCR. The irreversible change of the color of the solution from red to gray confirmed the existence of perfectly matched target, whereas the unchanged color indicated the existence of mismatched target. The limit of detection was expected to be 20 aM by theoretical modeling from experimental data (Shen et al. 2012b). Selectivity factor was 1,500 in the detection of wild-type KRAS genes from mutant type.

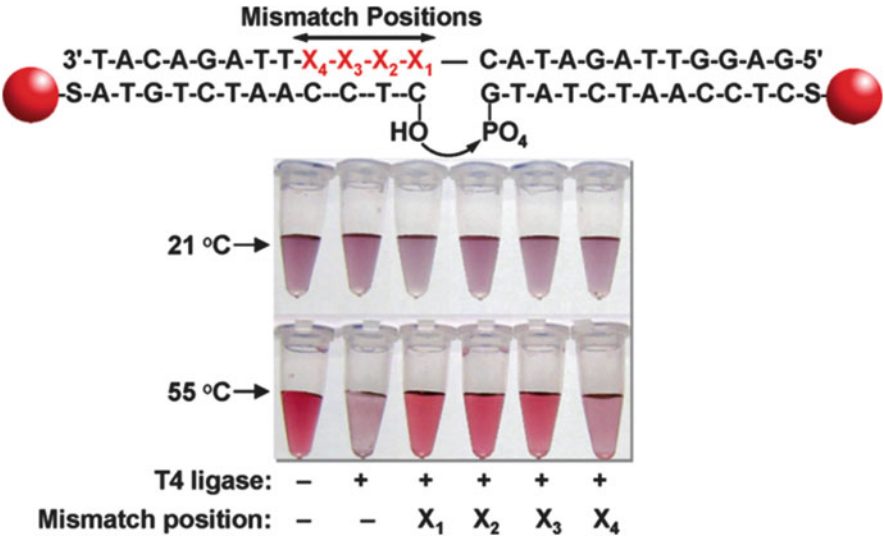

Fig. 4 AuNP-based assay on different SNP positions (reproduced with permission from Xue et al. 2009)

2.2 *Primer Extension-Based Genotyping of Single Nucleotide Polymorphisms*

Most of the SNP genotyping methods are based on known sequences available in order to specifically design the sequence of the probe to achieve the allele-specific discrimination. However, the construction of such a database does not have any references, which means most of the designed detection method for SNP is not applicable. It will be almost impossible to establish a SNP library on a whole-genome scale every time as the Human Genome Project did because of the unaffordable cost. Next-generation sequencing, however, provides the possibility to build the library with a much lower price (Shendurel et al. 2005; Shendurel and Ji 2008). Some representative methods are 454 pyrosequencing, Illumina, SOLiD, and HeliScope (Margulies et al. 2005; Shendurel and Ji 2008; Mardis 2008). Accuracy of the data was further increased by the framework of selection (Depristo et al. 2011; Nielsen et al. 2011). After the whole-genome sequencing, it is not always necessary to perform large-scale SNP detection if only one or few SNPs are of interest in consideration of cost and efficiency. Some methods based on the known library to detect SNPs are illustrated below.

2.2.1 Allele-Specific PCR

Common PCR procedures use the same primers for the extension of targets (Pelt-Verkuil et al. 2008). However, to determine the existence of SNPs, primers with

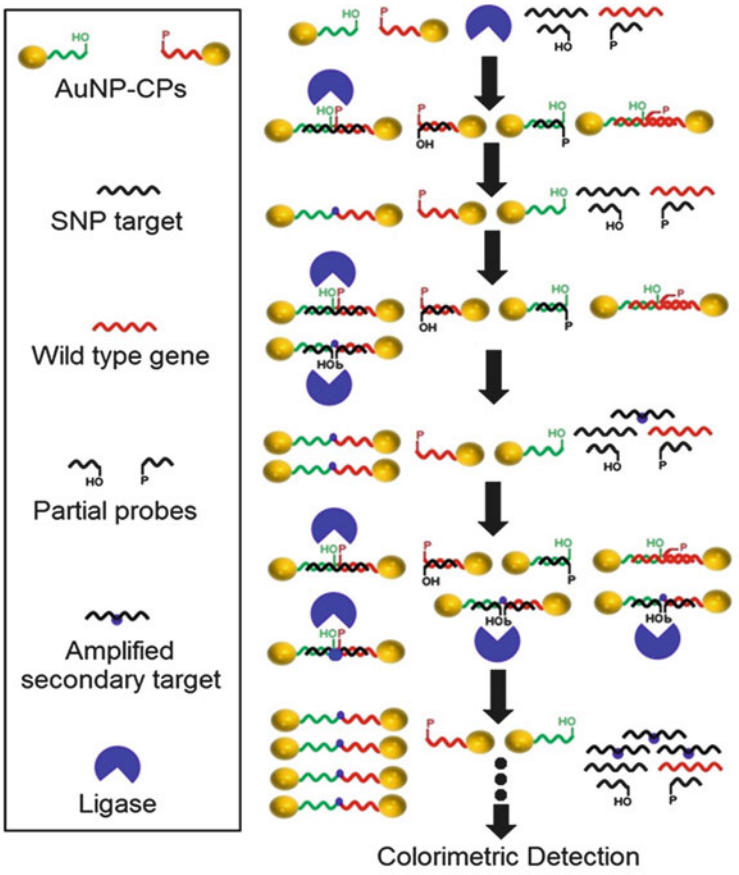

Fig. 5 Representation of SNP detection employing LCR and AuNPs (reproduced with permission from Shen et al. 2012b)

single-base differences at the 3′ end, which is deliberately designed at the SNP site, are utilized so that the extension reaction of PCR can only happen with perfectly matched sequences. Discrimination and genotyping of SNPs can be achieved with specific fluorophore functionalization and gel analysis (Kim and Misra 2007). A SNP assay with similar idea but no reverse primer extension performed is called sequence-specific primer PCR (SSP-PCR) (Hori et al. 2003). In 1989, the first successful application of SNP genotyping by allele-specific PCR was achieved in which a third primer (common primer) was introduced to identify the products with different "allele-specific primers" (Gibbs et al. 1989). High discrimination factor (100:1) and the success in the detection of SNP in low abundance (100-fold mismatched product) proved the robustness and reliability of the method.

As illustrated in Fig. 6, genotyping was successfully performed by combining cationic conjugated polyelectrolytes (CCP) with fluorescent PCR products to

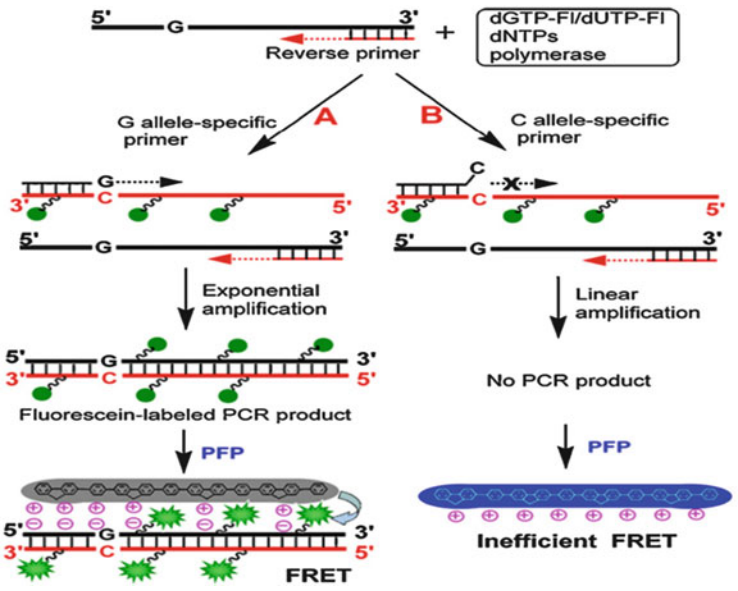

Fig. 6 Schematic illustration of SNP typing by CCP (reproduced with permission from Duan et al. 2009)

enhance the detection power of the assay by the fluorescence resonance energy transfer (FRET) from CCP to the dGTP-FI and dUTP-FI, which can make the intensity of fluorescence increased (Duan et al. 2009). SNP determination was successfully conducted with 50 ng of genome DNA from lung cancer cells. A colorimetric SNP detection scheme based on the optical properties of AuNPs was also reported (Jung et al. 2011). Extension reactions were carried out in four different tubes with all experimental conditions maintained the same, except that the four primers with the only difference at 3′ ends were chemically functionalized with sulfhydryl group (−SH), which promotes the aggregation of PCR product on AuNPs. Red color was observed in the tube containing perfectly matched primer, whereas the rest changed to blue because of the aggregation of AuNPs.

2.2.2 Single-Base Extension

The method of single-base extension (SBE) was initially invented by Goelet et al. as a form of US patent (Goelet et al. 1999). Primers of which the 3′ ends are immediately adjacent to the query site of mutation are designed to hybridize to the target DNA. SBE occurs with the introduction of four dideoxynucleotides, namely, ddATP, ddTTP, ddGTP, and ddCTP, labeling with distinctive reporters. Different detection techniques such as fluorometry, voltammetry, Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry, and gel electrophoresis, thereafter, can be conducted to identify the SNPs (Brazill and Kuhr 2002). SNP

detection based on SBE with MALDI-TOF mass spectrometry is one of the examples that has been used to determine SNPs in real targets such as trisomic DNA (Trewick et al. 2011). Application of ribonuclease was also performed (Mengel-Jorgensen et al. 2005). In addition, fluorescence discrimination by the incorporation of graphene oxide can type the SNPs with detection limit of 3 nM (Xu et al. 2013).

Introduction of microfluidic platforms for primers improves the detection by higher automation and multiplex SNP detection. SBE on glass slides (SBE-TAGS) applied this concept to achieve a highly accurate and sensitive assay (Hirschhorn et al. 2000). Two-color SBE reactions to score the SNPs were employed in the configuration of a whole-genome genotyping method (Steemers et al. 2006). Hybridization of targets and primers, immobilized on a BeadArray platform, happened before the execution of SBE during which four types of dideoxynucleotides with two kinds of fluorophores were used. After staining, genotype can be analyzed by the color of the image of the SBE products. Based on the same principle, the employment of gold-coated magnetic nanoparticles functionalized by streptavidin (SA-GMNPs) as the platform for the immobilization of primers was used to discriminate SNPs (Li et al. 2012). After SBE, fluorophore–GMNP complexes were formed which then are "printed" on a glass slide with the aid of an external magnetic field. Discrimination of SNPs was achieved by the comparison of the colors generated by different fluorophores. No purification of the PCR product and no background correction were needed in the assay.

2.3 *Enzymatic Cleavage-Based Genotyping of Single Nucleotide Polymorphisms*

Restriction endonucleases are a class of enzymes that specifically recognize and cleave certain DNA sequences (Roberts 1976; Kessler and Manta 1990). A systemic summary of the classification, function, and potential applications is available (Kessler and Manta 1990). When restriction endonucleases cleave, some of the recognition regions of the endonucleases contain SNPs, or other types of mutations often give rise to unsuccessful cleavage (Kim and Misra 2007). By applying such a strategy, the SNPs within the recognition regions of restriction endonucleases can be determined, of which one of the initial methods was the assay of restriction fragment length polymorphisms (RFLP) and amplified fragment length polymorphisms (Botstein et al. 1980; Nicod and Largiader 2003). However, the drawbacks of these methods are obvious because the restriction endonucleases can only recognize certain sequences of the genome, leaving the majority of SNPs untouched. Restriction endonuclease next-generation sequencing (NGS) can improve the performance of the previous methods by the construction of reducible representation of a genome by the endonucleases and the dedicated design of the assay (Davey et al. 2011). NGS has been applied to genotype whole-genome SNPs, and the results were promising. However, the problem caused by the limitation of

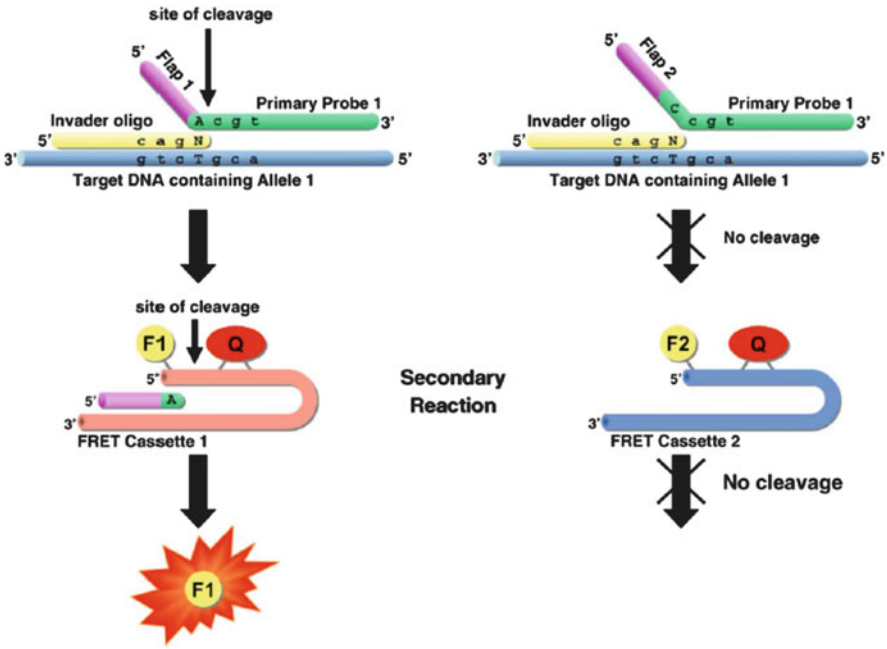

Fig. 7 Principle of invader assay (reproduced with permission from Olivier 2005)

restriction endonucleases may still cause a lower level of SNP detection than other methods (Kim and Misra 2007).

Invader assay is a SNP genotyping method using restriction endonucleases but has a different detection mechanism. The principle of the detection is based on a triplex structure formed among the target DNA sequence, the allele-specific probe, and the invader probe. If the allele-specific probe had perfect match with the target, the restriction endonuclease would cleave off the invader sequence from the triplex structure (Fig. 7) (Gut 2001; Olivier 2005). Detection can be achieved if the free invader probes are labeled. Based on the original method, an enhanced assay in which two invasive reactions were used for the amplification of the target gene has been developed (Hall et al. 2000). The power of amplification was much higher than that of the single invasive detection method (10^7 compared to 3,000–10,000).

2.4 Hybridization-Based Genotyping of Single Nucleotide Polymorphisms

The working principle of the hybridization-based genotyping relies on the detection of subtle differences in stabilities caused by the single-base mismatches, which was

first systemically studied in 1979 (Wallace et al. 1979). Some commercially available approaches are then developed, such as the GeneChip array assay and Qbead technique (Kennedy et al. 2003; Xu et al. 2003). Because hybridization-based genotyping does not require the involvement of complex enzymatic reactions, it is more convenient and easier to be conducted (Mir and Southern 1999; Gao et al. 2013). However, the thermodynamically driven reaction suffered from the problems of sensitivity and specificity (Shen et al. 2013a, b). Much effort has been devoted to overcome the problems on the assay, and some of the improvements are elaborated in the following section.

2.4.1 Molecular Beacon

Molecular beacon (MB) is a stem-loop structure that contains both a fluorophore and a quencher, which diminishes the fluorescence by FRET between them (Tyagi and Kramer 1996). With the stepwise improvement of MBs, many derivatives of MBs are invented, such as the superquencher MBs (Yang et al. 2005), quencher-free MBs (Venkatesan et al. 2008), and the stemless MBs (Gifford et al. 2005). All of the modifications aim at increasing signal-to-noise ratio and specificity. The first application of MBs in SNP genotyping appeared in 1998 in which two MBs labeled with fluorescein and tetramethylrhodamine were employed to selectively detect the wild type and mutant type (Kostrikis et al. 1998). Subsequently, a large variety of applications of MBs were developed. Some representative examples will be elaborated in here.

Base discriminating fluorescent (BDF) oligodeoxyribonucleotides, which are mainly pyrene-functionalized derivatives, can be applied to differentiate SNPs (Ostergaard and Hrdlicka 2011). Research based on this principle was later executed by many groups. The quencher-free MBs were discovered based on the pyrene-modified 2′-deoxynucleotides (Fig. 8) (Seo et al. 2005). When the loop is opened by the target, the fluorescence is quenched through photoinduced electron transfer and π–π interaction, which is mainly attributed to the coupling between the pyrene-labeled bases and the neighboring bases on the nucleotides. Probe A^{PY} was shown to have the best discrimination capacity, and S1 was proven to be suitable as the sequence of the probe. By using deoxyuridine with functionality of 9-fluorenone (FO) or fluorine (FL) as fluorescent probes on the base part, the quencher-free MBs are developed, and some of them showed excellent performance and were regarded as the promising candidates for SNP typing (Lee et al. 2013).

The detection of dual SNPs simultaneously employing two different quantum dot molecular beacon (QD-MB) probes using capillary electrophoresis (CE) for the detection of the SNPs was also reported (Li et al. 2011). The multiple SNP detection was achieved by the application of QDs instead of the normal fluorescence dyes, and CE minimizes the problem of false results which might be observed by the effectively separation of unreacted MB with the MB–target complex. Two QD-MBs hybridized to two SNP sites, which generate distinguishable signals.

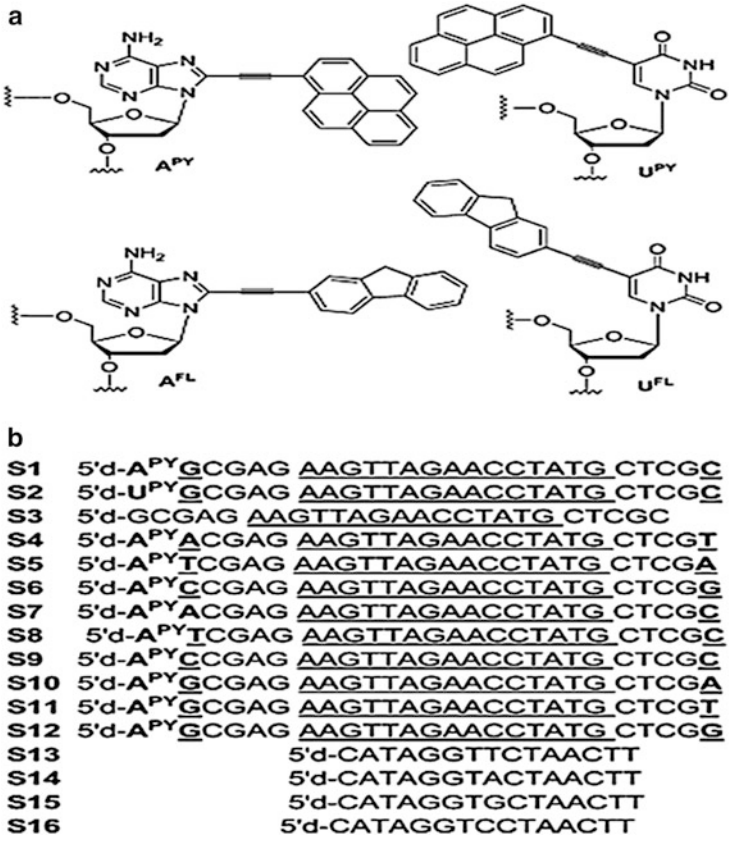

Fig. 8 Structure of fluorescent nucleoside derivatives for the assay (reproduced with permission from Seo et al. 2005)

The detection limit of this method is 14 nM (16.2 pg), an impressive improvement over that of the original method using QD-MBs (8 ng) (Kim et al. 2004).

2.4.2 Y-Shape Junction Probe

Y-shape junction probe is a special case of junction forming probes, which is isothermal and non-PCR based (Knez et al. 2014). The principle of the operation of the probe is template-enhanced hybridization process (TeHyP). The first application of the Y-shape conjugation probe to the detection of SNPs was conducted in 2008 by using the enzymatic cleavage of specific sequences (Nakayama et al. 2008). Afterward, some improvements of the method were achieved.

The effective SNP genotyping strategies of oral cancer by the junction probes with 2′-deoxyinosine nucleoside (dI-nucleotide) substitution on the SNP site using electrochemical detection were attempted (Zhang et al. 2010a). Different stabilities

were obtained when various nucleotides were complementary to the dI-nucleotide in the formation of a three-way junction complex, which can be differentiated by linear sweep voltammetry. Based on the same principle employing the DNA enzyme activity to oxidize 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonicacid) (ABTS) by hydrogen peroxide to the colored $ABTS^{-}$ radical, SNP discrimination can be achieved by the detectable color change by the naked eye (Tang et al. 2013). Both methods are easily operated, but the detection limits are still relatively high (0.13 pM for the first method and 0.25 nM for the second method).

A point-of-care detection of SNP based on the chameleon NanoCluster Beacon (cNCB) probe, which was divided into NC probe and G-rich probe, was also reported (Yeh et al. 2012). NanoCluster Beacons (NCBs) employed in the research are silver nanoparticles which serve as reporters, which can generate fluorescence with distinctive colors in different proximities of DNA sequences. When the targets were exposed to two parts of the cNCB probes, the Y-shape junction DNA structure was generated. Certain color was obtained when the target was the wild type. When the target had a SNP site within the hybridization region with the cNCB, the frameshift of the third arm caused the color change, which can be detected by the emission spectra or the naked eye. Three signals were obtained when using various bases in the SNP position. The method was further varified by the combination of different nucleotides in SNP sites, and all results were distinguishable, which means it is applicable for SNP typing.

2.4.3 Toehold

Toehold is a short, single-strand overhanging region of a double-stranded nucleotide (Knez et al. 2014). Fast and specific strand displacement reaction (SDR) will happen when an oligonucleotide binds to the toehold, with the replacement of the original “shorter” chain by the totally complementary one (Yan et al. 2014). SNP determination and genotyping based on the toehold-mediated SDR have been systemically studied in recent years.

The application of toehold in SNP detection was first performed in 2010 (Zhang et al. 2010b). Single-stranded DNA probes were initially hybridized with the practically complementary DNA reporter. The sequences, not forming the double-stranded structure with the reporter, were the toehold. The capture probes were connected by DNA origami, which showed red under an atomic force microscope. Streptavidin, generating white-bulge images, was incubated into the reporter as a contrast label to reveal the position of the reporter. When perfectly matched target was employed, complete strand displacement reaction happened, which can be visualized by the disappearance of the streptavidin feature on the DNA origami platform and vice versa, thus achieving genotyping of SNPs.

Optimization was almost necessary for the hybridization-based SNP genotyping in the abovementioned reports, which are tedious, time-consuming, and sometimes highly costing. Toehold exchange probes offer the possibilities for not performing the optimization procedure (Zhang et al. 2012). Three common factors are needed

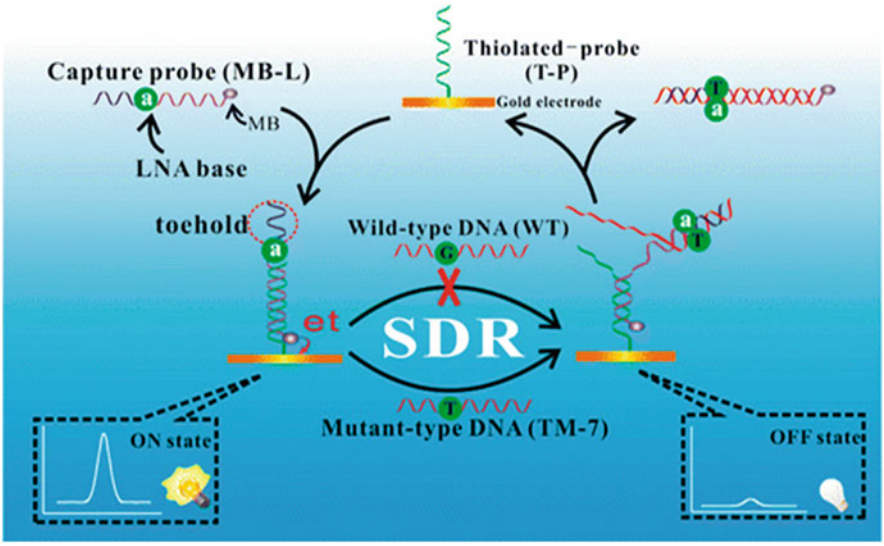

Fig. 9 Principle of SNP detection by toehold-mediated SDR (reproduced with permission from Gao et al. 2014)

for further optimization in previous studies, which are temperature, salinity, and oligonucleotide concentration. However, they did not influence the efficiency and specificity of the probes in a wide range according to the theoretical modeling and actual measurements. The near-optimal SNP discrimination can be maintained from 10 to 37 °C and from 1 to 47 mM Mg^{2+}, and the concentration of target oligonucleotide is from 1 nM to 5 μM. The performance of the probe on the SNP determination was promising with theoretical discrimination factor being as high as 343.

As illustrated in Fig. 9, an on–off switching biosensor based on locked nucleic acid (LNA) and toehold structure was recently proposed (Gao et al. 2014). Thiolated DNA probes (T-P) were first incubated with the capture probes (MB-L) labeled with methylene blue and integrated with LNA at the mismatched point, of which the position optimized the discriminating capability. By replacing the normal nucleotide with LNA, the ability of discrimination between the perfectly matched target and SNP-based target was significantly improved. The current was enhanced with the addition of MB-L by the electron transfer between AuNPs and MB, which is the ON signal. SDR was then initiated by the toehold when the target was introduced. With perfect matching between the MB-L and the target, T-P leaves the system, shifting the signal to OFF. When mismatched template was present, displacement was not executed completely, leaving the signal partially in the ON state. This approach was verified on complex samples such as urine and soil. It was shown that the discrimination ability was very robust permitting the coexistence 6,000-folds of irrelevant genomic DNA.

2.4.4 G-Quadruplex

G-quadruplex is a four-stranded structure assembled from G-rich nucleic acid oligomers (Neidle 2009). The sequence for the uniquely high-ordered DNA structure is mainly connected by Hoogsteen-type base pairing (Hoogsteen 1963). The existence of such a structure in human genome varies, with the occurrence approximately from 300 to 400,000. The morphology of G-quadruplex is diversified, but all are likely to coordinate with metal ions, particularly with high affinities to alkali metals. In fact, potassium ions have a great importance on the sensitivity and specificity of G-quadruplex-based assays (Ren et al. 2012). Coordination of other ions in G-quadruplex is also possible, which makes it possible for the application in genotyping of SNPs.

The first SNP determination strategy was based on the peroxidase-like DNAzyme with the G-quadruplex structure (Kolpashchikov 2008). The DNAzyme was separated into two parts,with four triple G sequences split into a 2 + 2 pattern, which would not be active after the combination with the template DNA and hemin. Each segment was modified by removing the deoxycytidine and connecting a binding arm with a triethylene glycol linker. In the presence of the perfectly matched target with binding arms of the two probes, the formation of G-quadruplex and the acquisition of the enzymatic activity occurred, and the colorless 3-3′-diaminobenzidine tetrahydrochloride was oxidized to a brown compound by hydrogen peroxide. If the mutant type was introduced, no color change was observed. Enabling the distinction by the naked eye, this method has the advantages of simplicity and low cost with relatively promising discrimination power(S/N ~10).

As shown in Fig. 10, a significant improvement was achieved with the incorporation of PCR-like temperature circles into the DNAzyme assay for amplification (Wang et al. 2011b). The probes contained three distinct segments—Domain I, II, and III. The amplification was carried out same as ordinary PCR reactions. At 60 °C, annealing reaction occurred with the Domain III connecting to the target, after which the addition of triple G at the 3′ end of Domain III happened. After the temperature rose at 95 °C, probe and template were separated, and the template was used in the next round of PCR reaction. The addition of hemin generated the G-quadruplex with catalytic reactivity, which was involved in the oxidation of 2,2′-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) with the colorless ABTS change to green $ABTS^{+}$. SNP typing can thus be achieved, but it is necessary to further improve the reaction conditions and probe design by increasing the annealing temperature and incubating competitive probes without G-rich sequences to improve the selectivity of the assay. In principle, shortening the matching sequence can also improve the performance.

Based on the same detection mechanism, the performance of the assay can also be improved by increasing the efficiency of signal amplification (Fig. 11). By utilizing isothermal strand displacement amplification (SDA) twice, better results were obtained (Wang et al. 2011a). Allele discrimination was accomplished by the combination of discrimination probe I and II with the common probe III by ligase. First round of SDA reaction was performed with the 3′ end of probe III as primer.

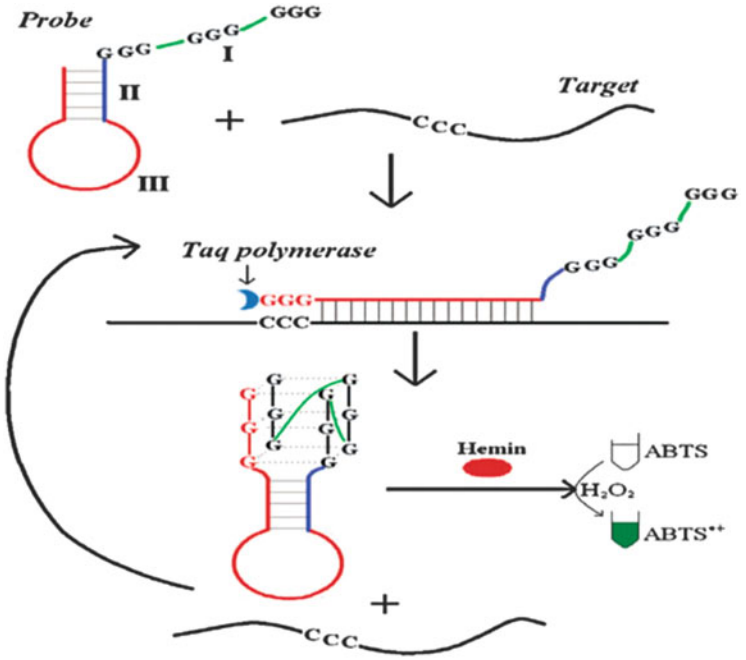

Fig. 10 DNAzyme-mediated SNP genotyping (reproduced with permission from Wang et al. 2011b)

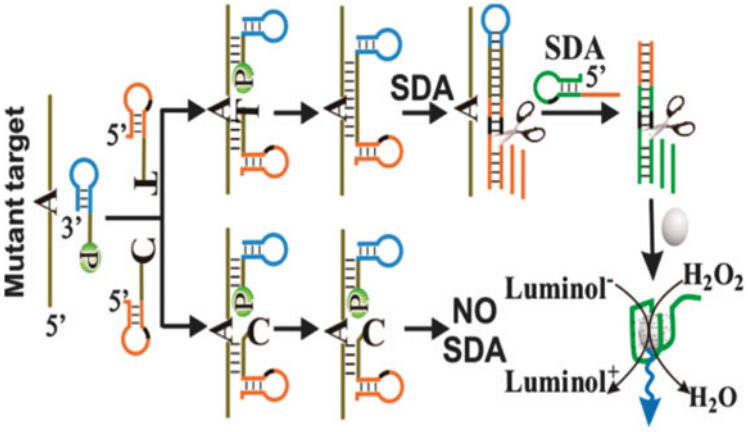

Fig. 11 Discrimination of SNPs by SDA using chemiluminescence (reproduced with permission from Wang et al. 2011a)

The product was further combined with probe IV, of which the product underwent the second round of SDA. The introduction of hemin into the amplicon produced the DNAzyme which triggered a chemiluminescence reaction. The selectivity of

the method is superior to other similar methods with the signal-to-background ratio around 150. Furthermore, the detection limit was 0.1 fM, indicating the procedure can be applied in genotyping SNPs in real-world samples.

3 Conclusions and Future Prospects

SNPs are the most abundant DNA variations in human genome and are closely related to differences in phenotypes and a large number of diseases. Robust, effective, and highly specific SNP genotyping techniques are therefore necessary for the studies of SNPs. Currently, two categories of SNP genotyping techniques—the allele-specific hybridization method and the allele-specific enzymatic method—are widely studied. Enzymatic assays based on ligation, primer extension, and enzymatic cleavage are systemically investigated, and the methodologies are usually more complex than the hybridization methods. However, based on the subtle difference in the stability between the wild-type and mutant genes, the hybridization method is still advantageous because of the comparably simple procedures and relatively low cost. Notwithstanding the current obstacles faced, such as the relatively low discrimination power and higher limit of detection, the hybridization-based SNP genotyping is still a promising way with much attention drawn to the field and promising progress made in recent years.

SNP genotyping technologies have been developed in the past 20 years. Much effort has been spent to the discovery of SNPs on the role they play that may cause certain diseases, such as the Hyperkeratosis Lenticularis Perstans gene variations (Wang and Moult 2001). Modules on the impact of protein that may cause genetic disease by SNP have also been investigated (Bakker et al. 2006). However, the expected destination of the study of SNP genotype should not just stop at the SNP discovery stage when it comes to the study of diseases. The pathogenic mechanism behind the existence of SNPs should be thoroughly investigated in addition to SNP genotyping, thus, enabling the development of molecular diagnostics and personalized therapeutics.

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