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Dna Mutation/Methylation Screening Method for Colon Cancer Screening

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DNA MUTATION/METHYLATION SCREENING METHOD FOR COLON CANCER SCREENING

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DEVELOPMENT OF HIGH SENSITIVE PROBE ENRICH MUTATION/METHYLATION-HIGH RESOLUTION MELTING ANALYSIS METHOD FOR COLORECTAL CANCER SCREENING

WEI MENG

ABSTRACT

Cancer is a result of unregulated cell growth. For all types of cancer currently studied, the transition from a healthy cell to a malignant tumor cell is a step-by-step process which requires mutation in at least several oncogenes and tumor suppressor genes together. Another cancer early event is DNA methylation. Cancer-related DNA methylation focuses on promoter hypermethylation of the certain genes. The DNA mutation and methylation profile can serve as biomarkers for diagnosing early stage of cancer.

Colorectal cancer is the third most common cancer type in the United States and has been well studied. The essential mechanism of cancer development is becomeing clear, so there are more approaches to diagnose early stage cancer and improve cancer treatment, which benefits colorectal cancer screening in recent years.

The current mutation/methylation detection techniques generally have two major categories which rely on the 1) physical property of double strand DNA or 2) enzyme

selectivity to survey the target sequence. Chapters I and III summarize the major methods used in the present DNA mutation and methylation analysis.

High Resolution Melting (HRM) is a simple, PCR-based method for detecting DNA sequence variation by measuring the melting temperature of a DNA duplex. In Chapter II, a robust and lower cost HRM assay for screening P53 and Kras mutations is discussed. In Chapter IV we developed Probe Enrichment Mutation/Methylation-High Resolution Melting (PEMM-HRM) assay. PEMM-HRM analysis is a simple and high sensitive post PCR technique which can be used for high throughput mutation scanning, genotyping and methylation analysis. PEMM-HRM analysis with enhanced sensitivity and specificity can have broad applications in clinical research.

In chapter V, We studied adenylosuccinate lyase deficiency, which is a defect of purine metabolism. We developed a method combining ESI-MS with solid-phase extraction to detect succinyladenosine (SA) and succinylamino-imidazolecarboxamide riboside (SAICAr) of patients with adenylosuccinate lyase (ADSL) deficiency urine samples. For the first time, we demonstrated that both SAICAr and SA biomarkers can be detected by Electrospray Ionization Mass Spectrometry.

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LIST OF ABBREVIATIONS

ADSL	adenylosuccinate lyase
AICAr	aminoimidazole carboxamide ribonucleoside
APC	adenomatous polyposis coli
DHPLC	denaturing high performance liquid chromatography
DGGE	denaturing gradient gel electrophoresis
dNTP	2'-deoxynucleotide
ddNTP	dideoxynucleotide
DNMT	DNA methyltransferases
ESI-MS	electrospray ionization mass spectrometric
HRM	high resolution melting
DNA	deoxyribonucleic acid
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MSP	methylation specific PCR
SA	succinyladenosine
SPE	solid phase extraction
SAICAr	succinylamino-imidazolecarboxamide riboside
PEMM	probe enrich mutation/methylation
SNP	single-nucleotide polymorphism
dsDNA	double stranded DNA

CHAPTER I

CURRENT DNA MUTATION ANALYSIS METHODOLOGY

1.1 Introduction

The origins of genome started with Frederick Sanger, a Noble prize winner, who developed an in vitro method to synthesize a ladder of DNA fragments differing by single nucleotides (Sanger and Brownlee 1970). Then the largest biological program, the Human Genome Project, provides an unprecedented opportunity to look into the information of the basis of disease, genes function, forensic purpose and evolutionary studies (Venter, Adams et al. 2001).

It was proposed that DNA sequences which show variations among individuals

could serve as reference points (DNA markers) to determine specific diseases. It shows increasingly significant importance for identifying and genotyping a large number of genetic polymorphisms in general populations.

1.1.1 Progression of Colorectal Cancer

Cancer arises from the accumulation of mutations in oncogenes and tumor-suppressing genes which can potentially lead to the unregulated cell growth. By far for all types of cancer studied, the transition from a healthy cell to a malignant tumor cell is a step-by-step process which requires mutation in at least several oncogenes and tumor suppressors together. An example is that tumors rarely develop among children but have higher frequency among older people. Because cells have a protective mechanism to kill the mutation-occurred cell, it is very rare for several mutations existing in the same cell to generate a cancer cell. The graph below shows colon cancer rates in the United States as a function of age (National Cancer Institute website).

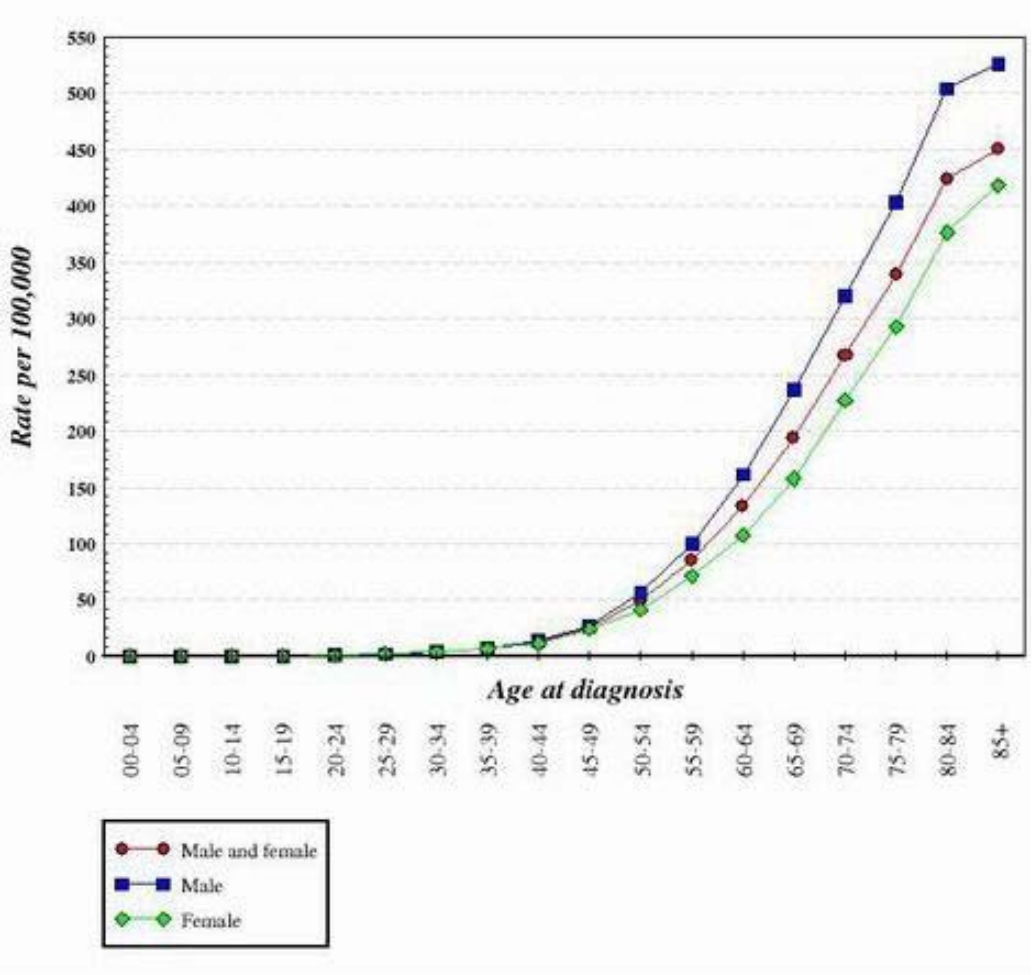


Figure 1 Colorectal cancer incidence rate increases with age. Before age of 40, the incidence of colorectal cancer is very low. After age of 50, men have a higher rate to develop colorectal cancer than women.

Colorectal cancer is the third most frequent cancer in the world in both sexes and also the third most frequent cause of cancer related deaths. Until age 50, men and women exhibit the same incidence, but after age of 50 men are more venerable to colorectal cancer. Colorectal adenocarcinomas are the most common type of colon cancer, but other types include melanoma, lymphoma, and carcinoids. Adenocarcinomas accounts for about 90-95 percent of all colorectal cancers.

1.2 Types of Mutations

The production of protein is based on reading the sequence information on mRNA, while the mRNA is synthesized from DNA via the process called transcription. As a result, any changes in DNA sequences will lead to alternations of the mRNA and then further may lead to the production of protein without its proper function. Even a single nucleotide change in a gene can lead to conformation change in protein which can't perform its function. There are several types of DNA alternation.

1.2.1 Point mutation

This is the most common type of DNA alternation. One or a few nucleotides along DNA strand are mutated which causes changes of the three-letter codons read by the ribosomes. If the changed codon causes the truncated and non-functional protein, this is called a nonsense mutation. If the changed codon causes a wrong amino acid to be incorporated into the protein, this is called a missense mutation.

1.2.2 Frame-shift mutation

In a frame shift mutation, one or several bases are inserted or deleted. Because the transcriptase read DNA sequence by each codon, adding or removing one or more bases can make the DNA information meaningless and often produce a protein without biological function.

Deletion and insertion also belong to frame-shift mutation. Mutations that result in missing one base are called deletions. Deletion affects a large number of genes on the chromosome. Mutations that result in the addition of extra base are called insertions. Insertions can also cause frame shift and generally result in a nonfunctional protein.

1.2.3 Neutral mutation

In a neutral mutation, one DNA codon is replaced by different codon, but this alternation doesn't change the amino acid which is introduced by ribosome. Neutral mutation has no biological advantage or disadvantage.

1.3 Resource of Mutation

It is well known that cancer cells result from normal cells with an accumulation of mutations. Generally, there are four types of mechanisms which induce the genetic changes. The first type is called Spontaneous Mutation which results from random molecular events during DNA replication. The second type is called Induced Mutation. Environmental factors such as radiation, chemicals, or oxygen radicals can alter the sequence of a DNA

strand. The third type results from the abnormal cell division which creates aneuploid cells. Because the aneoploid cells lose a great number of genes which may include tumor suppress genes, a higher possibility of cancer may occur during the unregulated cell division. The fourth type of mutation resource is the virus. The virus can damage, insert or alter DNA sequences of host cells which can activate potential oncogenes in host cells during development of certain cancers.

Colon cancer and rectal cancer are collectively known as colorectal cancer. In 2009, the American Cancer Society estimates that there would be approximately 146,970 new cases diagnosed and 49,920 deaths due to colorectal cancer in the United States. More than 95% of colorectal cancers are adenocarcinomas, which start from gland cells that make mucus to lubricate the inside of the colon and rectum. For most cases, colorectal cancer develops slowly within a period of several years. During a pre-cancerous stage, non-cancerous and benign polyps grow on the inner lining of the colon or rectum. Some of these polyps can develop into colorectal cancer. Although it is a life-long period for development of mutations that lead to cancer (usually more 50 years old), in most cases the first mutation starts from the APC gene. Inactivation of APC gene leads to uncontrolled cell proliferation and formation of polyps which accelerate further mutations of genes such as K-ras, p53 and Braf. These changes can lead the cells to grow and spread uncontrollably.

Extraction of DNA from stool is a feasible technology now. Several research groups have shown the feasibility of detecting colorectal cancer diagnosis using a panel of DNA markers like K-ras, APC, p53 and BAT26. The result shows high specificity and a

significant improvement over fecal occult blood tests. In addition, BAT26, a microsatellite instability marker, is useful for diagnosing sporadic colorectal cancer (Atkin 2003).

1.4 Current mutation detection method

There are two categories of mutation detection techniques, specific mutation detection and scanning mutation detection. Specific mutation detection techniques, including primer extension (Syvanen, Aalto-Setala et al. 1990)., allele-specific amplification (Ruano and Kidd 1989), and oligonucleotide ligation assay (Khanna, Cao et al. 1999), are used to identify characterized sequence changes in a known DNA sequence. These techniques are useful for examining common pathogenic mutated genes. Scanning mutation detection techniques are used to detect gene alterations in long stretches of unknown DNA sequencing. Scanning techniques include denaturing high performance liquid chromatography (DHPLC) (Underhill, Jin et al. 1997), denaturing gradient gel electrophoresis (DGGE) (Kuehn, Meyer et al. 1980), chemical cleavage of mismatches (CCM) (Cotton, Rodrigues et al. 1988), protein truncation test (PTT) (Roest, Roberts et al. 1993), single-stranded conformational polymorphism (SSCP) (Orita, Iwahana et al. 1989) and heteroduplex analysis (HA) (Prior, Papp et al. 1993).

1.4.1 Scanning mutation detection techniques

Chemical Cleavage of Mismatch (CCM)

The hemical cleavage of Mismatch method was introduced by Cotton et al and has

been widely used in diagnosis of genetic diseases (Cotton, Rodrigues et al. 1988). In CCM analysis, two commonly available reagents are used for detecting and modifying mismatched pyrimidine bases. Hydroxylamine (NH_2OH) modifies mismatched cytosine bases and potassium permanganate ($KMnO_4$) modifies mismatched thymine bases. Piperidine cleavage reaction is used to cleave the modified mismatched bases which produces separation of DNA fragments by gel-electrophoresis. The main advantage of CCM is that each mutation has at least two chances to be detected, which is a key for detecting single base substitution mutation.

Several modifications of the current CCM protocol provide a simpler and higher sensitivity approach than the original one. A toxic chemical, osmium tetroxide (OsO_4) can be replaced by potassium permanganate and give greater sensitivity (Roberts, Deeble et al. 1997). In addition, multiple fluorescent dye labeled DNA fragments improves the throughput of CCM method (Rowley, Saad et al. 1995).

Although the original report of CCM utilizes clone DNA for formation of DNA heteroduplexes, some modifications evolved over years. The invention of PCR technology provides a faster method than the traditional cloning genomic DNA method. The sample preparation time is significantly reduced which mades screening for mutations in large numbers of samples possible. Solid phase CCM uses biotinylated primers to generate PCR fragments, while avoiding time consuming ethanol precipitation steps, then streptavidin-coated magnetic beads and a magnet capture the PCR fragments (Hansen, Justesen et al. 1996).

CCM combined with fluorescence-based technology provides a simple and low cost method for screening the location and nature of sequence variations in a long range of DNA. Recent developments to chemical cleavage analysis ensured that CCM is still one of the most reliable methods of mutation detection.

Enzyme Mismatch Cleavage (EMC)

Some enzymes are more reactive with specific secondary structure of duplex DNA, such as bubbles and bends on mismatched duplex DNA. Heteroduplexes can be generated by heat denaturation of PCR products containing wild-type and mutant alleles. At first, researchers attempted to study potential power of the post replicative mismatch repair enzymes (Lu and Hsu 1992; Lahaska, Ostrander et al. 1994). MutS can recognize the mismatched sequences, but the specificity is not high enough for mutation screening (Lishanski, Ostrander et al. 1994; Parsons and Heflich 1997).

The use of phage T4 endonuclease 7 was one of the first methods which were used as a scanning method before complete sequencing. Phage T4 endonuclease 7 belongs to resolvase family which resolves a range of complex substrates like cruciform “Holliday Junctions” or single base pair mismatches. T4 endonuclease cleaves within 3 to 5 base pairs on the 5’ side of mismatch. The study showed that cleavage of at least one strand of the pairs of heteroduplexes occurred in 17 of the 18 known single-base-pair mutations tested but an A.A/T.T set could not be cleaved in any mismatched strand(Youil, Kemper et al. 1995). The problem of this method is that normal duplex DNA could be also cleaved by

T4 endonuclease..

CEL1 is also used in the EMC reaction. CEL1 is a mismatch cleaving enzyme from celery (Qiu, Shandilya et al. 2004). Members of the CEL family are homologs of S1 nuclease which prefers double-stranded mismatched DNA substrate and are not affected by high G/C content (Oleykowski, Bronson Mullins et al. 1998; Kulinski, Besack et al. 2000; Yang, Wen et al. 2000). The high specificity of CEL 1 nuclease is due to cutting the 3' phosphodiester bond immediately next to the mismatched base.

Single-strand conformation polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) is apparently the most frequently used procedure for point mutation analysis. This is due to its simplicity and relatively high specificity for the detection of sequence variation. Coupled to polymerase chain reaction (PCR), the sequence containing mutation sites can be analyzed.

SSCP was introduced by Orita and Colleagues (1989) as a simple and reliable method for the detection of sequencing alternation in genomic loci (Orita, Iwahana et al. 1989). Under the solution condition, single-stranded DNA fragments will form a special secondary structure. The secondary structure depends on the nucleic acid sequence. The principle of SSCP relied on the facts that single-stranded DNA fragments can migrate in a non-denaturing gel not only as a function of their size but also of their sequence. In other words, a single nucleotide substitution of the nucleic acid sequence may alter the secondary structure which cause differences in electrophoretic mobility. But there is no theoretical

calculation method established so far that could be used for predict the exact electrophoretic mobility of a given single strand DNA sequence. So SSCP should not be considered as the method of choice for the analysis of unknown sequences.

The first step of SSCP is selecting the range of PCR amplification. Preferably, the size of PCR amplification should be between 150 and 350 bps (Hayashi 1991; Hayashi and Yandell 1993). If the PCR product is too long, the point mutation may not generate detectable mobility difference. Kukita demonstrated that the sensitivity of SSCP can be greatly improved even for a PCR product as long as 800 bp if the electrophoresis is performed in low pH buffer systems and at a fixed temperature (Kukita, Tahira et al. 1997). The concentration of glycerol and the constancy of temperature play an important role in the sensitivity of SSCP assay.

In order to reduce radioactive isotope labeling, there is a variation of SSCP called fluorescent capillary electrophoresis SSCP (F-SSCP). F-SSCP has a higher specificity and sensitivity (~95%) (Yap and McGee 1992; Yap and McGee 1992).

Denaturing high-performance liquid chromatography (DHPLC)

Denaturing high-performance liquid chromatography, also called temperature-modulated heteroduplex analysis, provides a rapid method for scanning known or unknown mutations. The principle of denaturing high-performance liquid chromatography is similar to that of TGGE, but the UV detector is used to detect eluted fragments. DHPLC also needs heteroduplex formation and optimization of running

conditions (Underhill, Jin et al. 1997). DHPLC separates heteroduplex from homoduplex DNA fragments by means of ion-pair reverse phase HPLC.

The DHPLC is able to discover unknown genetic variants without sequencing at a lower cost. DHPLC can find low abundance mutations in heterogeneous samples which usually can't be found in sequencing method. DHPLC has been shown to detect mutations with a sensitivity ranging from 94% to 100%. (Oefner, Stoppa-Lyonnet et al. 1999; Wagner, Stoppa-Lyonnet et al. 1999; Harvey, Haynes et al. 2000). Cancer related mutation such as EGFR, CD14, p53 and BRCA1 genes were readily recognized by the DHPLC approach with high sensitivity (Mason, Ricks-Santi et al.; Oefner, Stoppa-Lyonnet et al. 1999; Harvey, Haynes et al. 2000).

1.4.2 Specific mutation detection techniques

Single base extension

Single base extension is the most widely used method for detecting SNP genotyping and point mutations (Syvanen, Aalto-Setala et al. 1990). In single base primer extension reaction, the primer anneals immediately next to the polymorphic site and is extended with a single nucleotide, using terminating dideoxynucleotides (ddNTPs) and a DNA polymerase. Various detection methods were coupled with the single base extension method such as florescence (Nikolausz, Chatzinotas et al. 2009), DHPLC, capillary electrophoresis, MALDI-TOF (Tost and Gut 2005) and tag array (Shoemaker, Lashkari et al. 1996).

Nucleoside triphosphates labeled with ^{32}P or ^{3}H were first used to detect the extension event (Kuppuswamy, Hoffmann et al. 1991). Multiplex single base extension was explored by Krook too (Krook, Stratton et al. 1992). The labeled primers were separated from the templates by gel electrophoresis, and visualized by autoradiography method. Later on, single base extension was utilized on a solid surface. Biotin is chemically linked to one of the primers used for SBE reaction, and streptavidin modified microtiter plate wells or magnetic beads were used to immobilize the extension template.

With the development of automated DNA sequencers, multiplex detection becomes possible when ddNTPs can be labeled with different fluorescent dye. The use of different target-specific primers with different lengths allows the analysis of more than one mutation in one reaction. Combined with the higher resolution power of capillary electrophoresis and laser-induced fluorescence, up to 8-12 extended products can be screened in one capillary (Nikolausz, Chatzinotas et al. 2009). A variation of multiplex SNuPE assay uses MALDI–TOF MS (matrix-assisted laser-desorption ionization–time-of flight MS) to detect extended products (Haff and Smirnov 1997).

DNA chip-based microarray is the latest development in the genotyping arena. Single-base extensions with a microarray format can generate highly multiplexed and parallel analysis of mutation. Cyclic single base extension reactions with fluorescently labeled dideoxynucleotides (ddNTPs) are performed in solution using multiplex PCR product as template and detection primers, designed to anneal immediately adjacent and upstream of the mutation space. The unique tag-sequences are attached to the 5’ end of

detection primers and oligonucleotides complementary to the unique tag-sequence (cTag) are printed on a microarray plate. The extension product can then be sorted by hybridizing the ‘tags’ to complementary ‘cTags’ attached to an array surface. Fluorescent scanning instruments can be used for subsequent analysis.

Ligase based method

The DNA ligase discovered in 1967 by the Gellert, Lehman, Richardson, and Hurwitz laboratories was a watershed event in molecular biology (Lehman 1974). The ligase can join 3’-OH and 5’-PO_4 termini to form a phosphodiester which is an essential step in DNA replication and repair such as nucleotide excision repair, base excision repair and single-strand break repair (Tomkinson, Vijayakumar et al. 2006). Because of its high specificity, DNA ligase plays an important role in genomic integrity. Two types of ligases were discovered: ATP-dependent and NAD+ dependent ligase. All known eukaryal cellular DNA ligases are ATP-dependent (Ellenberger and Tomkinson 2008). ATP-dependent ligases are also found in archaea, consistent with a common ancestry for the archaeal/eukaryal DNA replication machinery (Shuman 2009). NAD+-dependent ligases contain a unique domain which is responsible for binding NAD+ and required for the reaction with NAD+ to form the ligase-AMP intermediate (Gajiwala and Pinko 2004; Shuman 2009).

The high fidelity of ligation detection reaction (LDR) can distinguish matched target in the presence of a 10^2 to 10^3-fold excess of mismatched target. The presence of a 100-fold

excess of mismatched template depressed ligation of matched template only 2-fold Thermostable ligase has evolved to search and seal damaged DNA which has been nicked and corrected, while rejecting nicked DNA containing mismatches at the junction (Khanna, Cao et al. 1999).

Ligation assays have several features that make them ideally suited for typing point mutations. The specificity of the ligation between two oligonucleotide primers is determined by three factors: (i) the specificity of hybridization of the oligonucleotide primers to their complementary sequences on the template, (ii) the need for these primers to hybridize in a head-to-tail orientation on the template, and (iii) the fact that the oligonucleotides must have perfect base pairing with the target at their junction.

Sequencing Technology

Although conformation-based mutation screening methods are a cost-effective way to detect an unknown mutation site, once the potential regions have been confirmed to contain a putative mutation, these regions should be sequenced to confirm it. Due to the development of sequencing technology, direct sequencing methods has been divided into three fields, traditional Sanger sequencing, pyrosequencing, and next generation sequencing methods.

Sanger Sequencing

Electrophoresis-based, Sanger sequencing technology is the most commonly used

technology for sequencing and was the mainstay of the Human Genome Project. With the improvement of computer analysis capacity and florescence detection systems, traditional Sanger sequencing has become a fully automatic DNA sequencing process. Over the past 10 years, significant improvements in Sanger technology have cut the cost of sequencing from ~$10/kb to ~$1/kb. Over the same period of time, the throughput for a state of the art instrument has increased from <10 kb/h to ~100 kb/h.

The Sanger sequencing is a mixed-mode process using 2′-deoxynucleotides (dNTPs) to extend the complementary primer and using 2′,3′-dideoxynucleotides (ddNTPs) as the terminator by DNA polymerase synthesis (Sanger, Nicklen et al. 1977). There are several important breakthroughs which promote the fully automatic DNA sequencing technique. Prober and Smith applied a novel set of four chain-terminating dideoxynucleotides with each dideoxynucleotides carrying a different dye distinguished by its fluorescent emission (Smith, Sanders et al. 1986; Prober, Trainor et al. 1987). The DNA fragments can be resolved by polyacrylamide gel electrophoresis in one sequencing lane rather than 4 lanes in none fluorescent system. The separation matrix, linear polyacrylamide (LPA), also greatly elevated the resolution of CE system. Replaceable LPA solution has been used to read more than 1000 bases with a run time of 80 min (Carrilho, Ruiz-Martinez et al. 1996; Madabhushi 1998). The capillary array electrophoresis (CAE) increased the throughput of Sanger sequencing method (Kheterpal, Scherer et al. 1996).

Pyrosequencing

Pyrosequencing is a real-time sequencing method that detects light signal during DNA synthesis. During DNA synthesis single-stranded primer complementary to the DNA template will be added a deoxynucleotide triphosphates and generate one inorganic pyrophosphate (Ronaghi, Uhlen et al. 1998). Then inorganic pyrophosphate is converted to adenosine triphosphate which is the catalyst of luciferin oxidation reaction. The luciferin can be oxidized by luciferase and emits visible light. The visible light signal is proportional to the number of nucleotide incorporated into the extending primer.

Pyrosequencing technology is important for its high sensitivity, the flexibility of assay design, the capability for high throughput analysis and the quantitative data that makes analysis more accurate. Pyrosequencing can be also applied to screen mutations or SNP. The sensitivity for mutation sites is around 5% (Dufort, Richard et al. 2009).

Next Generation Sequencing

The high demand for low-cost sequencing has driven the development of high-throughput sequencing technologies that parallelize the sequencing process, producing thousands or millions of sequences at once. Roche, illumina and ABI all developed their unique way to perform sequencing-by-synthesis. The Genome Sequencer FLX by 454 Life Sciences and Roche depends on an emulsion PCR followed by parallel and individual pyrosequencing of the clonally amplified beads in a Picotiter Plate (Ellegren 2008). Emulsion PCR is a clonal amplification performed in an oil-aqueous

emulsion. By the addition of general adaptor sequences to the fragments, only one primer pair is required for emulsion amplification. In emulsion PCR, a sequence specific primer attached to a bead, a general primer and PCR component are isolated in a water micro-reactor. Once the emulsion is broken the amplified and enriched beads are then distributed on the Picotiter Plate, of the 1.6 million wells on the Picotiter Plate, not all will contain a bead and not all of those that do will give a useful sequence. The Illumina Genome Analyzer is relying on clonal bridge amplification on a solid surface. The sample DNA fragments ligated with an adapter can be attached to the solid support. The DNA fragments on the solid support are amplified because the dense adaptor primers and the primers complementary sequences on the surface, which will form colony like local clusters, each containing approximately 1000 copies and with a diameter of about 1 μm. Then sequencing is then carried out with fluorescently labeled nucleotides on solid surface. Although Illumina gives shorter read lengths than the 454 system, the throughput is much higher. By combining Roche and Illumina technology, Applied Biosystems developed a different SOLiD system (Sequencing by Oligonucleotide Ligation and Detection). The clonal amplicons on 1 μm beads are generated by an emulsion PCR. The beads are attached on a glass surface forming a very high-density random array. The amplicons on beads are sequenced by adding a florescence labeled di-deoxynucleotide at each step. The two-base encoding system greatly increases the accuracy of sequencing result.

Although next generation sequencing technologies is quite expensive now, once the technology is widely available, enormous impact on research fields will take place in a

short time frame

1.5 Summary

Current knowledge demonstrates the genetic changes are closely related to cancer development. The chapter I briefly reviewed the molecular genetic change in colon cancer and current method for mutation screening. In the review, two groups of mutation detection methodology are described: scanning mutation detection and specific mutation detection. With the development of those existing and under development diagnostic platform, especially the high throughput sequencing and microarray technology, the relationship between mutation and cancer will be revealed in a more deep and comprehensive view.

CHAPTER II

HIGH RESOLUTION MELTING ANALYSIS BASED MUTATION SCANNING MEHTOD

2.1 Introduction

HRM is a simple, PCR-based method for detecting DNA sequence variation by measuring changes in the melting of a DNA duplex. The annealing and melting properties of double-stranded DNA have enabled the development of several genetic assays. The fluorescence dye labeled DNA probe targeting mutation or SNP sequence is widely used in Taqman Real time PCR, FISH and microarray analysis. Recent advances in thermal cycler temperature control, fluorescent DNA intercalating dye and data acquisition method make

more accurate assessment of sequence variations based on melting curve analysis. First Idaho technology, then ABI, Roche and Bio Rad, developed the commercial high resolution melting platform based on their real-time PCR instruments.

The principle of HRM analysis is based on measuring a signal change of the fluorescent dye which indicates the annealing status of double-stranded DNA fragment. After PCR HRM is carried out in the presence of a suitable dye, the HRM method uses high data-density acquisition, and detects small sequence differences in PCR fragments while the product is heated. As the temperature rises and the duplex passes through its melting temperature, dye is released and fluorescence intensity is reduced (Figure 2).

2.1.1 Melting Curve Analysis

Usually a homozygous sequence will have a defined melting temperature (Tm). If there is a base change on this sequence (G:C to T:A), the Tm will decrease approximately 0.8-1.2ºC (Liew, Pryor et al. 2004). A heterozygous sample contains four types of duplex species: A:T, G:C, C:A, T:G. The observed melting curve (dashed line) is a composite of the four individual melting curves (Figure 3). The down shift of composite melting curve is the contribution from the relatively unstable heteroduplexes. Overall changes in fluorescence intensity are small and need specifically designed instrument for High Resolution Melting analysis to ensure the maximum sensitivity and specificity.

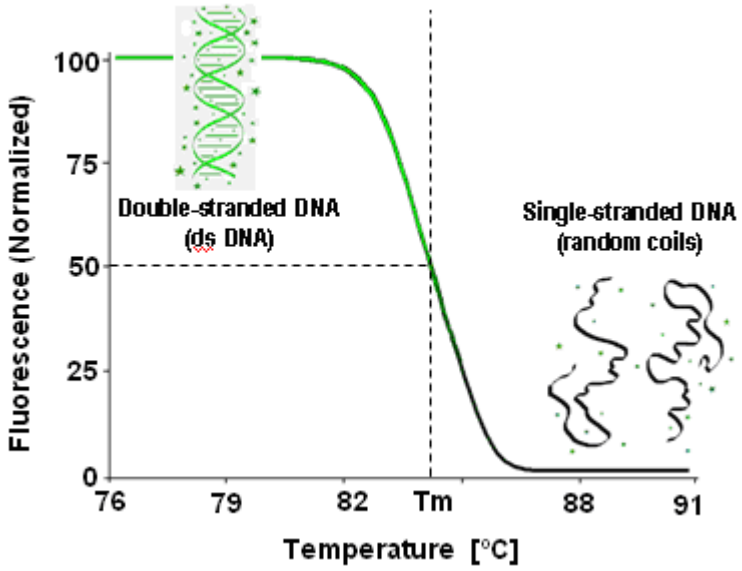


Figure 2 Fluorescence signal monitors the transition from double-stranded DNA to single-stranded DNA. As the temperature rises, fluorescence dye is released and fluorescence signal intensity is reduced (http://hrm.gene-quantification.info/).

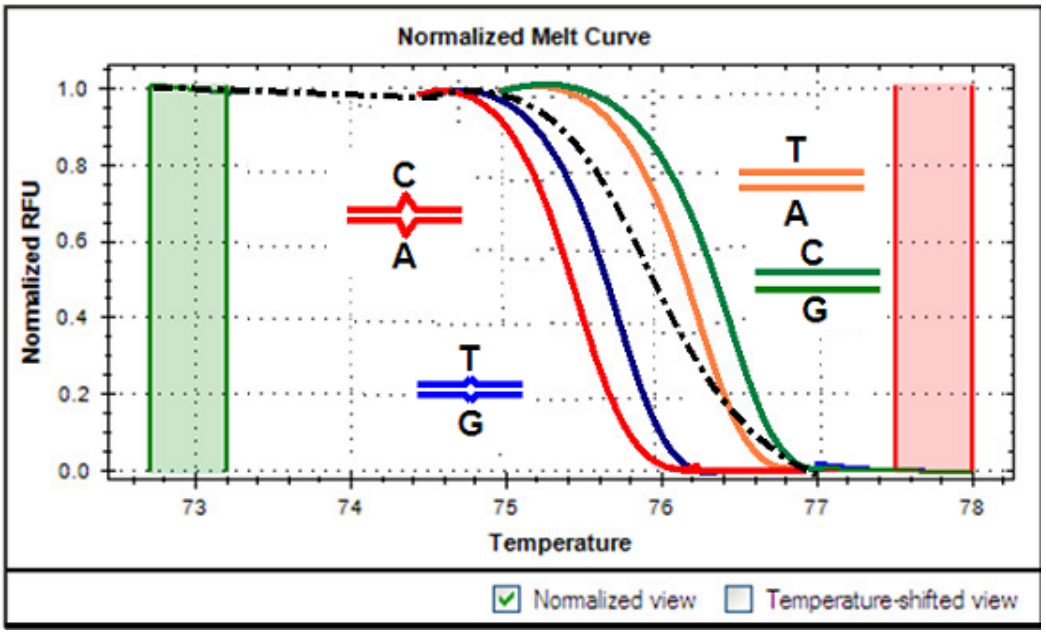


Figure 3 Composition of an observed melting curve. A heterozygous sample combining fluorescence signals of two heteroduplexes and two homoduplexes (www.bio-rad.com).

For the mutation screening, a different plot should be chosen to distinguish the homozygous and heterozygous sequences (Gundry, Vandersteen et al. 2003). The 3 steps of HRM analysis are displayed in Figure 4. During the first steps, a pre-melting and post-melting zone are selected which normalizes the starting and ending fluorescent signal level. The second step applies a temperature shift to each normalized the melting curve. This step is optional. It is easier to distinguish heterozygous samples from the now superimposed wild type homozygous samples. The third step magnifies curve differences by subtracting each curve from the most abundant type (usually wild type sample). This helps cluster samples automatically into groups that have similar melting curves.

2.1.2 Dye selection

"Saturation" dyes are less toxic to PCR amplification. These dyes show differential fluorescence emission dependent on their association with double-stranded or single-stranded DNA. SYBR Green I is a first generation dye for HRM. It fluoresces when intercalated into dsDNA and not ssDNA. Because it may inhibit PCR at high concentrations, it is used at sub-saturating concentrations. Recently, some researchers have discouraged the use of SYBR Green I for HRM (Gudnason, Dufva et al. 2007).

LCGreen was the first saturating dye available (Wittwer et al., 2003) ; LCGreen dyes are specifically designed for high-resolution melting curve analysis to detect DNA sequence variants. The addition of LCGreen dyes increases the melting temperature of DNA by 1-3 °C (Idaho Technology).

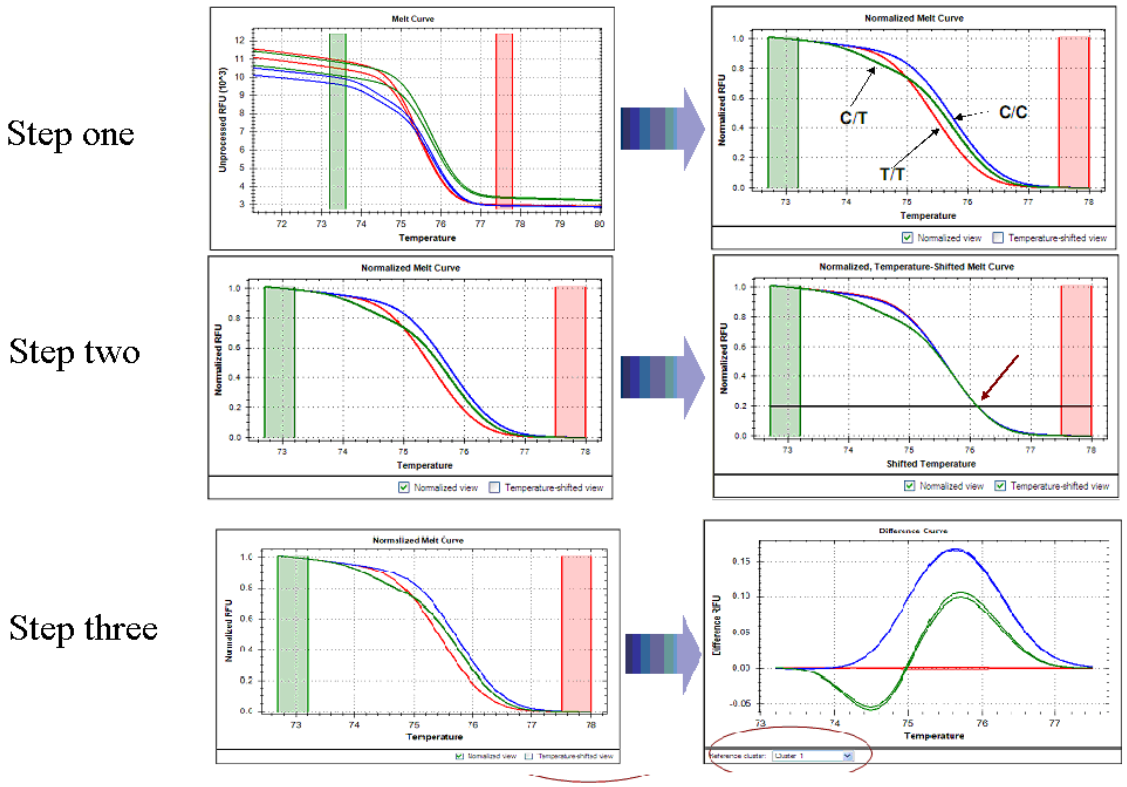


Figure 4 the workflow of HRM analysis. In step one, a pre-melting and post-melting zone are selected and all melting curves are normalized. In step two, a temperature shift is performed to each normalized melting curve. In step three, curve differences are displayed by subtracting each curve from the wild type sample (www.bio-rad.com)

Now there are many more new dyes, including SYTO9 (Invitrogen, Carlsbad, CA), EvaGreens (Biotum) and LightCycler 480 ResoLight Dye (Roche, Indianapolis, IN). SYTO 9 green fluorescent nucleic acid stain has been used to stain Gram-positive and Gram-negative bacteria. SYTO dye has some other special characteristics as well. SYTO dye can penetrate all mammalian and bacteria cells. The fluorescence signal of SYTO dye significantly enhance when SYTO dye binds to the nucleic acids.

EvaGreen dye is extremely stable both thermally and hydrolytically. This dye has no fluorescent signal by itself, but shows strong fluorescent signal upon binding to double strand DNA. EvaGreen dye is completely impermeable to cell membranes and has no mutagenic effect.

Relocation effect

As with any dye used in melting experiments, the HRM dye fluoresces strongly only when bound to dsDNA. This change of fluorescence during an experiment can be used both to measure the increase in DNA concentration during PCR amplification and, subsequently, to measure temperature-induced DNA dissociation during High Resolution Melting.

The “dye jumping” effect (Figure 5) may reduce accuracy during HRM analysis, which means dye from a melted duplex may get reincorporated into regions of dsDNA which had not yet melted (Reed, Kent et al. 2007).

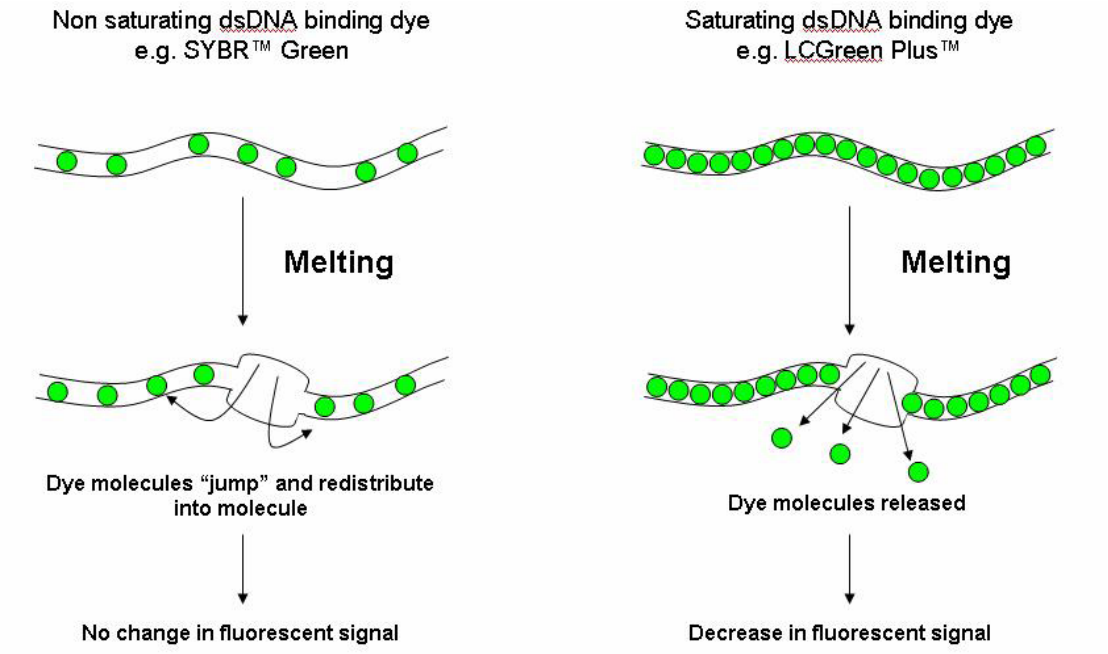


Figure 5 Non-saturating dye and saturating dye. Non-saturating dsDNA binding dye "jumps" when dsDNA melts. Saturating dsDNA dye doesn't redistribute in DNA duplex and generate accurate fluorescent signal.

A new generation of saturation dyes, specifically developed for high-resolution melting can avoid this situation. These new dyes like LCGreen, EVA Green and SYTO9 are fully compatible with PCR with a wide range of concentration and sensitive enough for single-base variants and small insertions or deletions.

2.1.3 HRM Instrumentation

For many years, researchers have examined the utility of high resolution DNA dissociation analysis. Idaho Technology was first to market an instrument made specifically to perform an HRM analysis which helps researchers understand the potential of HRM by acquisition of an individual melt curve. But as the first generation of HRM platform, the HR-1 is not capable of thermal cycling and can only analyze a single sample from within a glass capillary per run making data analysis time consuming.

Multi-well instruments were introduced into the market soon after the HR-1. Although Corbertt Life Science and Idaho Technology use the same fundamental principle, different techniques are applied to realize HRM analysis. Rotor-Gene 6000 (Corbertt Life Science) was the first multi-well instruments capable of both thermal cycling and HRM. The main design advantage of the Rotor-Gene 6000 is that all samples are arranged on a round rotor and fluorescent signals can be recorded by a common optical detector. This design can minimize the thermal and optical variation between wells which is critical to HRM.

Recently the LightCycler 480 (Roche), 7000 serial (ABI) and CFX system (Bio Rad)

were designed for both HRM and thermal cycling. Roche, ABI and Bio Rad all adopted the block design.

2.1.4 Primer design

Successful amplicon design is an important consideration for successful HRM. Well-optimized PCR is needed for maximum sensitivity and specificity. If designed primers produce the nonspecific PCR product or primer dimmer, it will significantly reduce the sensitivity of the HRM method. The length of the resulting DNA amplicon may also impact the sensitivity and specificity of subsequent HRM analysis. Generally speaking, HRM primers with anneal temperatures of 60°C and generating 100–250 bp products should be adopted. The short amplicons generate a greater change of fluorescence intensity than long amplicons which may benefit the sensitivity of HRM. However, care should be taken to not make an amplicon too short. Relativly short amplicons tends to bind less dye because fewer double-stranded products are amplified. However using products above 250 bp can reduce sensitivity due to the increased potential for multiple melt domains with complicated melt curves.

2.2 Materials and Methods

2.2.1 HRM Machine specifications

ABI 7300 fast

The Applied Biosystems 7000 instrument is a block-based 96-well thermal cycler.

The 7300 system has a tungsten-halogen lamp for excitation of fluorescent dye. The dye which can be detected include FAM, SYBR Green I, VIC, JOE and TAMRA. The Passive Reference Dye, ROX, is used to normalize real-time PCR reactions. A Charge-Coupled Device (CCD) camera is used for signal detection.

Lightcycler II 480

The LightCycler 480 Real-Time PCR System is a high throughput gene quantification or genotyping real-time PCR platform with exchangable blocks for 96 and 384 samples in multi-well plates. LightCycler II 480 showed high specificity and yield because the innovative silver thermal block design rapidly and accurately arrives at and maintains reaction temperatures. The LighCycler is a rapid cycler which pumps air through a chamber to heat and cool the silver thermal block. Usually the LightCycler 480 System enables completion of a PCR run within 40 minutes without Sacrifice of well-to-well homogeneity.

The LightCycler 480 Real-Time PCR System employs a high-intensity Xenon lamp that emits light over a broad wavelength range (430–630 nm). The five excitation and six emission filters of the system can be used in any combination.

Bio-rad CFX 96

The CFX 96 system is a 96-well block system. Six independently controlled thermal electric modules control the heating and cooling elements of the thermal cycler to maintain

tight temperature uniformity. CFX 96 uses the Bio-rad 1000-series cycler which exhibits high average ramp rates, rapid settling time and tight thermal uniformity.

The optics design of CFX 96 adopts a shuttle system and the optics scanner reproducibly centered above each well without affection of light path. CFX system can detect multiple dyes at the same time and include the FAM, SYBR Green, HEX, Texas Red, Cy5 and Quasar 705. Further details can be obtained from the respective company websites:

Roche: www.roche.com/

Applied Biosystems: www.AppliedBiosystems.com

Bio-rad Laboratory: www.bio-rad.com/

2.2.2 DNA sample

Colorectal tissue and cell line samples were prepared at the Cleveland Clinic by standard methods. Genomic DNA was extracted using a QIAamp Tissue Extraction kit (Qiagen, Hilden, Germany). Mutation in primary tumors and cell line were determined using sanger sequencing method. Wild type human genomic DNA samples were purchased from Promega (Madison, Wisconsin, USA). The samples containing low-abundance mutated DNA were created by diluting the mutated cell line DNA samples (copy numbers were measured by Real time-PCR) with wild type DNA.

2.2.3 HRM Primer

HRM primers contain 20-42 nucleotides in length; a reverse oligonucleotide primer was designed starting 90-200 bp downstream on the target site. The sequences of oligonucleotide primers for mutation scanning are provided. We studied Kras and P53 mutations. Kras gene primers were designed to span codon 12 and 13. Kras primers were designed with 3 different lengths for the best sensitivity. The T3 and T7 tails are introduced for further Sanger sequencing. This significantly reduces the number of pipetting steps in the DNA sequencing because all of the samples can be sequenced using T3 and T7 sequencing primers and don’t require specific primers for each amplicon. The influence of T3 and T7 universal tails on HRM Primer result was also studied.

Table I Sequences of primer pairs used in HRM analysis

Name	The size of amplicon	Primer Sequence (5’ to 3’)
P53 175F with tail	108 bp	GTAATACGACTCACTATAGG TACAGCACATGACGGAGGTTG
P53 179R with tail		CCCTTTAGTGAGGGTTAATT GCTCACCATCGCTATCTGAG
P53 196F with tail	164 bp	GTAATACGACTCACTATAGG ACTGATTGCTCTTAGGTCTGG
P53 213R with tail		CCCTTTAGTGAGGGTTAATT GGCGGCTCATAGGGCACCACC
P53 245F with tail	114 bp	GTAATACGACTCACTATAGG CAACTACATGTGTAACAGTTCC
P53 248R with tail		CCCTTTAGTGAGGGTTAATT CTTCCAGTGTGATGATGGTG
P53 273F with tail	150 bp	GTAATACGACTCACTATAGG CCTATCCTGAGTAGTGGTAATC
P53 282R with tail		CCCTTTAGTGAGGGTTAATT CCTTTCTTGCGGAGATTCTC
P53 175F	68 bp	TACAGCACATGACGGAGGTTG
P53 179R		GCTCACCATCGCTATCTGAG
P53 196F	124 bp	ACTGATTGCTCTTAGGTCTGG
P53 213R		GGCGGCTCATAGGGCACCACC
P53 245F	74 bp	CAACTACATGTGTAACAGTTCC
P53 248R		CTTCCAGTGTGATGATGGTG
P53 273F	110 bp	CCTATCCTGAGTAGTGGTAATC
P53 282R		CCTTTCTTGCGGAGATTCTC
Kras 80F	80 bp	AGGCCTGCTGAAAATGACTG
Kras 80R		GCTGTATCGTCAAGGCACTC
Kras 92F	92 bp	TTATAAGGCCTGCTGAAAATGACTGAA
Kras 92R		TGAATTAGCTGTATCGTCAAGGCACT
Kras 189F	189 bp	TCATTATTTTTATTATAAGGCCTGCTGAA
Kras 189R		CAAAGACTGGTCCTGCACCAGTA
Kras F with tail	120 bp	GTAATACGACTCACTATAGG AGGCCTGCTGAAAATGACTG
Kras R with tail		CCCTTTAGTGAGGGTTAATT GCTGTATCGTCAAGGCACTC

2.2.4 Fluorescent Dye Comparison

The HRM kits from Applied Biosystem and Qiagen were chosen for comparison. The HRM kit from ABI contains SYTO 9 dye and the kit from Qiagen contains EvaGreen. The characteristic melting curves of two types of non-saturate dyes are analyzed on the Roche LightCycler 480 instruments.

2.2.5 Real-time PCR and Fluorescent Melting Curves

The HRM assay was performed in the LightCycler 480 (Roche Diagnostics; Switzerland). The same primers that amplify the genomic DNA were used for the HRM assay. PCR reactions were performed in triplicate in 10 µl final volume using Type-it® HRM PCR kit (Qiagen, Maryland/USA). 0.7 µM primer and 1 µl probe enriched PCR template were added into each HRM reaction. The thermocycling consisted of an initial incubation at 95ºC for 5 min followed by 50 cycles of 95ºC for 10 s, 55ºC for 30 s and 72ºC for 10 s and a final extension step of 10 min at 72ºC.

High resolution melting analysis was performed after PCR process with a florescence acquisition setting of 95ºC for 30s, then 60ºC for1min, florescence change was recorded during temperature ramping from 65-95ºC, rising by 0.1ºC/s. All the reactions were performed in triplicate.

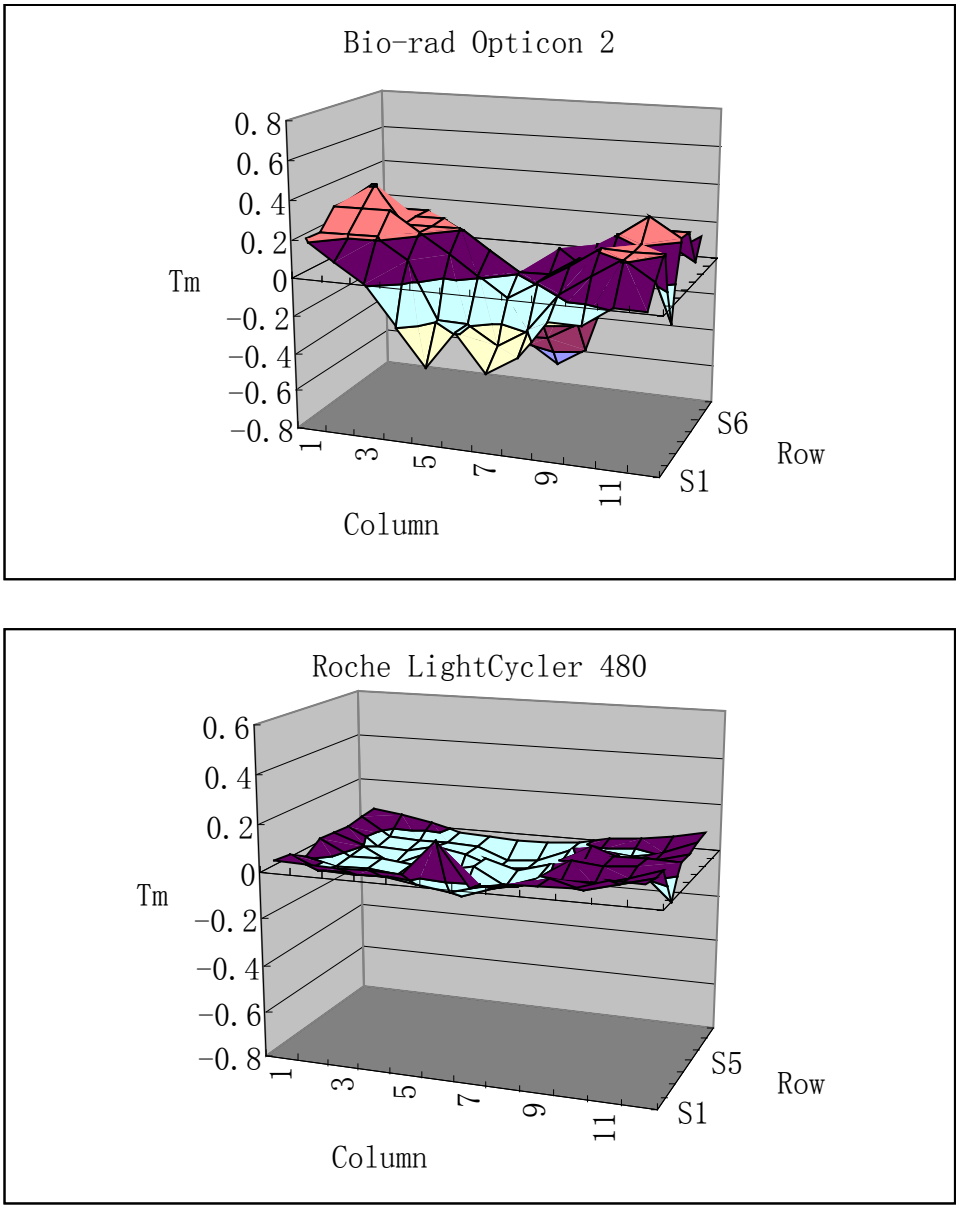


Figure 6 Well-to-well difference between regular and HRM real-time PCR system. The new generation real-time PCR system Roche LightCycler 480 display the accurately controlled temperature comparing to regular Bio-rad Opticon 2 real-time PCR system.

2.3 Results

2.3.1Performance of HRM instrument

For comparison purposes, two standard real-time PCR instruments were compared (Figure 6). The reproducibility of real time PCR instrument is a significant factor which determines the reliability of the HRM method. The well-to-well difference should be minimized in order to get a compact curve group. Two 48 replicates which contain one mutation sample and one normal sample was added into the 96 well plate. The melting curves of a 108 bp P53 175-2 G>A mutation amplicon were normalized by Bio-rad Precision Melt Analysis software.

In Figure 7, the mutation sample (red) can be clearly distinguished from normal sample (green) on CFX96 system. The CFX 96, LightCycler™ 480, and ABI 7300 fast are real-time PCR machines which have HRM capability. We tested the reproducibility and PCR variation of LightCycler 480, ABI 7300 fast and CFX 96 systems. The LightCycler 480 show more uniformity of the PCR reaction and melting curve. Also, the HRM software has better performance for mutation calling.

The reproducibility and well-to-well difference have a significant impact on the reliability of data. The standard deviation of the Tm shows the distribution of well-to-well difference among different real-time PCR systems, including the 7300 fast, Opticon 2, HRM instrument CFX 96 and LightCycler 480 by analyzing 10 to 96-well wild-type samples. Genomic DNA was amplified by Kras 189 primer. The ABI 7300 fast had the

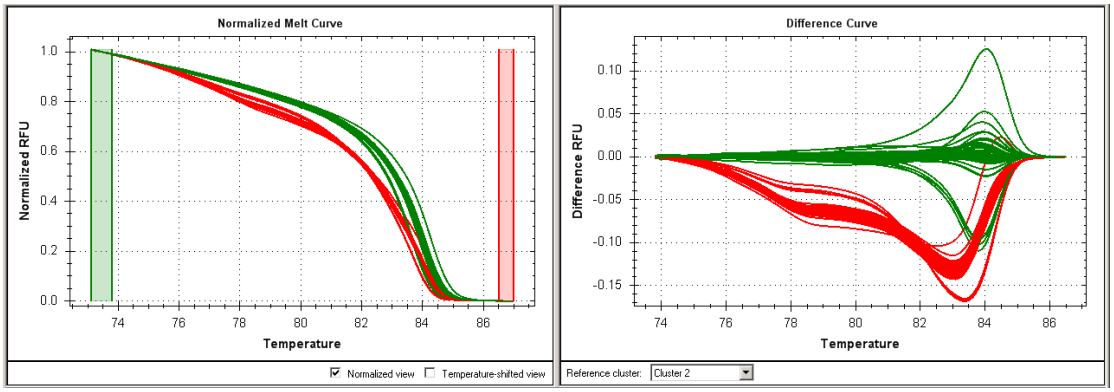


Figure 7 the reproducibility of Bio-rad CFX 96 real time PCR instrument. P53 175-2 G>A mutation sample and wild type samples are analyzed in 48 parallel experiment on a 96 well plate. The result shows that P53 mutation sample (red) can be distinguished from wild type sample (green) on CFX96 system.

Table II Well-to-well difference of Real-time PCR systems

	ABI 7300 Fast 10 well	Bio-rad Opticon 2 96 well	Bio-rad CFX 96 48 well	Roche LightCycler480 96 well
Range	0.17ºC	1.1 ºC	0.4 ºC	0.49ºC
Standard deviation	0.09	0.241	0.139	0.059

smallest Tm range at 0.40 °C across the plate (due to the smallest sample number). The standard deviation of LightCycler 480 is the smallest (0.058) which shows the higher reproducible and smaller well-to-well difference.

The Table II provides the range and standard deviation of the 3 different instruments. Because of the highly controlled temperature of well-to-well difference, the Roche LightCycler 480 was chosen for the rest of study.

2.3.2 Performance of intercalating dye

There are several commercial available HRM kits containing highly specific and non-saturated fluorescent dyes. The new double strand DNA binding dyes avoid “dye jumping” effect during DNA melting. The LCGreen, EvaGreen and SYTO dye are newly developed for high sensitivity and specificity of mutation detection.

The Melt-Doctor^{TM} HRM kit from Applied Biosystem and Type-it TM HRM kit from Qiagen were compared side-by-side on LightCycler 480 system.

2.3.3 Primer design in HRM mutaton analysis

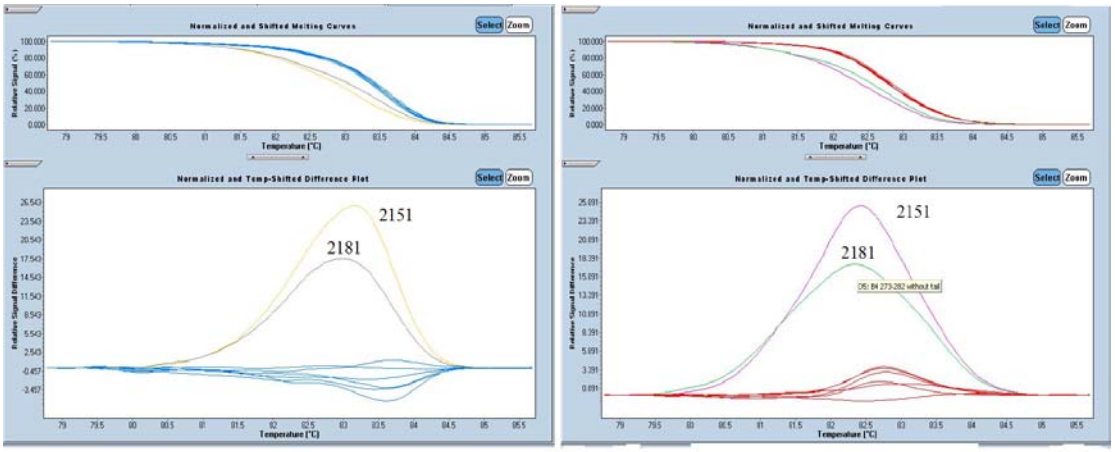
The primer design of HRM mutation analysis follows the general rule of PCR amplification. The Primer 3 software was used to minimize the internal secondary structure and primer dimers. The melting temperatures of primers are around 60 ^{o}C and melting temperature differences between forward and reverse primers are less than 2 ^{o}C. Amplicon size and GC content are also critical parameters for successful HRM analysis. Small

amplicons with low GC content (80-200 bp) are adopted in the assay for two reasons. 1) Short amplicons with low GC content shows more melting temperature difference between homozygote samples. 2) Because of degradation of genomic DNA in stool samples, short amplicons have higher chance to be amplified (Olson, Whitney et al. 2005; Colotte, Couallier et al. 2009). A wild type DNA template is always included in the HRM analysis to evaluate the amplification results of tissue or stool DNA samples. The specificity of PCR is confirmed by comparing the single melting peaking plots. For each sample, 3 technical replicates are prepared.

The effect of universal tail

Because the original primers which flank the p53 and Kras mutations are not designed for HRM analysis, although the 5 primer pairs perform excellent mutation screening, the assessment of primer without attached T3 and T7 tails was carried out on the LightCycler 480 system (Figure 8).

Colon cancer tissue samples 2151 and 2181 which contain the p53 273 codon CGT> TGT mutation confirmed by sequencing method were amplified by primer 273F and 282R.



p53 primer 273F and 282 R with tail

p53 primer 273F and 282R without tail

Figure 8 the effect of universal tail in HRM analysis. Universal tail will produce a longer PCR amplicon. The difference curves shows that without T3 and T7 universal tail P53 primer pair didn't lost its discrimination capability.

The data are shown for the same target amplified using PCR primers with and without T3 and T7 universal DNA tail appended to the 5' ends. The melt profiles of homozygous wild type and heterozygous mutant can be distinguished from each other, but since the amplicons have different sequences, the curves are slightly different.

The influence of amplicon length

Kras primers giving 80, 92 and 189 bp PCR amplicons were designed to test the effect of amplicon size on the sensitivity of detecting mutant sequence in a background of normal DNA. The sensitivity of HRM assay is the key to successfully identifying certain mutations.

Generally smaller amplicons gave better discrimination between mutation and wild type amplicons. But in our study, the result shows that this rule doesn’t always apply. Two Kras mutation samples 1186 which contains 35G>T mutation and 34G>T mutation individually were screened by three pairs of Kras primers. The longest 189 bp amplicons with 37% G+C content gaves the best resolution. The 80 bp amplicons with 49% G+C content can discriminate the mutant from wild type at a low resolution. The 92 bp with 43 % G+C content amplicons showed no melting curve difference between wild types and mutants. The sensitivity of mutation detection using the 80, 92 and 189 bp PCR amplicons shows that the length of the PCR amplicon is not the only factor determining the HRM sensitivity, the GC content and secondary structure of DNA duplex also have significant influence.

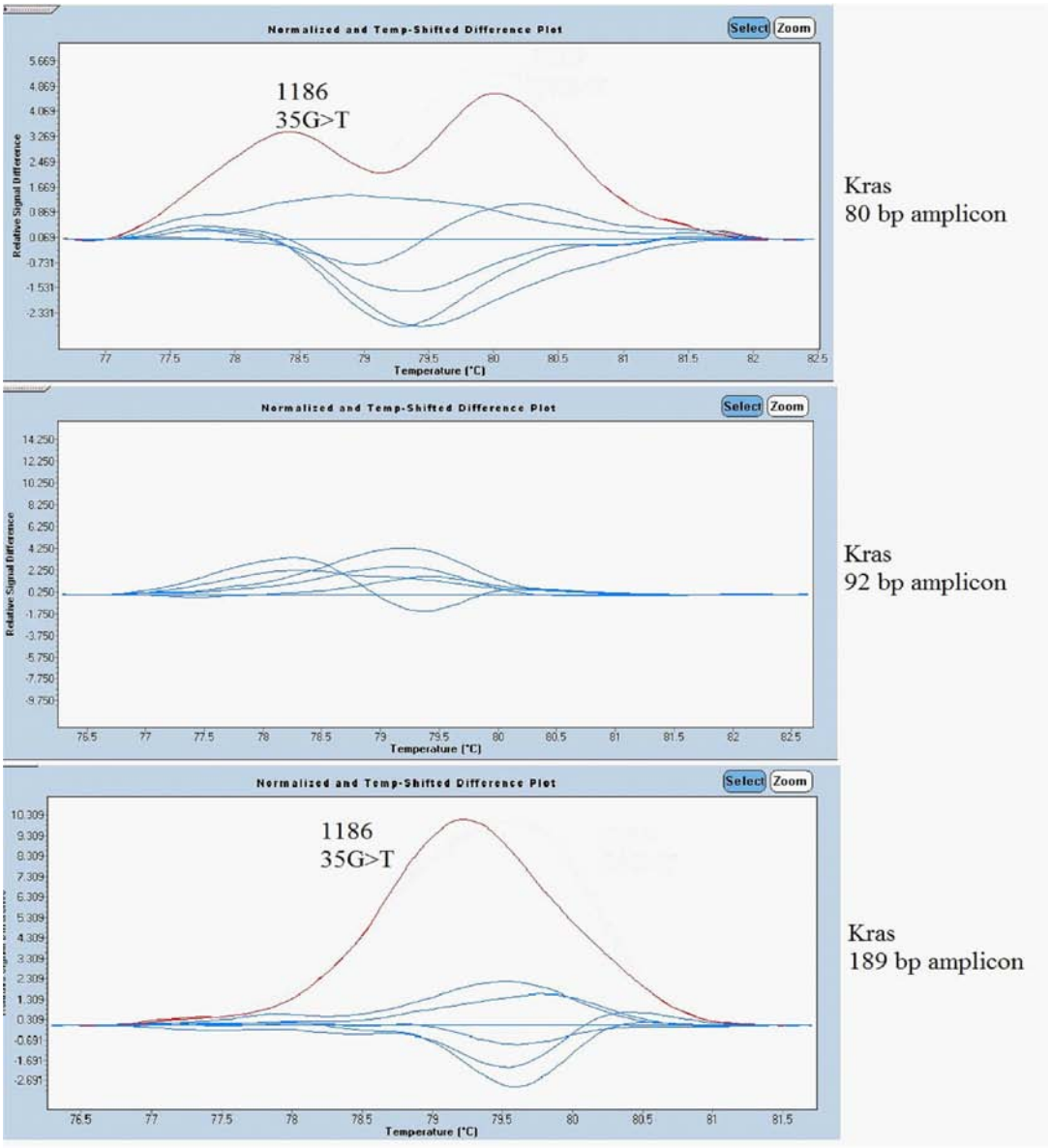


Figure 9 the effect of amplicon length in HRM analysis. The 80 bp amplicons show slightly difference between mutation and wild type sample. The 92 bp amplicons show no difference. The 189 bp amplicons have best discrimination ability to identify the mutation samples.

2.3.4 Assay validation

Cancer tissue samples with known p53 mutations were first used to test the HRM assay. The mutation types are listed in the Table III. Four pairs of p53 HRM primer were able to discriminate between wild-type DNA and different mutations. Both these colon cancer tissue samples showed typical heteroduplex melting patterns and were readily distinguishable from wild-type samples.

Table III List of eight P53 mutation samples

Tissue sample	Type	Mutation
B1	colon carcinoma	p53 codon175 CGC>CAC
B2	colon carcinoma	p53 codon213 CGA>TGA
B3	colon carcinoma	p53 codon273 CGT>TGT
B4	colon carcinoma	p53 codon273 CGT>TGT
B5	colon carcinoma	p53 codon196 CGA>TGA
B6	colon carcinoma	p53 codon213 CGA>TGA
B7	colon carcinoma	p53 codon248 CGG>CAG
B8	colon carcinoma	p53 codon196 CGA>TGA

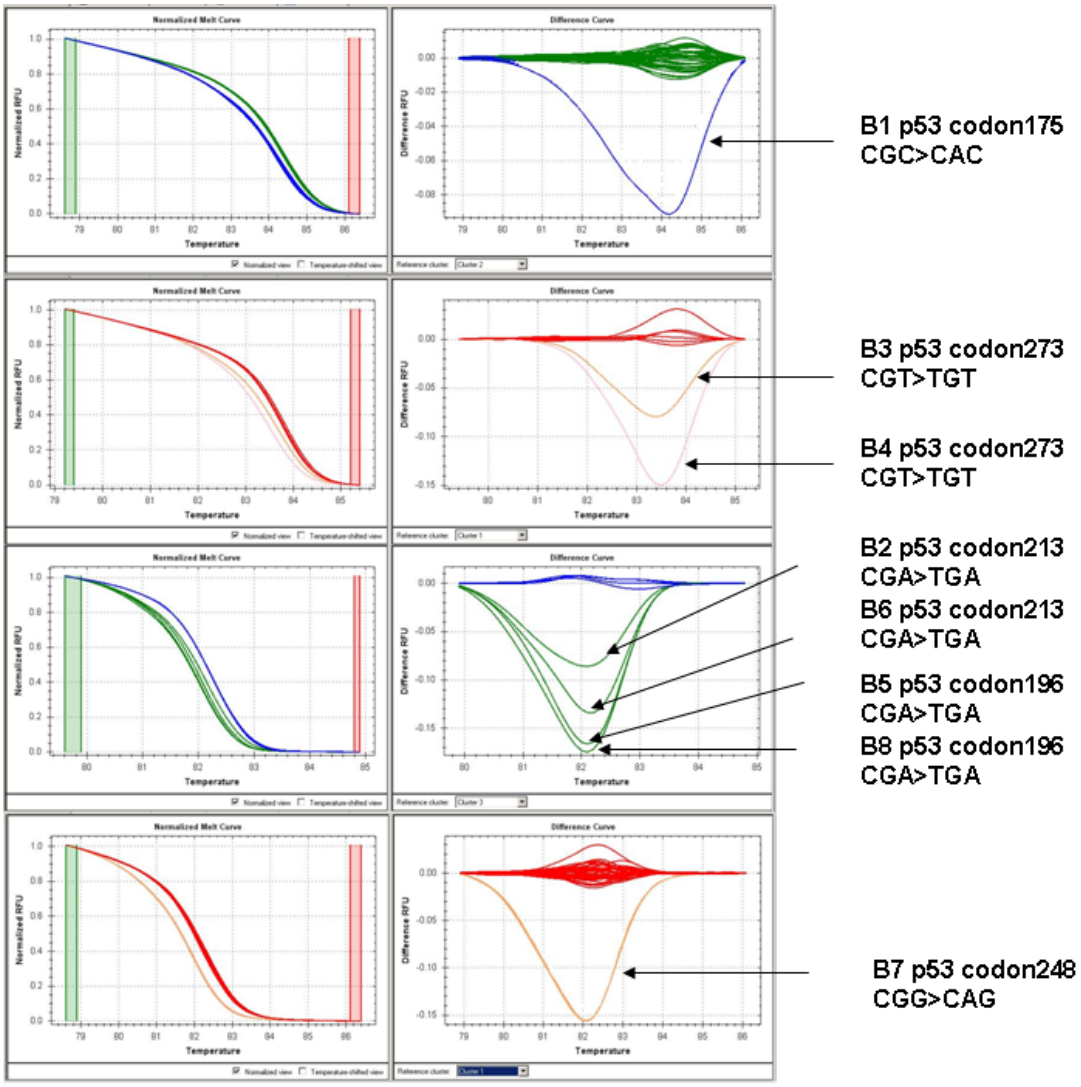


Figure 10 HRM analysis results of eight p53 mutation samples. From top to bottom, HRM curves showed one P53 mutation from codon 175 to179, two P53 mutations from codon 273 to 282, four mutations from codon 196 to 213 and one mutation from codon 245 to 248.

2.3.5 High resolution melting analytical sensitivity testing

The cancer tissue DNA containing mutation in p53 273-1 codon was mixed with wild-type DNA in dilution of 100%, 50%, 20%, and 10%. The dilutions were tested by using 150 bp amplicons. Figure 11 shows the difference plots for both PCR amplicons with the cell line dilutions. Using the 150 bp amplicon, we were able to detect 20% mutation in wild-type DNA.

2.3.6 Kras and p53 mutation detection in colorectal cancer tissue and cell line DNA samples

Table IV shows a table for cell line control, wild-type control, and a selection of patient tissue sample with Kras, p53 mutations. The Kras and p53 primers were used for screening mutation in DNA of 20 colorectal tissue sample and 11 cell lines. We detected the presence of 19 variant curves in the 32 samples. Sixteen mutations were identified by HRM using p53 primer and 7 mutations were identified using Kras 189bp primer. After HRM analysis, the PCR amplicons with aberrant curves were subject to the Sanger Sequencing method. The summary of all mutations detected in the panel of 20 colon cancer biopsy samples and 11 cell line samples is presented in Table IV.

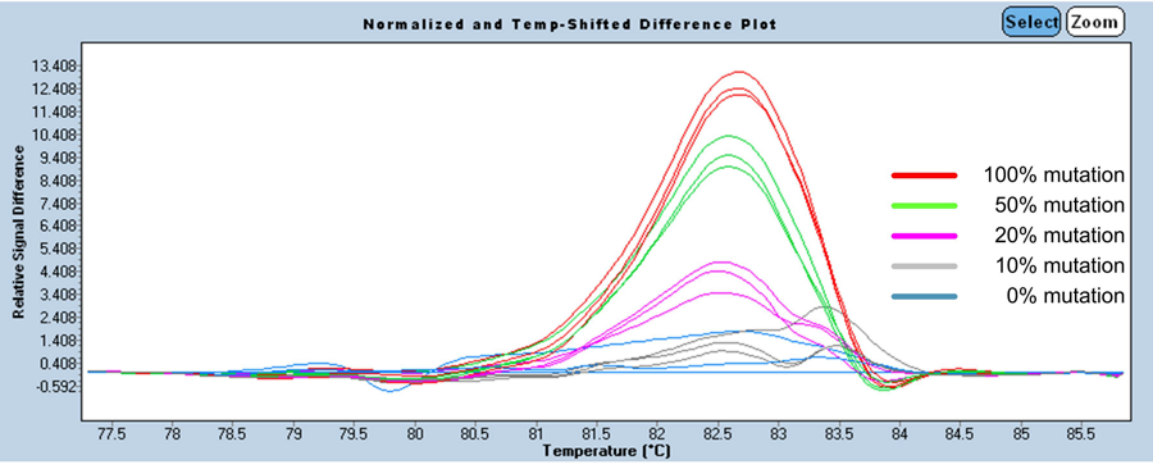


Figure 11 Sensitivity of high resolution melting analysis. Depend on the primer design and mutation type, the sensitivity of high resolution melting analysis ranges from 10% to 20%.

Table IV P53 and Kras mutation results determined via HRM and sequencing

Sample	Histology	HRM	Sequencing
779	Adenocarcinoma	WT	WT
864	Adenocarcinoma	Not Covered	P53 190 CCT>CTT
788	Adenocarcinoma	P53 mutation	P53 267 CGG>CCG
946	Adenocarcinoma	P53 mutation	P53 274 GTT>TTT
		Kras mutation	Unsequenced
955	Adenocarcinoma	Kras mutation	Kras 12 GGT>GAT
1012	Adenocarcinoma	Kras mutation	Kras 12 GGT> TGT
		P53 WT	P53 248 CGG>TGG
1122	Adenocarcinoma	Kras mutation	Kras 12 GGT> TGT
1186	Adenocarcinoma	Kras mutation	Kras 12 GGT> GTT
1278	Adenocarcinoma	Kras mutation	Kras 12 GGT> GTT
1319	Adenocarcinoma	WT	Kras 12 GGT> TGT
1166	Adenocarcinoma	P53 mutation	P53 175 CGC>CAC
1719	Adenocarcinoma	P53 mutation	P53 248 CGG>CAG
1913	Adenocarcinoma	WT	P53 175 CGC>CAC
2088	Adenocarcinoma	P53 mutation	P53 213 CGA>TGA
2151	Adenocarcinoma	P53 mutation	P53 273 CGT> TGT
2181	Adenocarcinoma	P53 mutation	P53 273 CGT> TGT
2373	Adenocarcinoma	P53 mutation	P53 196 CGA>TGA
2381	Adenocarcinoma	P53 mutation	P53 213 CGA>TGA
2418	Adenocarcinoma	P53 mutation	P53 248 CGG>CAG
2779	Adenocarcinoma	P53 mutation	P53 196 CGA>TGA
V8	Adenocarcinoma	WT	WT
HCT 116	Adenocarcinoma	WT	WT
V410	Cell line	WT	WT
V425	Cell line	P53 mutation	P53 248 CGG>TGG
V441	Cell line	P53 mutation	P53 193 CAT>CGT
V451	Cell line	WT	WT
V456	Cell line	WT	WT
V478	Cell line	P53 mutation	Unsequenced
V489	Cell line	WT	WT
V576	Cell line	P53 mutation	P53 273 CGT>CTT
V531	Cell line	WT	P53 205 TAT>TTT
Normal	Cell line	WT	Normal
Sensitivity		19/23	
Specificity		8/8	

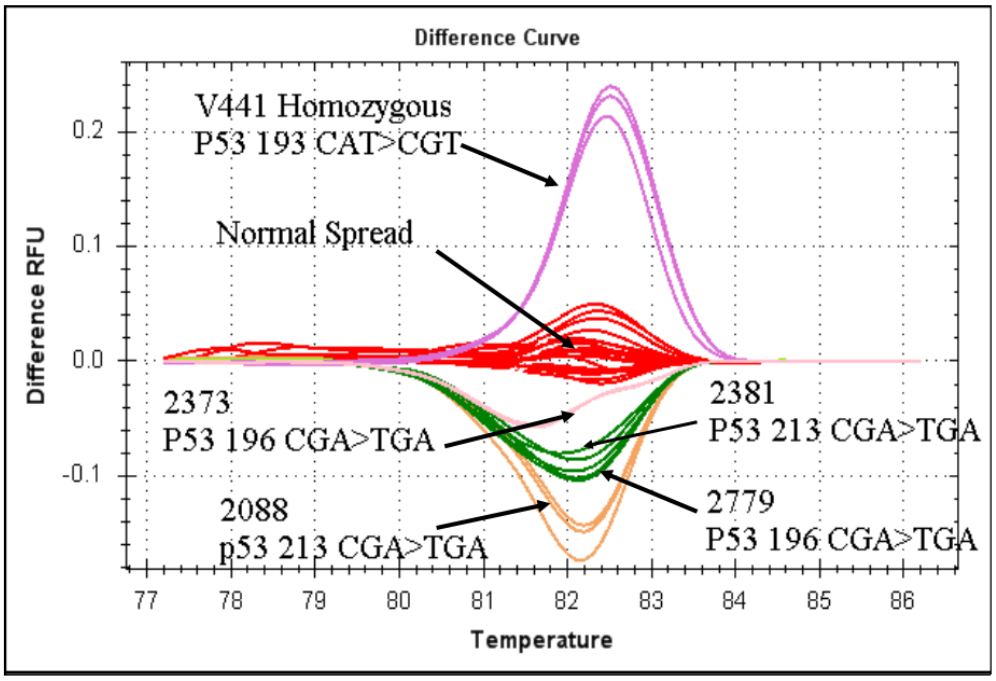


Figure 12 Comparing HRM difference plot and Sanger sequencing results in wild type sample and P53 196-213 mutations samples. 2373, 2381, 2088 and 2779 are colorectal cancer tissue samples. V441 is cell line DNA sample. The 10 µl HRM test of each sample was performed in triplicate.

Figure 12 shows a difference plot and sequencing results for cell line controls, wild-type samples and a selection of patients with P53 196-213 mutations. Cell line sample V441, which had HRM curves above the wild-type curves, showed the presence of a homozygous p53 193 CAT>CGT mutations with prominent mutant peaks. Sample 2381 and 2088 all contained P53 codon 213 CGA>TGA mutation which had a HRM curve that deviated less from the wild-type plot. Sample 2381 showed a relative small peak for the low mutation to wild type ratio in DNA template. The same situation applied to sample 2373 and 2779 with P53 196 codon CGA>TGA mutation.

2.4 Discussion

The principle of HRM is to measures the fluorescent signal changes of double-stranded DNA molecules melting. Usually the template will be amplified by PCR reaction which will generate a strong enough signal for detection. The new fluorescent dyes can bind more specifically to the double-stranded but not single-stranded DNA at a high concentration without suppressing the PCR reaction. Once the dyes bind to the DNA, they don't redistribute along the DNA duplex like old non-saturation dyes which can show biased melting curve and omit the difference between the homoduplex and heteroduplex.

The sensitivity and accuracy of heterozygote detection were both high. Most of research groups can determine close to 100% of mutations, though there may be a small amount of false positive results. Point mutations share the same pattern. The conducted study showed the lowest level of mutation detection by HRM is 5% or lower; the majority

of detection limit is around 10-20%.

The volume change due to PCR buffer evaporation from badly sealed membrane or calibration of auto pipette can cause difficulties to analyze sample. Theoretically, the fluorescence signal change is the only factor for HRM and normalization can minimize the data deviation due to volume change. But from our experience, a 10 % volume change causes the problem of a wide melting curve distribution.

There are not established rules for HRM primer design. All categories of mutations, insertions, and deletions can be detected as long as the PCR reaction can be performed. Location of mutation positions in the PCR amplicon can clearly affect the shape and magnitude of melting curve for certain primer pairs. Mutations are detectable at any position at any location in the amplicon, even close to or within the PCR primers. Sample V441 with a mutation at P53 codon 193 CAT>CGT is a perfect example (Figure 12). Amplicon length and GC content may also influence the sensitivity of HRM assay, but the relationship among them is not clear enough to help primer design. The type of mutation is also important for successful detection. The switch between GC base pair to AT base pair usually shows the highest sensitivity. A>T mutation generate comparatively small signal change, and for our assay of the sample V531 with P53 codon 205 TAT>TTT, this mutation was unable to be detected. But carefully designed primers do detect A to T or T to A mutations. The melting domains within the amplicon and secondary structure of the amplicon may have more impact on the HRM analysis.

Single Nucleotide Polymorphism (SNP) within an amplicon is a challenge for the

HRM method too. False positive results may result from SNP existence. As a result, positive results from HRM assay always need the sequencing method to confirm the identity of variants.

In conclusion, we have developed a robust assay for screening P53 and Kras mutations that is found in clinical samples. The main limitation of HRM analysis is that the precise mutation cannot be readily identified, and the Sanger sequencing method is needed to confirm the result. HRM is a closed tube method with less possibility of contamination and template purification. Comparing to the other scanning method, the HRM analysis has a lower cost and easily prepare samples. The sensitivity and specificity is higher than the most of traditional enzyme based method. The high throughput of HRM is possible in the future with development of nano-scaled PCR reaction.

CHAPTER III

ALTERATIONS OF DNA METHYLATION IN COLORECTAL CANCER

3.1 Introduction

Colorectal cancer is the third leading cancer type for males in the United States. According to the statistic data from Center for Disease Control and Prevention (CDC), 139,127 people were diagnosed with colorectal cancer, and 53,196 people died from it in 2006. Because of the long study time, the essential mechanism about cancer is becoming clear. There are more approaches to diagnose cancer at early stage and improve treatment.

Gene mutations of cancer have been associated with the main focus during the last decades. Two sorts of genes control the proliferation of human cells: 1) proto-oncogenes, which serve as accelerators to activate the genes of the cell; and 2) tumor suppressor genes, which can slow down the growth of cells. When proto-oncogenes are mutated, they will lead to cancer. But recent research found that epigenetic events might be the origin of human cancer. Different from genetic changes, epigenetic changes have a higher incidence rate than genetic change. Epigenetic changes include DNA methylation and histone modification (methylation or acetylation). Those epigenetic changes directly affect secondary structures of chromatin and promoter sequence of certain genes without influencing the native DNA sequence. When the promoter region is heavily methylated, the downstream gene will not be transcripted. The aberrant methylation of gene promoter regions may result in loss of tumor-suppressor gene function and lead to cancer.

3.1.1 Definition of CpG island

CpG islands are genomic regions that contain a high frequency of CG dideoxynucleotides. A definition of CpG island is the base pair number greater than 500, the GC content is no less than 55% and observed CpG/expected CpG ratio of greater than or equal to 0.65. CpG island is the target of methylation. Recent research results have shown that methylation of promoter CpG islands plays an important role in gene silencing, genomic imprinting, X-chromosome inactivation, and carcinogenesis. Based on this definition, more than 35% of the human protein coding genes have their own CpG islands. In mammalian genomes, CpG islands are typically 300-3,000 base pairs in length.

CpG islands typically occur at or near the transcription start site of genes,

particularly housekeeping genes. Normally a cytosine base followed immediately by a G (guanine) base (a CpG) is rare in vertebrate DNA because the cytosines in such an arrangement tend to be methylated. This methylation helps distinguish the newly synthesized DNA strand from the parent strand, which aids the final stages of DNA proofreading after DNA duplication (Bishop 1995; Jones and Laird 1999; Baylin and Herman 2000).

3.1.2 Methylation modification of Cytosine

Cytosine methylation occurs after DNA synthesis by transferring a methyl group from methyl donor S-adenosylmethionine to the carbon-5 position of cytosine. The enzymatic reaction is performed by DNA methyltransferase (DNMTs). Four active DNA methyltransferases have been identified in mammals: DNMT1, DNMT2, DNMT3A and DNMT3B. DNMT1 is the major enzyme in mammals, which can methylate the newly synthesized DNA strand after replication. DNMT1 is responsible for maintenance methylation in mammal (Saxonov, Berg et al. 2006; Yamada, Shirakawa et al. 2006).

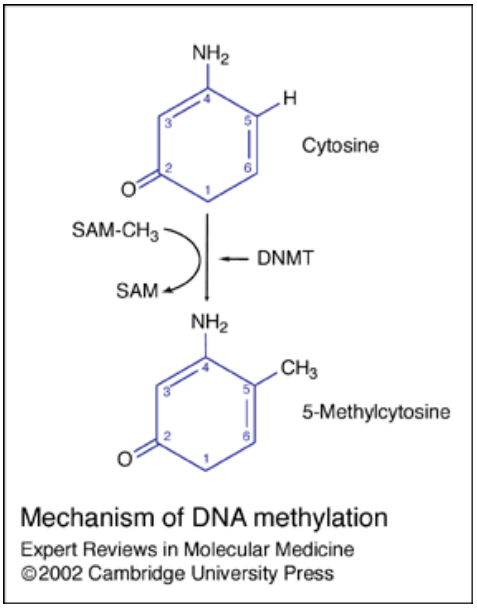


Figure 13 Mechanism of DNA methylation. 5-Methylcytosine is produced by the DNA methyltransferases (DNMT) that transfer a methyl group (CH3) from S-adenosylmethionine (SAM) to the carbon-5 position of cytosine.

Most methylated cytosine residues are located outside of CpG islands. Methylation of some CpG islands in non-cancer tissue increases with age, but the genomic 5-methylcytosine level decreases. Hypomethylation has been hypothesized to contribute to carcinogenesis by transcriptional activation of oncogene or activation of latent transposons. At the same time, hypermethylation of CpG island and histone modification leads to gene silencing, which is an early sign of carcinogenesis. Right now cancer-related DNA methylation research focuses on the hypermethylation status of the certain gene. Researchers find some stable and reliable methylated CpG islands as biomarkers for diagnosing early stage cancer (Robinson, Bohme et al. 2004; Holm, Jackson-Grusby et al. 2005).

3.1.3 Methylation and transcription regulation

Cytosine-5 DNA methylation occurs in mammals at CpG dinucleotides. About 70% of the CpG dinucleotides in the mammalian genome are methylated. According to Hanahan and Weinberg, six new phenomenon of a cell should take place before a tumor become malignant: (1) limitless replicative potential, (2) self-sufficiency in growth signals, (3) insensitivity to growth-inhibitory signals, (4) evasion of programmed cell death, (5) sustained angiogenesis, (6) tissue invasion and metastasis. The most studied genes are categorized by these six events. The transcriptional silencing of tumor suppressor genes by promoter CpG island hypermethylation can contribute to oncogenesis. New methylation markers are discovered every year.

Methylation of promoter CpG islands causes terminated transcription of downstream genes, which means loss of corresponding gene expression that regulates

tumor suppression, cell growth, differentiation and apoptosis.

3.1.4 DNA methylation in colon cancer

During cancer development two different changes in DNA methylation take place. One is the genome-wide hypomethylation and the hypermethylation in promoter-associated CpG islands related to tumor suppressor genes. Since both hypermethylation and hypomethylation are early events in cancinogenesis, detection of DNA methylation may greatly help to the early diagnosis of cancer.

The role of methylation in oncogenesis has been explored during the past 10 years. There are there possible mechanisms that determine the development of cancer development. First, hypermethyaltion of CpG islands in promoter regions can delay or stop the transcription of tumor suppress genes. P16, MGMT, E-cadherin and RB are identified as methylation inactivated genes (Chen, Mao et al. 2004; Krakowczyk, Strzelczyk et al. 2008; Attaleb, El hamadani et al. 2009; Moreira, Guimaraes et al. 2009). Second, mutations are closely associated with DNA hypermethylation patterns. Hypermethylation of tumor suppressor genes like APC, MGMT, and hMLH1 is very common in pre-invasive colorectal neoplasia causing the increased mutation rate among the oncogenes (Nagasaka, Sasamoto et al. 2004; Judson, Stewart et al. 2006). Finally, Chromosomal instability and microsatellite instability can be initialized by DNA methylation (Kaina, Ziouta et al. 1997; Derks, Postma et al. 2008).

Jones and Baylin point out that the tumor development can be triggered by promoter hypermethylation of tumor suppressor genes. Genome-wide loss and regional gains of DNA methylation are associated with inappropriate gene silencing, which significantly affect tumor progression.

3.1.5 Methylation frequency of ALX4, UNC5, EYA, BMP3 and GATA5

Aristaless-like homeobox-4

Aristaless-like homeobox-4 (ALX4) gene controls parietal bone growth, which is localized in human genome 11p11.2 (Wu, Badano et al. 2000). Mutation of ALX4 gene may cause skull defects (Wuyts, Cleiren et al. 2000) and craniofacial development as in skin and hair follicle development in human (Kayserili, Uz et al. 2009). ALX4 also plays a major role in anterior-posterior pattern formation during bovine limb development (Wohlke, Kuiper et al. 2006). Lymphoid Enhancer Factor-1 gene also can interact with ALX4 gene during early embryonic development, especially for embryonic vasculogenesis (Boras-Granic, Grosschedl et al. 2006). Chang‘s group displays that loss of ALX4 expression in epithelial and stromal cells may be a potential marker for breast cancer (Chang, Mohabir et al. 2009). Recently research results support ALX4 could be a sensitive methyaltion marker for cancer diagnosis. In 2006 Ebert and Model first pointed out that heavily methylated ALX4 genes could be a novel genetic and epigenetic marker indicative of changes in the pathogenesis of colon cancer. They used methylation-specific arbitrarily primed polymerase chain reaction to identify 41/60 colon adenoma and primary colorectal cancer samples (Ebert, Model et al. 2006). This result was confirmed by study conducted by Hongzhi Zou in 2007 (Zou, Harrington et al. 2007). Another research result shows that methylation frequency of ALX4 gene in bladder cancer was around 25% which is much lower than colorectal cancer, but still could be a potential methylation marker when combining with other genes (Yu, Zhu et al. 2007). The frequency of methylated ALX4 gene in colorectal cancer is 56%-68% (He, Chen et al.; Ebert, Model et al. 2006; Zou, Harrington et al. 2007).

UNC5 family

UNC5 gene belongs to a family of netrin-1 receptors, but UNC5C only shows limited expression in certain adult human tissues (Ackerman and Knowles 1998). Netrin-1 receptors mediate several critical neuronal and vascular development (Moore, Tessier-Lavigne et al. 2007; Bernet and Fitamant 2008; Freitas, Larrivee et al. 2008; Low, Culbertson et al. 2008). Recent study shows that UNC5C gene generated UNC5 receptors and these UNC5 receptors facilitate chemorepulsion away from Netrin source (Dillon, Jevince et al. 2007). The molecular mechanisms of UNC5C gene expression are systematically studied. Netrin-1 and its receptors deleted in colorectal cancer play an important part in tumorigenesis by inhibiting p53-depended apoptosis (Arakawa 2004). Recent research indicated that UNC5C methylation was significantly correlated with colorectal cancer development. Bernet and Mazelin's results show that UNC5C expression is down-regulated in human colorectal cancers through promoter methylation and loss of UNC5C expression observed in human colorectal cancer is the cause of increased intestinal tumor progression and a decrease in tumor cell apoptosis (Bernet, Mazelin et al. 2007). Comparing with the 6% methylated UNC5C gene in normal mucosa, UNC5C was significantly methylated in colorectal cancer (76.2%) and adenomatous polyps (63.5%) (Shin, Nagasaka et al. 2007). The research result from 36 patients' primary carcinomas and the corresponding normal tissues in 2009 showed that aberrant methylation of the UNC5C gene is as high as 69% (Hibi, Mizukami et al. 2009).

Eyes absent (EYA) family

EYA2 and EYA4 are key regulators of ocular development. Lost expression of EYA gene will cause absence of eye development (Borsani, DeGrandi et al. 1999). It is also found that EYA4 is important for the maturation of organ of Corti (Wayne, Robertson et al. 2001). An EYA4 point mutation caused hearing loss was reported, which was thought because of long-term exposure to loud noise (Hildebrand, Coman et al. 2007). EYA4 expression also shows strong correlation with premalignant esophageal cancer (Li, Diao et al. 2009). Zou and Harrington showed that the EYA2 gene was highly methylated in colorectal neoplasia (Zou, Harrington et al. 2007). Another study from Osborn and Zou showed that EYA4 gene promoter methylation in colorectal tissues can be used for discrimination of neoplasia in chromic ulcerative colitis. The EYA4 gene promoter is heavily methylated commonly in sporadic and colitic neoplasia and may be a potential methylation marker for neoplasia in chronic ulcerative colitis. EYA4 promoter shows hypermethylation in 80% of colorectal cancers (Osborn, Zou et al. 2006).

Bone morphogenetic protein 3 (BMP3)

Bone morphogenetic protein (BMP) is responsible for regulating cell proliferation, differentiation and apoptosis in different cell types. BMP mediates two signaling pathways: 1) BMP binds to transmembrane receptors and activates their kinase activity, then the kinase phosphorylates SMAD protein(Euler-Taimor and Heger 2006); 2) Epigenetic silencing of BMP antigrowth signal can activate Ras/MAPK pathway and further induce the carcinogensis. BMP induces endochondral bone formation in human skeletal(Luyten, Cunningham et al. 1992). BMP signaling has multiple purposes of chondrogenesis and

endochondral bone formation, especially for the earliest stages of skeletogenesis (Gamer, Cox et al. 2009). Lost function of BMP genes may cause several disorders of cartilage and bone formation (Tabas, Zasloff et al. 1991). BMP3 acts as a negative regulator of bone formation by limiting BMP signal transduction, and BMP3 has a unique expression pattern in the development of perichondrium (Gamer, Ho et al. 2008). Because BMP proteins are one of the TGFB growth factor families in bone formation, inactivation of BMP3 gene can be served as an early event in colorectal cancer development (Loh, Chia et al. 2008). 55-66% methylated BMP3 gene was observed in different colorectal cancer study. (Zou, Harrington et al. 2007; Loh, Chia et al. 2008)

GATA transcription factor

The GATA family of zinc finger proteins is transcriptional regulators which regulate lineage differentiation and embryonic development. The GATA5 is present essentially in the heart and gut (Nemer, Qureshi et al. 1999). GATA5 regulates expression of Nkx2.5 transcription factor by antagonizing transcription of Nkx2.5 (Jiang, Drysdale et al. 1999). Molkentin and Tymitz showed that during mouse embryogenesis GATA5 expressed in the heart and lung, vasculature, and genitourinary system. Mutation in GATA5 will cause abnormal development of the female genitourinary system (Molkentin, Tymitz et al. 2000). Huggins and Wong's study confirmed that GATA5 can activate the progesterone receptor gene promoter in breast cancer cells (Huggins, Wong et al. 2006). GATA5 is silenced by epigenetic events in cancer development, which are shown by several studies (Akiyama, Watkins et al. 2003; Guo, Akiyama et al. 2004; Guo, House et al. 2006). Derks and Postma comprehensively analyzed promoter methylation status of hMLH1, O6MGMT,

APC, p14F, p16, RASSF1A, GATA4, GATA5, and CHFR using methylation-specific PCR technology. Results show significant correlation between promoter methylation of APC, P16, GATA4 and GATA5 and colorectal cancer (Derks, Postma et al. 2006). Fu's research also displays that GATA4 and 5 play a role in carcinogenesis of human tumors derived of endodermal and mesodermal origin. GATA4 and GATA5 methylation are related to occurrence of human pancreatic cancer (Fu, Guo et al. 2007). Other studies show that GATA methylation may cause the development of sporadic gastric carcinomas. 53.8% GATA4 and 61.3% GATA5 methylation are observed in sporadic gastric carcinomas (Wen, Akiyama et al.; Akiyama, Watkins et al. 2003).

3.2 Laboratory methods for detecting methylation

There are two directions to analyze the methylation: 1) non-specific DNA methylation analysis and 2) gene specific analysis.

Before the bisulfite-modification method was invented, there were no useful methods to analyze the methylation status of a single gene. Therefore global methylation content, also called non specific DNA methylation, was the only factor which could be analyzed. The non-specific DNA methylation analysis plays an important role in the development of carcinogenesis. The nonspecific DNA methylation analysis can only give information about the total methylated cytosine in whole genomic DNA. But this factor can't help identify the cancer marker.

The current gene specific analysis is based on two approaches, restriction enzyme and bisulfite-modification. Restriction enzymes like Hpa II can cut unmethylated DNA but not methylated DNA. The limitation of restriction enzymes is that they only recognize certain DNA sequences. Bisulfite-modification is the standard method to analyze cytosine

methylation. The principle of bisulfite-modification is that sodium bisulfite deaminates cytosine located on single-stranded DNA. After the base treatment, the unmethylated cytosine will be converted to uracil and methylated cytosine will not be converted. Designing proper primers can recognize the modified DNA sequence. Combined with other techniques, detailed methylation status can be determined by the bisulfite-modification method. The bisulfite-modification method is the basis of present methylation analysis (Pollack, Stein et al. 1980; Oakeley 1999; Shames, Minna et al. 2007).

3.2.1 Global methylation content

The distribution of 5-methylcytosine residues from genomic DNA can be analyzed in different ways.

HPLC and TLC

LC-mass spectrometry is considered the gold standard for DNA-methylation analysis as it provides the user with the exact amount of methylated cytosines in a sample. However it is costly and requires a certain amount of expertise. In addition the DNA must be digested to the single nucleotide level prior to analysis. HPLC based DNA methylation analysis has several variations. Using single nucleotide primer extension (SNuPE) assays in combination with ion pair-reverse phase-high performance liquid chromatography (IP-RP-HPLC) separation techniques, methylated and unmethylated CpGs can be discriminated and quantified based on the different masses and hydrophobicities of the extended primer products. This method needs bisulfite converted DNA as the template. The HPLC and TLC rely on DNase to hydrolyze DNA to deoxyribonuleoside. Because of

the different mobility of 5-methylcytosine and cytosine, their intensity can be measured (Kuo, McCune et al. 1980; Bestor, Hellewell et al. 1984).

Chloracetaldehyde Reaction

Chloracetaldehyde reaction is a fluorescent assay of the DNA methylation level. 5-methylcytosine and chloracetaldehyde form ethenocytosine which is a fluorescent compound. Comparising genomic DNA from cell lines known to differ in their methylation levels, the fluorescence intensity can be interpreted to assess the level of 5-methylcytosine in the genome. One drawback of the chloroacetaldehyde reaction is the use of quite toxic reagent (Oakeley, Schmitt et al. 1999).

Immunological techniques

Immunological techniques make use of the highly specific reaction between monoclonal antibodies and 5-methylcytosine to study DNA methylation (Golbus, Palella et al. 1990; Bernardino, Lamoliatte et al. 1996). The shortcoming of immunological techniques is that they are only quantitative when the 5-methylcytosine is on the single strand DNA. Weber used an immunocapturing approach followed by DNA microarray analysis to generate methylation profiles of human genomic chromosomes CpG islands. The resolution of this method is around 80kb which can detect epigenetic modification of primary and transformed cells genome-wide (Weber, Davies et al. 2005).

The global methylation studies always need large amounts of high quality genomic DNA. These methods can’t give satisfactory data on trace amounts of DNA. Likewise the specificity and sensitivity is too low to be used in clinical samples.

3.2.2 Gene-specific methylation analysis

When the methylation status of a single gene is involved in research, there are two major kinds of methods to study methylation: 1) methylation-sensitive restriction enzymes, 2) bisulfite-conversion based method.

Methylation-sensitive restriction enzymes methods

Restriction enzyme methods do not change the sequence of DNA strand, but use methylation-sensitive restriction enzyme that can't cut the methylation DNA sequence. After incubation with restriction enzyme, the unmethylated DNA is digested and methylated DNA is left untouched. This method is only useful for probing a very limited number of potential methylation sites, so there is only limited information that can be gained. Another disadvantage of this method is that if incomplete digestion takes place, a false-positive result will be generated from the experiment.

Many variations of restriction enzyme-based methods have been used in conjunction with genomic analysis and they are discussed below.

Restriction Landmark Genomic Scanning (RLGS)

RLGS takes advantage of methylation sensitive restriction enzymes like NotI and AscI which specifically cleave the unmethylated genomic DNA and leave the methylated DNA untouched (Costello and Vertino 2002). The advantage of the RLGS method is that the gene sequence information can be unknown (Smiraglia, Fruhwald et al. 1999). RLGS employs direct labeling of the genomic DNA fragments digested by a restriction enzyme and two-dimensional electrophoresis with high-resolution (Hayashizaki, Hirotsune et al.

1993). As long as genome wide scanning for aberrant DNA methylation patterns of different human cancers is performed, the methylation status can be resolved.

Methylated CpG island amplification

Methylated CpG island amplification (MCA) can be applied to both methylation analysis and cloning differentially methylated genes. Firstly unmethylated SmaI sites are eliminated by digestion with SmaI. Then methylated SmaI sites are digested with the SmaI isoschizomer XmaI, which digests methylated CCCGGG sites which form a ligated ends. Adaptors are ligated to these sticky ends and PCR is performed to amplify the methylated sequences. The MCA amplicons can be analyzed in a dot blot assay, and the methylation status of a specific gene is determined by an oligonucleotide probe (Toyota, Ho et al. 1999).

Combination of methylated-DNA precipitation and methylation-sensitive restriction enzymes (COMPARE-MS)

COMPARE-MS rapidly and quantitatively detects CpG hypermethylation in hundreds of samples simultaneously. Genomic DNA is digested with AluI with or without the methylation-sensitive restriction enzyme HpaII and then DNA is precipitated by methyl-binding domain polypeptides immobilized on magnetic beads. Either the magnetic beads captured methylated DNA or all digested DNA is subjected to real-time PCR at a gene-specific locus. Enrichment of methylated DNA by methylation-sensitive restriction enzyme digestion and by magnetic beads capture of methylated DNA can increase the sensitivity and specificity of methylation detection (Yegnasubramanian, Lin et al. 2006).

Methylation-sensitive arbitrarily primed polymerase chain reaction (MS-AP-PCR)

Arbitrarily primed polymerase chain reaction (AP-PCR) is a method for fingerprinting genomes. AP-PCR uses various arbitrary primers which bind to different sites on the two DNA strands and amplify the DNA located in between the primers (Menard, Brousseau et al. 1992).

For the MS-AP-PCR, methylation sensitive restriction enzymes with different recognition sequences were used to digest genomic DNA samples from cell lines, tumors and normal tissues before AP-PCR. After resolving the PCR products on high-resolution polyacrylamide gels, fragments that showed differential methylation were cloned and sequenced (Gonzalgo, Liang et al. 1997).

Methylation-specific multiplex ligation-dependent probe amplification

MLPA (Multiplex Ligation-dependent Probe Amplification) is a multiplex PCR method detecting copy number changes of up to 40 different genes. MLPA probes are hybridized to the denatured single strand DNA sequence and ligated by thermoligase. Ligated probes are exponentially amplified during the subsequent PCR reactions and separated using capillary electrophoresis (Schouten, McElgunn et al. 2002). Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is very similar to the MLPA method, except that during ligation reaction half of the samples are undigested for copy number detection and the other half sample is digested by methylation-sensitive endonuclease HhaI. MS-MLPA probes for methylation detection all contain the restriction site of the methylation-sensitive endonuclease HhaI. Because

digested probes cannot be amplified during PCR reaction, comparing the CE data from the digested sample with the undigested sample shows the methylation signal (Scott, Douglas et al. 2008).

There are several benefits of MS-MLPA: (1) a large number of genes can be studied using only trace amounts of sample DNA; (2) a large number of samples can be analyzed simultaneously; (3) MLPA is quantitative and can discriminate between methylation of one, both, or none of the alleles.

Bisulfite-based Method

Bisulfite-based method distinguishes the methylated and unmethylated DNA sequence by converting unmethylated cytosine to uracil. This method is a revolutionary tool to study methylation status. Based on the bisulfite conversion method, different detection methods are developed to amplify and analyze the bisulfite-converted template. This method only needs trace amount of DNA. The sensitivity and specificity is higher than previous nonbisulfite-conversion methods (Shapiro, DiFate et al. 1974).

The bisulfite method in Figure 14 can be used to correctly identify all methylated and unmethylated CpG islands present in the genomic DNA. Sodium bisulfite can only deaminates unmethylated cytosine located on single-stranded DNA. Sodium 5, 6 -dihydrocytosine-6 sulphonate forms at low pH condition. Raising the pH to basic conditions causes the degradation of sodium bisulfite and the transformation of unmethylated cytosine into uracil. 5-Methylcytosine may also undergo such a reaction but the rate of this reaction is very slow and inhibits the formation of the final product (Sono, Wataya et al. 1973, Piperi, Farmaki et al. 2008).

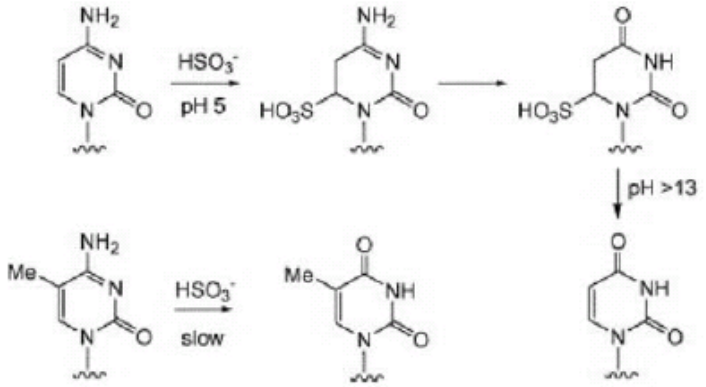


Figure 14 Bisulfite modification reaction. The unmethylated cytosines form sodium 5, 6 -dihydrocytosine-6 sulphonate at low pH, and then generate uracil at the basic condition. The methylated cytosine has a slow reaction rate in the bisulfite modification reaction.

PCR product of bisulfite, converted single strand DNA replace the uracil with thymine. The PCR product can be analyzed by various methods including Sanger or pyro-sequencing (McDonald and Kay 1997; McDonald, Paterson et al. 1998), single-nucleotide primer extension (Gonzalgo and Jones 1997; Gonzalgo and Liang 2007; Boyd and Zon 2008), and ligation detection reaction (Dahl and Guldberg 2007; Scott, Douglas et al. 2008; Figueroa, Melnick et al. 2009).

Sequencing method

Bisulfite genomic sequencing generates methylation maps with single-base resolution which is still the gold standard method for methylation measurement. The procedure is the same as common sequencing. The method is based on the selective deamination of cytosine to uracil by treatment with bisulfite and the sequencing of subsequently generated PCR products. Different from cytosine, 5-methylcytosine does not react with bisulfite. Thereby the methylated CpG site is still read as CpG, while the unmethylated CpG site is read as TpG. Bisulfite sequencing also is the standard of other bisulfte-based methods, usually used to check validity (Loebel and Johnston 1996; McDonald and Kay 1997).

Pyrosequencing is a relatively new method for real-time nucleotide sequencing. It has been widely applied in DNA sequencing, genotyping and single nucleotide polymorphism analysis. The pyrosequencing for methylation detection involves five major steps: 1) bisulfite reaction treats the genomic DNA; 2) DNA is amplified using PCR; 3) double-stranded DNA is converted to single-stranded DNA templates; 4) oligonucleotide primers are hybridized to a complementary sequence of interest; 5) the pyrosequencing

reaction itself (Colella, Shen et al. 2003). The pyrosequencing assays shows a sensitivity and specificicty around 100% and can provide a quantitative approach of methylation detection of all CpG sites

PCR based method

Methylation Specific PCR (MSP) was described in 1996. Methylation-specific PCR (MSP) is a simple, quick and cost-effective method to analyze the DNA methylation status of virtually any group of CpG sites within a CpG island. The first tests included p16, p15, VHL and E-cad genes. After bisulfite modification, DNA was amplified with two pairs of primers: the methylated and the unmethylated sequences. The advantages of this method include a short time of analysis and the possibility of obtaining results from small amounts of DNA. MSP reveals the methylation status of the CpG sites within the two short sequences targeted by MSP primers. Specificity and sensitivity of primers are significantly related to the primer design. MSP eliminates the false positive results generated from incomplete restriction enzyme cleavage. Methylation is detected even when only 0.1% of alleles are methylated. The limitations of this method are connected to the possibility of contamination of the analyzed sample and strict PCR condition are needed to avoid false-positive results (Herman, Graff et al. 1996).

HeavyMethyl is real-time PCR assay which measures methylation biomarkers. Cottrell designed a set of methylation-specific oligonucleotide blockers and a methylation-specific probe to achieve methylation-specific amplification and detection (Cottrell, Distler et al. 2004). Distler extended the application to a wider level, including analysis of frozen, formalin-fixed samples. He presented a assay for quantitative

methylation measurement of GSTP1 gene (Distler 2009).

MSP primers are normally designed to flank one or more CpG sites at or near the 3' end. This makes the primers highly selective for the methylated template. However if there is an incompletely converted sequence in the bisulfite-treated DNA, a false positive result is difficult to avoid. SMART-MSP used melting as a quality control to avoid false-positive results caused by incomplete bisulfite conversion (Kristensen, Mikeska et al. 2008).

Single-nucleotide primer extension

Single nucleotide primer extension, or so-called single base extension, was first used to identify genetic disease with high frequency of occurrence. Fluorescent dye or radioactive isotope labeled nucleotide is added to the 3’ end of a primer (Kosaki, Yoshihashi et al. 2001). Single nucleotide primer extension products are then carried out and analyzed by denaturing polyacrylamide gel electrophoresis (Sokolov 1990), autoradiography (Kuppuswamy, Hoffmann et al. 1991) , capillary electrophoresis (Piggee, Muth et al. 1997), or mass spectrometry (Fei and Smith 2000). The extension of the primer by a single base will show the targeted base pair of either the mutant or the wild type.

Methylation-sensitive single nucleotide primer extension (MS-SNuPE) measure methylation differences at specific CpG sites based on bisulfite treatment of DNA followed by single nucleotide primer extension (Gonzalgo and Jones 1997). Gonzalgo’s group first reported SNuPE assay on the DNA methylation determination. The advantage of the SNuPE assay is easy to achive high throughput and supply a relatively simple measurement of methylated cytosine content. The generated C vs. T signal strength strands for original methylated cytosine vs. unmethylated cytosine positions (Boyd, Moody et al.

2007).

Ligation detection reaction

Ligation-mediated polymerase chain reaction (LM-PCR) was the first method to combine the methylation detection with ligation reaction. Genomic DNA is cleaved simultaneously with one methylation sensitive restriction enzymes and one methylation insensitive restriction enzyme. After cleavage, a gene-specific oligonucleotide primer is extended on the template followed by linker ligation and then conventional PCR (Steigerwald, Pfeifer et al. 1990). This method does not use the high discrimination of ligase itself but depend on the methylation sensitive restriction enzymes.

Dahl established a multiplex, simple and cost-effective approach to analysis of CpG methylation. In Ligation Detection reaction/PCR assay, a pair of methylation-specific oligonucleotide probes hybridize to the bisulfite treated DNA template. Because the high specificity of ligase on double stranded DNA, the matched probe pair can be linked together and then amplified by a common primer pair in a PCR reaction. The PCR product mixture which stands for up to 6 genes can be resolve in an agarose gel (Dahl and Guldberg 2007).

After bisulfite treatment, two ligation-based microarray methods were developed for methylation analysis. Briefly, it first utilizes multiplexed PCR to amplify multi-promoter areas, followed by ligation chain reactions. One common upsteam proble is designed along with two downstream probes targeting methylated and unmethylated sequence are labeled with two different dyes (Cy3/Cy5). The ligation products are then hybridized on the probe microarray to determine the methylation status at each promoter via measuring ratio of

Cy3/Cy5 fluorescence signals (Cheng, Shawber et al. 2006).

Physical property

Methylation-dependent fragment separation (MDFS) is a methylation detection method based on Capillary electrophoresis. Electrophoresis is conducted after sodium bisulfite modification of genomic DNA and amplification with specific primers labeled with fluorescent dye. The migration speed of polymorphic sequences is different, which allows for the identification of methylated and unmethylated DNA (Boyd, Moody et al. 2006; Boyd, Moody et al. 2007). After bisulfite conversion, methylated genomic DNA differs from unmethylated genomic DNA by multiple methylated cytosines versus uracils. A region of interest is then amplified using fluorescence dye-labeled primers. Since the primers don't cover any CpG sites, the methylated and unmethylated DNA can be amplified unbiasely. The presence of the multiple polymorphisms (C vs. T) leads to differential migration times during fragment analysis by CE, therefore an amplicon from fully methylated genomic DNA is readily resolved from an amplicon from fully unmethylated genomic DNA.

High resolution melting analysis (HRM) is a sensitive and low cost method for the detection of methylation profile. Methylated DNA and unmethylated DNA generate different sequences after bisulfite treatment which causes a significant change in the melting curve between methylated and unmethylated PCR products. MS-HRM can estimate the methylation level by comparing the melting profiles of unknown PCR products to the melting profiles of PCR products from a serial standard DNA mixture of methylated and unmethylated templates (Wojdacz and Dobrovic 2007).

High throughput methylation methods

The Human Epigenome Project uesd standard sequencing approaches to sequence bisulfite-converted DNA from human tissues and primary cell lines to determine all the possible tissue-specific differentially methylated regions (Bradbury 2003; Rauscher 2005). There are a number of methods for large-scale screening of DNA methylation, but the DNA microarray and next generation sequencing are the most reliable and informative methods that can be compared easily by different laboratories (Lippman, Gendrel et al. 2005).

DNA microarray

Tampa first took microarray as a genome-wide mapping of DNA methylation patterns. The genomic DNA is cleaved by a methyl-sensitive restriction endonuclease and then followed by size fractionation and hybridization to microarrays. This study demonstrated that methylation patterns can be characterized by microarray (Tompa, McCallum et al. 2002). Lippman from Cold Spring Harbor laboratory subsequently utilized the genomic microarrays to profiling DNA methylation patterns (Lippman, Gendrel et al. 2005).

At the early stage of microarray studies on DNA methylation, individual laboratories prepared their own arrays. Today the high-quality commercial microarray products from Illumina, Affymetrix, NimbleGen and Agilent are more popular in methylation research. Bead array from Illumina are capable of single base resolution. Two primers targeting methylated and unmethylated bisulfite-treated sequence are labeled different fluorescent dyes. The ratio of two primers ligated on the common primer immobilized on solid surface

is used to evaluate methylation status (Bibikova, Lin et al. 2006). Affymetrix GeneChip arrays achieved a high density spot pattern by photolithographic method. Millions of probes can be synthesized on the chip surface. Each spot contains a 25-mer oligonucleotides that provide the specificity of the assay (Dalma-Weiszhausz, Warrington et al. 2006). Restriction enzyme processed DNA from human tissue has been ligated with a linker, then methylation binding protein is applied to enrich the methylated DNA fragments. The enriched DNA fragments are labeled with fluorescence dye via PCR and hybridized to methylation arrays for data analysis (Ho and Tang 2007). Each Affymetrix chip is designed for one sample at a time. To compare different samples, each sample is hybridized to a separate array and the resulting signals are compared. NimbleGen and Agilent adopt long 60-mer oligonucleotide probes. The only difference between the two companies is the way to that oligonucleotide probes are synthesized (Nuwaysir, Huang et al. 2002; Wolber, Collins et al. 2006). Two samples are labeled with different fluorescent dyes, methylated DNA and control DNA, are hybridized on a single chip. The signal difference between the two samples hybrizided on the same chip indicates the variation pattern of methylation. The longer probes provide a better balance between specificity, sensitivity and noise.

Next Generation Sequencing

High-throughput sequencing is the newest and most promising methodology for genome-scale analysis of DNA methylation in the future. There are a number of platforms available on the market or under development (Shaffer 2007; Rusk and Kiermer 2008; Shendure and Ji 2008). High-throughput sequencing can be an alternative way for analyzing DNA methylation with oligonucleotide arrays. There is no need of labeling and

hybridizing control samples in array experiments, the sequencing result can be directly interpreted. High throughput sequencing methods provide a quantitative measure of methylation abundance rather than the relative measure in array-based methods. Next-generation sequencing is poised to incur dramatic changes in every area of molecular biology (Park 2008). This approach often measures billions of base pairs per run, compared with the millions of base pairs per day generated by automated capillary DNA sequencers (Chan 2009; Gargoyle and Mince 2009).

3.3 Conclusion

5-Methylcytosine, commonly referred to as DNA methylation, is a modified base that adds heritable information upon the DNA code, which is important for regulating many cellular processes. DNA methylation research is entering a new phase after years of study. For the first time we have the ability to analyze methylation patterns of the whole genomes and it provides the most basic type of information about the cytosine methylation signal. The genome-wide and gene-specific methylation analysis methods were briefly reviewed in this short review. Each method has distinct advantage and application area. Fast developments in high-throughput sequencing may replace microarrays within a few years for genome wide methylation analysis. High resolution melting curve shows more potential in gene specific studies.

CHAPTER IV

SENSITIVE MEULTIPLEX MUTATION AND METHYLATION DETECTION IN A LARGE BACKGROUND OF Normal DNA

4.1 Introduction

The analysis of genomic mutation or methylation in cancer-related DNA markers plays an increasingly significant role in the fields of early cancer diagnosis. Since there are large numbers of genomic mutations and methylations to be screened to yield accurate information for cancer diagnosis/prognosis, a sensitive and low-cost method is needed for clinical laboratories.

Despite the best advances in the imaging technology, cancer is still hard to detect before metastasis has occurred. The cancer screening is still the most challenging and promising strategy for saving lives from cancer. Molecular diagnostics, which takes the molecules in human body like DNA, RNA, or protein as the biomarkers to detect cancer, is emerging as a promising method in early cancer screening. DNA mutation and methylation are believed to be the leading cause for the development of cancer. The mutation in p53 and APC genes initiate 60-80% of colorectal neoplasia (Smith, Carey et al. 2002; Traverso, Shuber et al. 2002). Promoter methylation also plays an even more important role in development of cancer (Nagasaka, Sasamoto et al. 2004; Bazan, Bruno et al. 2006). However it has been challenging for the current screening methods to screen cancer. There remain two challenges in the current screening methods: 1) find a sensitive method to detect mutation and methylation which can detect small amounts of target sequences in a vast excess of wild-type DNA; 2) the screening method should be simple and cost-effective. Since cancer screening focuses on the general population, the cost-effectiveness determines the feasibility of the method.

There are different technologies which fit for allele specific point mutation discrimination including single base extension (Huang, Arnheim et al. 1992; Greenwood and Burke 1996; Gonzalgo and Jones 1997), allele-specific hybridization (Iitia, Mikola et al. 1994), and restriction enzyme cleavage and ligase based assay (Giunta, Youil et al. 1996; Tsuji and Niida 2008). Combining these methods with microarray (Tebbutt 2007), mass spectrometry (Li, Butler et al. 1999; Bocker 2003), and fluorescence detection (Chen, Iannone et al. 2000; Dempsey, Barton et al. 2004) will greatly enhance throughput and sensitivity of the method.

Variations in DNA molecules are difficult to directly detect from body fluids, and current methods of discrimination do not have this capability. Nucleic acids in bodily fluids are present at concentrations on the order of 10–100 ng/mL. These nucleic acid sequences must be amplified before applying the final discrimination assay. Enrichment methods are employed to detect low abundance mutations. COLD-PCR (co-amplification at lower denaturation temperature) showed a wide and promising future (Li and Makrigiorgos 2009). This method enables enrichment of PCR amplicons containing unknown mutations at any position. After strict temperature controlled PCR cycles, the ratio of mutation sequence to normal sequence can be elevated significantly and subsequently subjected for sequencing method to identify the exact nucleotide change (Luthra and Zuo 2009). This clever amplification process utilizes two fundamental technologies. The first technology is an asymmetric design of the primers which flank the target sequence. The folding primer and turn-back primer are designed to amplify the target through a self-priming mechanism. The second is to use Thermus aquaticus (Taq) MutS for reduction of background amplification reaction in the isothermal amplification procedure (Tatsumi, Mitani et al. 2008).

The greatest challege for cancer screening by mutation and methylation analysis is the lack of methods that can survey hundreds of DNA mutation and methylation makers in a sensitive way. In this study, a method called the Probe Enrich Mutation/Methylation HRM analysis (PEMM-HRM) is developed to detect low abundance mutation. We demonstrated that a single PEMM-HRM assay could easily identify one mutation sequence in 1,000 wild-type DNA copies.

4.2 Materials and methods

4.2.1 DNA sample

Probes and primers used in our experiments are ordered from MWG/Operon. DNA samples were prepared at John Hopkins University by using the standard methods. The mutations in primary tumors and cell line were determined using conventional sequencing methods. The fully unmethylated control DNA and fully methylated control genomic DNA samples were purchased with CpGenome DNA modification Kit (Millipore, Billerica, MA). The samples containing low-abundance mutated DNA were created by diluting the standard mutation/methylation DNA samples (copy number was measured by Real Time-PCR) and then the standard mutation/methylation DNA samples were mixed with wild type/unmethylated DNA. Samples containing 1% and 0.1% mutated DNA were tested.

4.2.2 Bisulfite Treatment

The CpGenome DNA modification Kit (Millipore, Billerica, MA) was used for bisulfite treatment in this study. Bisufite treatment was performed according to the protocol from the manufacturer. Briefly, the DNA samples were mixed with bisulfite buffer and maintained at 50 ºC for 15 h. Then bisulfite buffers containing DNA sample were filtrated by 50K Microcon centrifuge column (Millipore, Billerica, MA). The DNA left on the centrifuge column is washed 3 times with 500 μL 1xTE buffer (pH=7.4) on Eppendorf Centrifuge 5415C (Eppendorf North America, Westbury, NY) at 10,000 RPM. The desulfonation reaction was performed by transferring 500 μL of 0.1 M NaOH solution to the column, which slowly went through the centrifuge column at 3000 RPM. The column

was then washed 3×500 μl TE buffer. In the end, the bisulfite modified DNA was eluted from the column with 200 μL TE buffer. The modified DNA was used immediately or stored at -20 ºC for further analysis.

4.2.3 Multiplex PCR

About 10 ng of genomic DNA/bisulfite converted DNA was added in to 20 μl 1×ABI PCR buffer (providing a final concentration of 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μM each dNTP and 1 unit AmpliTaq Gold DNA Polymerase). All primer pairs were mixed equally together with a final concentration of 0.01 μM each. Reaction mixtures were incubated at 95 ºC for 10 min to activate AmpliTaq DNA Polymerase, and then subjected to 16 cycles consisting of 30 sec at 95 ºC, 30 sec at 65 ºC (-0.5 ºC /cycle) and 45 sec at 72 ºC, followed by 30 cycles of 30 sec at 95 ºC, 30 sec at 57 ºC, and 45 sec at 72 ºC. The sequences of 6 pairs of primers are listed in Table V and Table VI.

4.2.4 Mutation/Methylation Specific Enrichment in Solution

Multiplex PCR amplicons were incubated with a mixture of the enriching and blocking probes for mutation-specific enrichment. This is carried out by first denaturing DNA at 95ºC for 5 min, followed by stepwise by reducing the temperature at about 0.1ºC/3 sec to 25ºC. Then the sample was incubated with the streptavidin-coupled Dynabeads for 30 min at room temperature to capture the mutant DNA carried by them. After washing, the beads were resuspended by water and heated at 95 ºC for 5 min to release the captured DNA.

4.2.5 Optimization Mutation Enrichment condition

A mixture of enriching probes (1 μM of each) and corresponding blocking probes (7.5 μM of each), and was incubated with the PCR products for hybridization. This was carried out by first denaturing DNA at 95ºC for 5 min, followed stepwise by reducing the temperature at about 0.1ºC/3 sec to 25ºC. Then the sample was incubated with the streptavidin-coupled Dynabeads for 30 min at room temperature to capture the mutant DNA carried by them. The enriching probe captured the PCR products were washed three times with 1ml 1xTE, at 30, 40, 50, 60 and 70ºC to remove the unspecific binding. The enriched templates were eluted at 95ºC. The templates were experimented with HRM assay with normal DNA as internal standard. The sequences of primers and probes are listed in Table VII and Table VIII.

Table V PCR Primer Sequences for Mutation Detection

Primer	Forward Primer 5'-3'	Reverse Primer 5'-3'	Size (bp)
p53 175-179	T7-TACAGCACATGACGGAGGTTG	T3-GCTCACCATCGCTATCTGAG	108
p53 196-213	T7- ACTGATTGCTCTTAGGTCTGG	T3-GGCGGCTCATAGGGCACCACC	164
p53 245-248	T7-CAACTACATGTGTAACAGTTCC	T3- CTTCCAGTGTGATGATGGTG	114
P53 273-282	T7-CCTATCCTGAGTAGTGGTAATC	T3-CCTTTCTTGCGGAGATTCTC	150
Kras 12/13	TCATTATTTTTATTATAAGGCCTGCTGAA	CAAAGACTGGTCCTGCACCAGTA	189

Table VI PCR Primer Sequences for Methylation Detection

Primer	Forward Primer 5’-3’	Reverse Primer 5’-3’	Size (bp)
BMP3	T7-GTTTTYGTTTTAGTTGGTTTGGAGTT	T3-TACTCYCCCCAACCATAACTAAATAC	178
ALX4	T7-GAGTAGGTYGGGTAAGGAGTGTATAG	T3-CAAATCTCAACATTCATACCTAACTTAC	189
EY4	T7-GGTTTTAYGAGTTYGTAGTAGTYGGTGG	T3-CCTCTCTAAAACAACRACAACTTCAC	208
UNC5	T7-GAGTTTTATTGGATATAGTTTAGTGG	T3-CCCAAAAAACCAACTATAAATTTACCC	218
GATA5	T7-AGGGAGGTAGAGGGTTYGGGATT	T3-ACRTAACCCTAACAAACCCTACTC	229

Table VII Oligonucleotide Probe for Methylation Enrichment

Probe	Methylation Enriching probe 5'-3'	Blocking Probe 5'-3'
BMP3	Biotin-AGTCGACGCGTCGCGCGG	AGTTGATGTGTTGTGTGG
ALX4	Biotin-TCGCGTTTTCGTTCGTCG	TTGTGTTTTTGTTTGTTG
EY4	Biotin-CGAAACGTACGCCGCGCT	CAAAACATACACCACACT
UNC5	Biotin-GTTCGGGGCGCGTTCGTT	GTTTGGGGTGTGTTTGTT
GATA5	Biotin-CGCACGACACGAAACGAC	CACACAACACAAAACAAC

Table VIII Oligonucleotide Probe for Mutation Enrichment

Probe	Mutation Enriching probe 5'-3'	Blocking Probe 5'-3'
p53 175-1	Biotin-GTTGTGAGGDGCTGCCCC	GTTGTGAGGCGCTGCCCC
p53 175-2	Biotin-GTTGTGAGGCHCTGCCCC	
p53 196-1	Biotin-ATCTTATCWGAGTGGAAGG	ATCTTATCCGAGTGGAAGG
p53 213-1	Biotin-AAACACTTTTKGACATAGTGT	AAACACTTTTCGACATAGTGT
p53 213-2	Biotin-AAACACTTTTCHACATAGTGT	
p53 248-2	Biotin-GATGGGCCTCDGGTTCAT	GATGGGCCTCCGGTTCAT
p53 245-1	Biotin-CCTGCATGGGCHGCATGA	CCTGCATGGGCGGCATGA
p53 245-2	Biotin-CCTGCATGGGCGHCATGA	
p53 273-1	Biotin-TTTGAGGTGDGTGTTTGTG	TTTGAGGTGCGTGTTTGTG
p53 273-2	Biotin-TTTGAGGTGCHTGTTTGTG	
p53 282-1	Biotin-GAGACDGGCGCACAGAGG	GAGACCGGCGCACAGAGG
p53 282-2	Biotin-GAGACCHGCGCACAGAGG	
Kras 12-1	Biotin-TTGGAGCTHGTGGCGTAGGC	TTGGAGCTGGTGGCGTAGGC
Kras 12-2	Biotin-TTGGAGCTGHTGGCGTAGGC	
Kras 13-1	Biotin-TTGGAGCTGGTHGCGTAGGC	
Kras 13-2	Biotin-TTGGAGCTGGTGHCGTAGGC	

4.2.6 HRM Assay

The HRM assay was performed in the LightCycler 480 (Roche Diagnostics; Switzerland). The same primers that amplify the genomic DNA or bisulfite converted DNA were used in the HRM assay. PCR reactions were performed in triplicate in 10 µl final volume using Type-it® HRM PCR kit (Qiagen, Maryland/USA). 0.7 µM primer and 1 µl enriched PCR template were added to each HRM reaction. The thermocycling consisted of an initial incubation at 95ºC for 5 min followed by 50 cycles of 95ºC for 10 s, 55ºC for 30 s and 72ºC for 10 s and a final extension step of 10 min at 72ºC.

The melting curve analysis was performed by the Ligthcycler 480 Gene Scanning Program. The melting curves of each primer were normalized at its temperature range. The value of shifted temperature was set at 0%. Finally the HRM difference plot was generated by subtracting the curves from known wild-type DNA sample. The grouping software uses a curve shape-matching algorithm to determine the wild type and mutation. The same preparation protocol was applied to the wild type and tissue samples. The grouping sensitivities of 0.3, 0.2 and 0.1 were compared to determine the optimal mutation calling.

The methylation specific HRM was performed in the same thermocycling condition and HRM kit. The only difference was that PCR reactions were performed on Opticon 2 instruments (Bio-Rad Laboratories, Hercules, CA) instead of the Lightcycler 480 system.

4.3 Results

4.3.1 Mechanism of PEMM-HRM

PEMM-HRM assay consists of five steps. First, DNA sample from human tissue, blood and stool samples are extracted and multiplex amplified by mutation targeting

primers. Second, the PCR products containing mutation and wild-type alleles are subjected to competitive hybridization. The enriching probes complementary to the mutation alleles are biotinylated and further captured by streptavidin-coupled Dynabeads. The blocking probes complementary to the wild-type alleles are non-biotinylated and stay in the solution. Third, the supernatant containing wild-type alleles are removed, but the streptavidin-coupled Dynabeads are kept by the magnet in the same vial. This step leads to the enrichment of mutation alleles. Fourth, the mutation alleles that are captured by streptavidin-coupled Dynabeads are eluted at 95 ºC for 5 mins. Finally, the enriched mutation alleles are subjected for high resolution melting analysis. Figure 15 shows a schematic representation of whole assay.

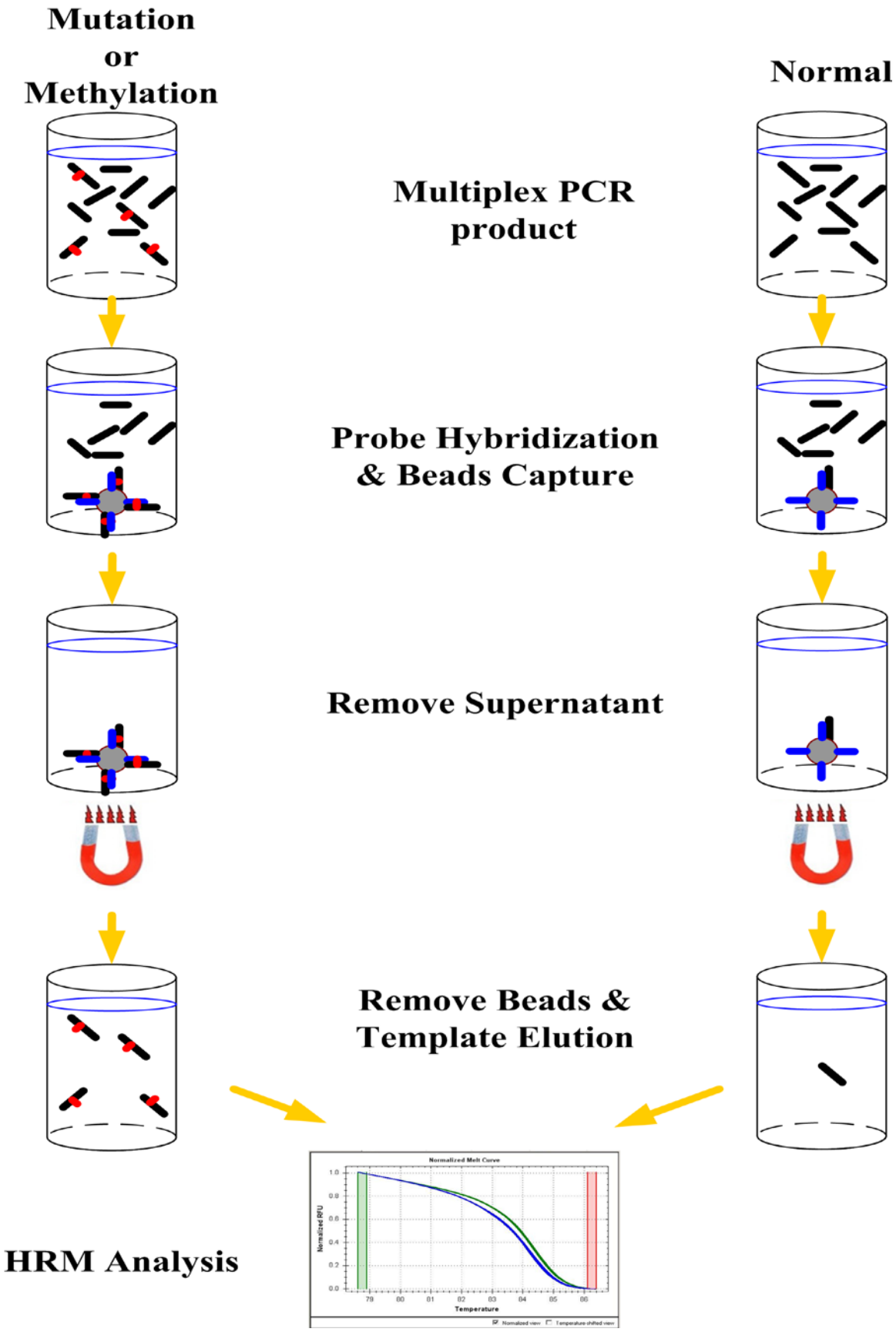

Mutation
or
Methylation
Normal
Multiplex PCR
product
Probe Hybridization
& Beads Capture
Remove Supernatant
Remove Beads &
Template Elution
HRM Analysis
Normalized Melt Curve
Normalized RFU
Temperature

Figure 15 the Workflow of PEMM-HRM assay. PEMM-HRM assay has five steps. In the first step genomic DNA containing mutation sites are amplified by multiplex PCR. In the second step mutation amplicons are hybridized to mutation enriching probes and captured by streptavidin-coupled Dynabeads. In the third step, the normal amplicons are removed by washing the magnetic beads. In the forth step, the captured amplicons are released from the magnetic beads by simple disrupting the biotin and streptavidin complex at high temperature. In the last step, HRM curves of mutation enriched sample are compared with a normal control sample and determine if there is any mutation site.

This assay adopted a competitive hybridization approach. The principle of competitive hybridization is illustrated on the Figure 16. Briefly, the PCR products are hybridized with the biotinylated enriching probes to selectively capture mutant DNA, followed by depleting wild-type DNA. After separation of from enriching probes, the enriched DNA can be analyzed by melting curve analysis. The second and third steps are the key to the PEMM-HRM. The mutation allele enrichment needs an enriching probe and a blocking probe which are fully complementary to the mutation and wild-type sequences respectively. As a result, the blocking probes which are complementary to the wild-type sequence can tightly bind to wild-type DNA and prevent the nonspecific binding to the enriching probe. For the normal sample, the nonspecific binding can be suppressed at a very low level. The following high resolution melting analysis shows there are not difference before or after the enrichment. In contrast, when mutated DNA is present, the blocking probe will not bind the targeted mutation sites and enriching probe can capture the low abundance mutation alleles. In this method, we used solid phase extraction to extract the mutation PCR product.

The methylation enrichment followed the same principle. The difference is that the extracted DNA subjected to bisulfite modification. The primers and probes were designed to match to the bisulfite-converted sequence.

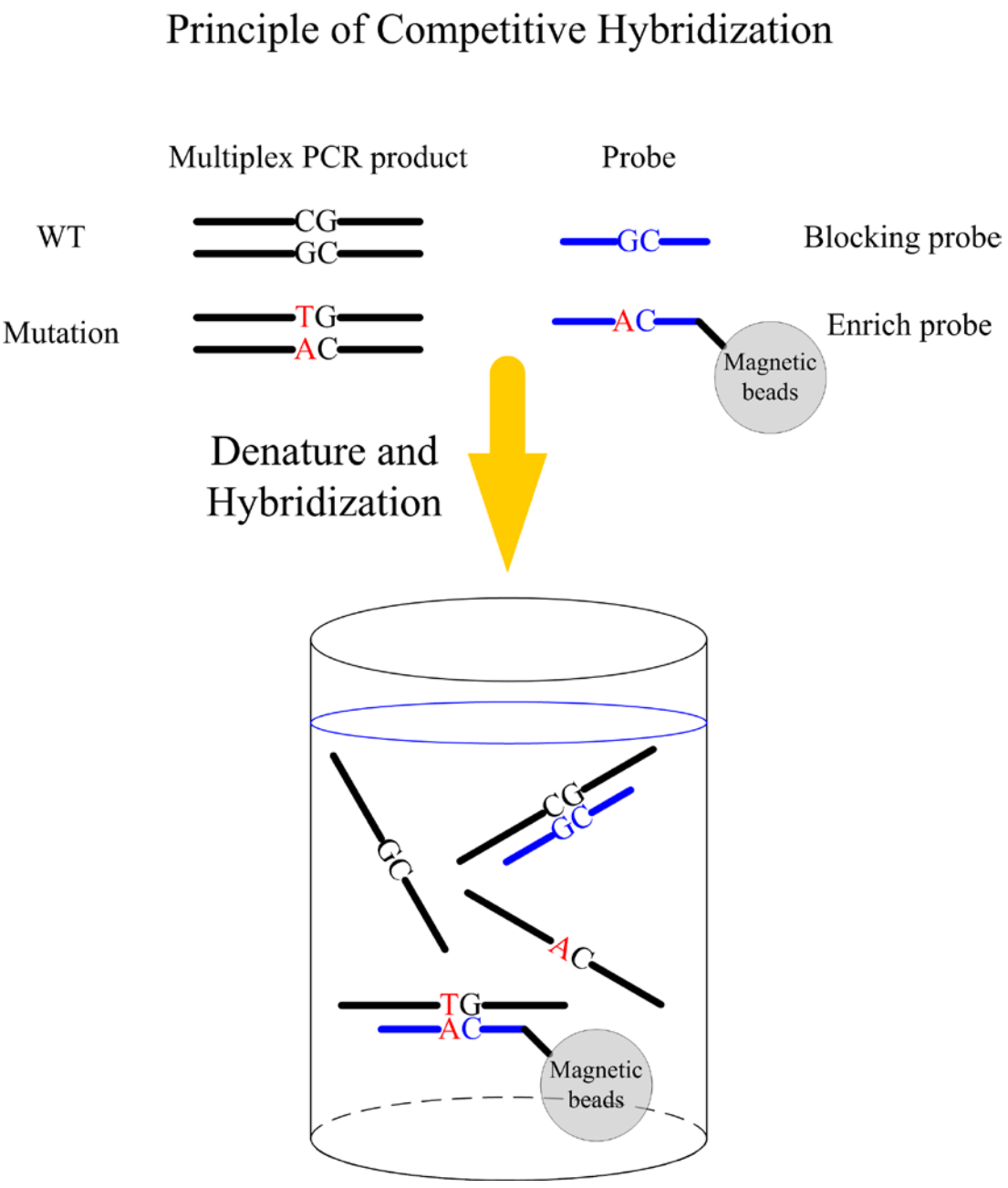


Figure 16 Principle of Competitive Hybridization. PCR products containing mutation amplicons are selectively hybridized with the enriching probes and blocking probes, which are fully complementary to the mutation and wild type sequences individually. The blocking probes hybridize to the wild type amplicons and suppress the nonspecific binding between wild type amplicons and the enriching probes. The mutation amplicons will be captured by mutation enriching probe on streptavidin-coupled Dynabeads surface.

4.3.2 Absolute Quantification

Cell lines DNA V425 and V576 (containing p53 248 CGG>TGG and 273 CGT>CTT individually) were used for study of the sensitivity of the PEMM-HRM method. The absolute DNA copy number of each cell line was measured by the standard Real time PCR method. Human genomic DNA was serially diluted in 50, 5, 0.5 and 0.05 ng per vial to construct a standard curve. The copy number of V425 and V576 is calculated by the LightCycler 480 software. The amplification curves were recorded as shown in Figure 17.

4.3.3 Sensitivity of PEMM-HRM

We used the LightCycler 480 Gene Scanning software to automatically call the TP53, APC, Kras mutation status from PCR amplicons fluorescent melting curves. Sensitivity thresholds of each primer which have been determined according to the experiment in Chapter II were based on the best discriminating between normal and mutation cell line DNA.

We assessed the sensitivity and specificity of PEMM-HRM by diluting mutation cell line DNA V425 and V576 (containing p53 248 CGG>TGG and 273 CGT>CTT) into wild type human DNA. The absolute DNA copy number of each cell line was measured the by standard Real time PCR method. The dilution factor is 100%, 1%, 0.1% and 0%. Figure 18A showed that after the probe enrichment process we can readily detect mutation in 1% and 0.1% mutation which can not be detected by regular HRM method.

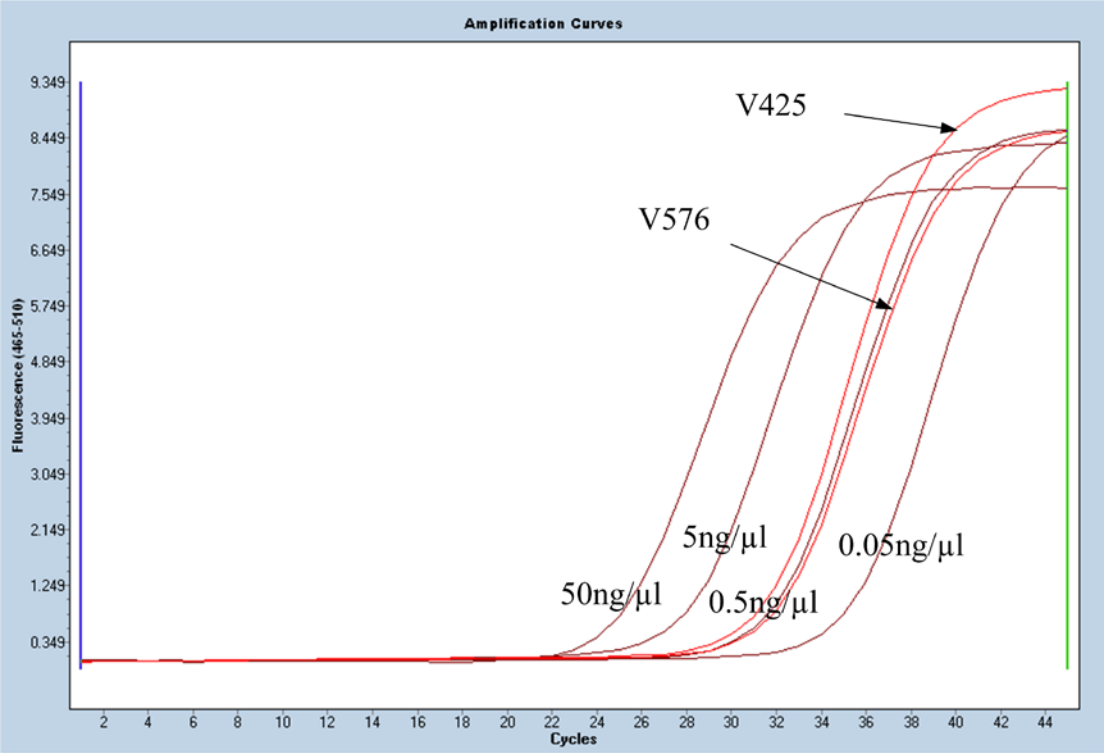


Figure 17 Quantification of mutation template. The copy numbers of cell line DNA V425 and V576 are measured against the serially diluted normal human genomic DNA.

The amount of human DNA extracted from a clinical specimen may vary from patient to patient; thus, the assay must have a good dynamic range. The mutation enrichment step is very robust, the volume variation from PCR template showed no dependece on mutation enrichment. Figure 18 B displays the result of analyzing the sample containing 50 ng, 10 ng and 2 ng DNA samples which contain 1% p53 273 248 CGG>TGG mutation were amplified by multiplex PCR assay, after performing the mutation enrichment. The results showed that the mutation enrichment assay was not influenced by the quantity of initial DNA. Even if the concentration ratio of mutation template to wild type template was decreased to 1:1000, detectable fluorescence melting curve differences between the mutated and normal templates were observed.

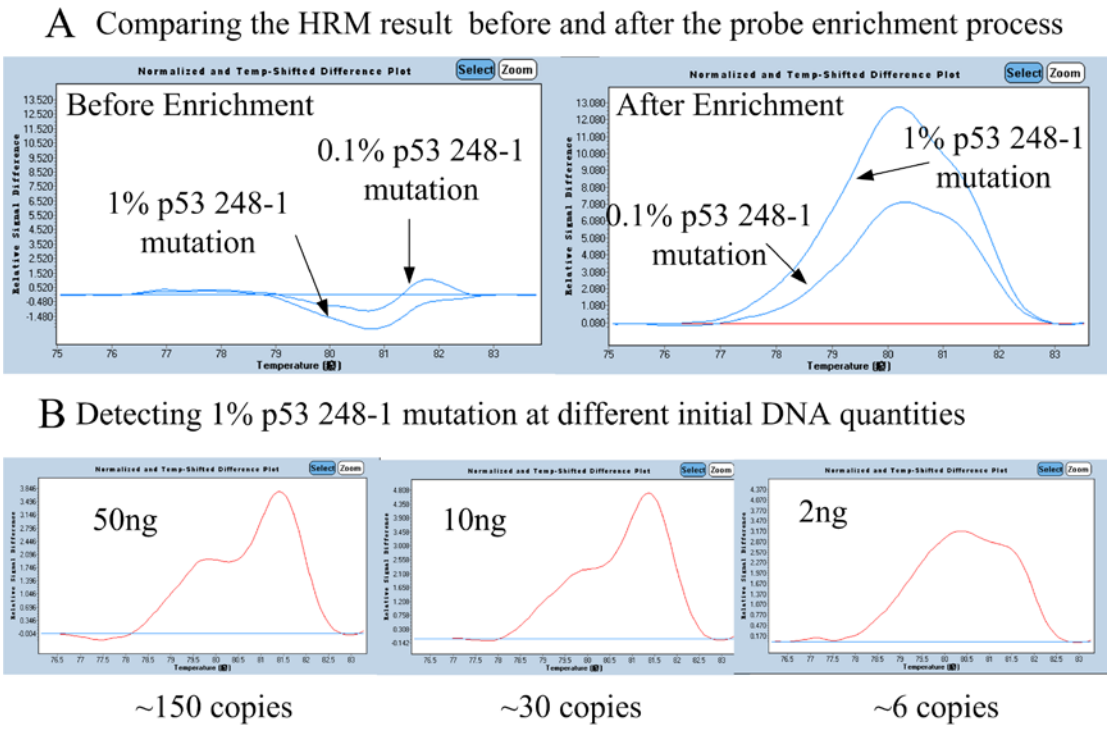
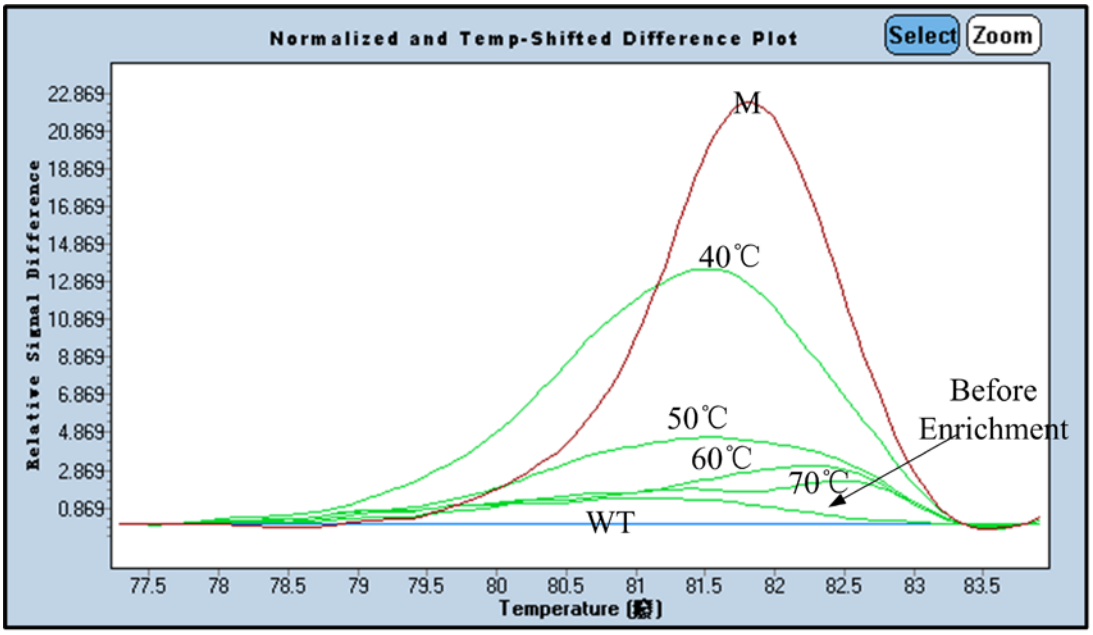


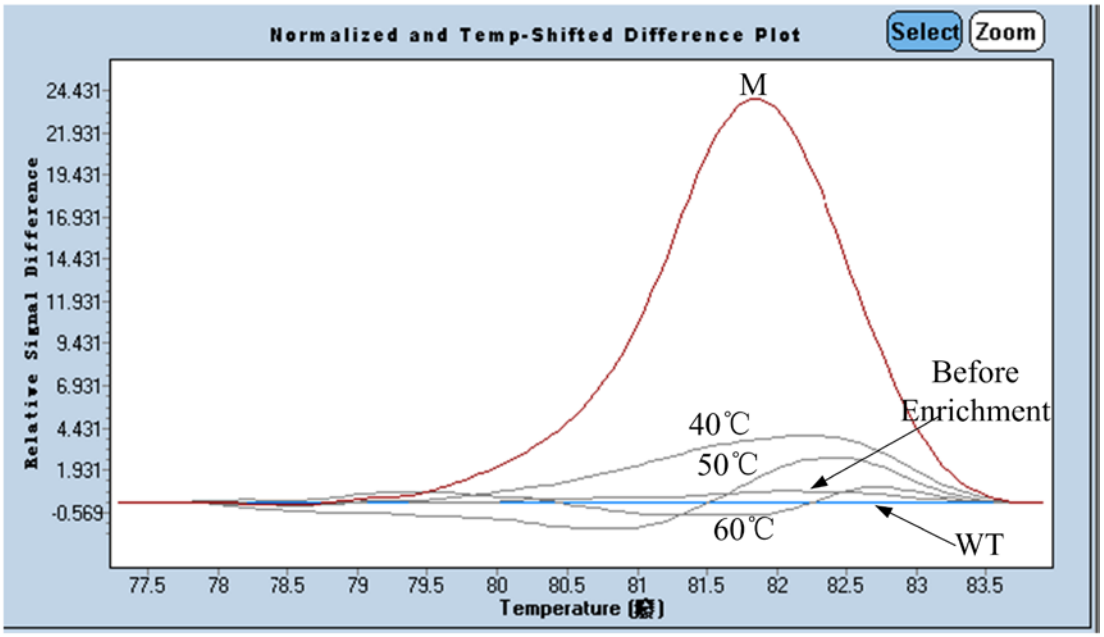
Figure 18 Sensitivity of PEMM-HRM assay. A) Before probe enrichment process, there was no difference among 0.1%, 1% mutation and wild type samples. After probe enrichment process, 0.1%, 1% mutation samples were readily identified by HRM assay. B) The results showed that as low as 6 copies initial mutation template can be identified by PEMM-HRM assay. The base line is the normalized melting curve from wild type DNA.

4.3.4 Temperature effect on hybridization

Because the sensitivity in the PEMM-HRM assay depends on the selective hybridization between the enriching probe and amplified DNA template. The best hybridization condition is important to mutation enrichment. In most cases, the destabilizating effect of a single base pair mismatch is insufficient to reduce nonspecific binding between enriching probe and wild type DNA. The hybridization can be influenced by the temperature, buffer, and detergent. In order to find the optimum conditions, the streptavidin-coupled Dynabeads were washed from 40 to 80ºC, the TE buffer and DI water were also compared as a streptavidin-coupled Dynabeads washing buffer. The 5 µl streptavidin-coupled Dynabeads which captured mutation alleles were incubated in 3x 1 ml of TE buffer at 40, 50, 60, 70 and 80ºC for 5 mins, then the supernatant was removed. The eluted DNA was subjected to PCR amplification and HRM analysis. Although the theoretical annealing temperatures between enriching probes and mutation amplicons are around 60ºC, the result showed that the higher temperature didn't provide better discrimination effect for enriching probe. There were always certain amounts of nonspecific binding between enriching probe and wild-type DNA. Figure 19 displayed the difference plot for 0%-100% p53 248 CGG>TGG mutation after enrichment process with increased hybridization temperature.



1% p53 248-1 mutation enriched by PEMM-HRM assay at different temperature



0.1% p53 248-1 mutation enriched by PEMM-HRM assay at different temperature

Figure 19 Probe enrichment efficiency at different hybridization temperatures. The difference plot showed probe enrichment efficiency of 1% and 0.1% 248 CGG>TGG mutation with increased hybridization temperature. The DNA sample before probe enrichment, the wild type DNA and the 100% mutation DNA were used as the references.

4.3.5 Singleplex and Multiplex Probe Enrichment

Oligonucleotide probes showed excellent discrimination power with single-base mutation enrichment. In many cases, a sequence amplified by PCR may contain multiple potential mutation sites. For example, the K-ras sequence amplified covers 4 potential mutation spots. As a result, the question must be addressed if we can use the same condition to enrich a variety of mutation sequence in a satisfactory manner. In this work, we examined the enrichment of each mutation sequence in the presence of all other probes to determine the degree of nonspecific hybridization.

Figure 20 displays analysis of the samples containing 0.1% and 1% p53 codon 273 CGT>CTT mutation which were carried out by using 36-probe mixture to survey p53 codons 175 196 248 213 245 273 and 282. After one round of probe enrichment, 0.1% and 1% of p53 codon 273 CGT>CTT mutation were successfully identified. Other studies also showed the nonspecific hybridization between the probes targeting other mutations within the same PCR product and wild-type DNA could affect on enrichment and lower the sensitivity. However this nonspecific problem can be minimized by increasing the ratio of the blocking probe to enriching probe or altering the sequence of the probe.

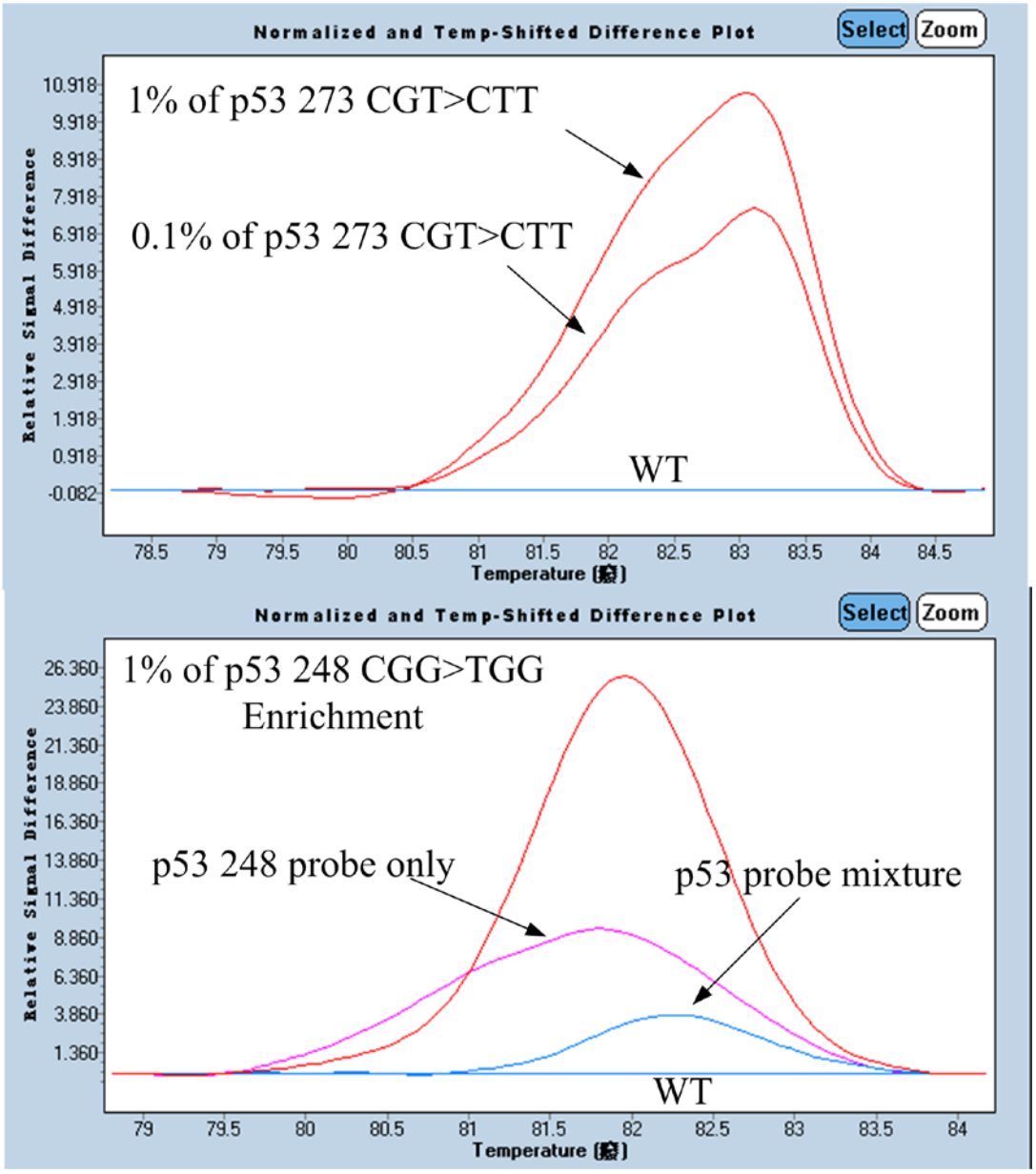


Figure 20 singleplex and multiplex probe enrichment efficiency. Top, 0.1% and 1% p53 codon 273 CGT>CTT mutation were enriched by 36 mutation enrichment probes. Bottom. 1% of P53 248 CGG>TGG mutation samples are enriched by singleplex and multiplex P53 enriching probes.

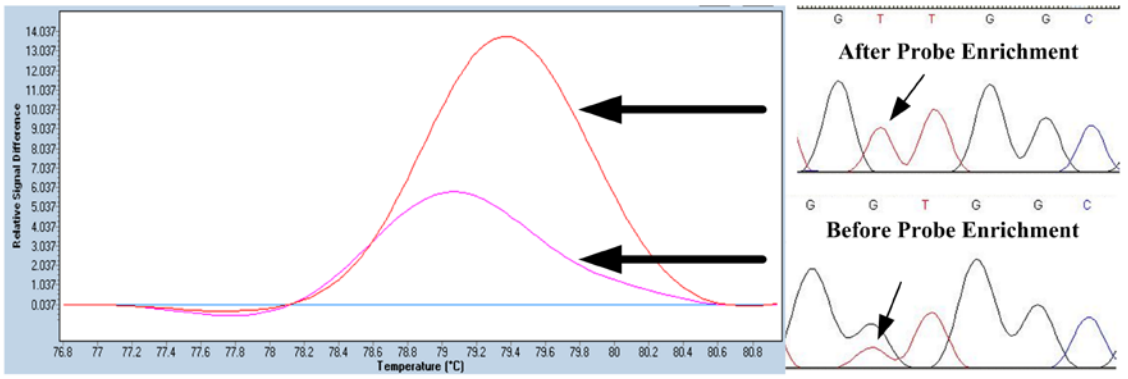
4.3.6 PEMM-HRM Assay Validation

Several mutation enrichment methods are developed for enriching known or unknown mutations. COLD-PCR (co-amplification at lower denaturation temperature) enables enrichment of PCR amplicons containing unknown mutations at any position (Li and Makrigiorgos 2009). This method needs strict temperature control of PCR cycles. SMART-amplification needs specially designed folding primers and turn-back primers and also needs Thermus aquaticus (Taq) MutS to suppress the normal template amplification (Tatsumi, Mitani et al. 2008).

The PEMM-HRM assay exhibits excellent enrichment of the mutation template. This assay improves the sensitivity of original HRM assay. We used a conventional Sanger sequencing method to validate the result of probe enichment process. Kras codon 12 and 13 are frequently mutated in colorectal cancer. Tissue DNA samples were amplified by 189 bp Kras primer pairs and P53 primer pairs. Then the mutation templates were enriched by standard Probe Enrich process as described before. The PCR templates which were processd before and after the enrichment were subjected to HRM analysis and Sanger sequencing methods. The results are compared side by side in Figure 21.

The sequence result of colorectal cancer tissue 1186 sample in Figure 21 A shows about 30% Kras codon 12 GGT>GTT mutation. The HRM analysis can directly detect the mutation from the wild type sample without probe enrichment process. After the probe enrichment, the sequencing data shows that the wild type templates containing Kras codon 12 GGT are depeletd by the blocking probe. Only mutation templates containing Kras codon 12 GTT mutations are present. The sequence result of colorectal cancer tissue 1319 sample in Figure 21 B shows less than 5% Kras codon 12 GGT>TGT

A



B

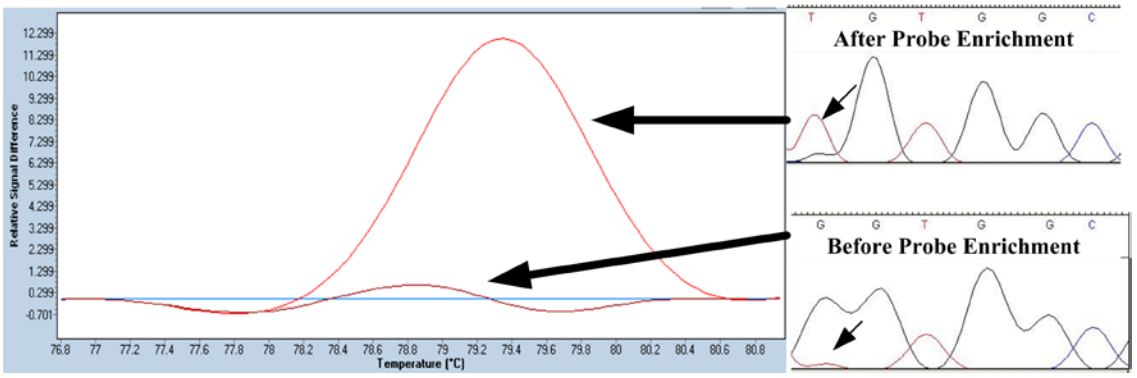


Figure 21 Examples of probe enrichment of mutant K-RAS codon 12 are demonstrated. A. the 1186 colorectal tissue sample contains Kras codon 12 GGT>GTT mutation. After probe enrichment, templates containing Kras codon 12 GGT are depleted by blocking probe. Only templates containing Kras codon 12 GTT templates are present sequencing result. PEMM-HRM result shows increased deviation between mutation and wild type melting curves than the original HRM assay. B. 1319 colorectal tissue sample contains low abundance Kras codon 12 GGT>TGT mutation. The sequencing data show that Kras 12 TGT Codons are significantly enriched by the PEMM-HRM assay. In addition, the low abundance mutations are more readily detected in PEMM-HRM assay than original HRM analysis. The baselines are the melting curves of wild type DNA (blue).

mutation, which is below the detection limit of regular HRM analysis. The HRM plot show a false negative result. The sequencing data shows that the ratio of Codon GGT to Codon TGT templates is significantly reversed by the probe enrichment process. In addition, PEMM-HRM assay can avoid the false negative results usually generated from original HRM analysis and detect the low abundance mutation.

4.3.7 Stool DNA extraction and primer design in methylation-specific HRM

DNA extraction from fecal samples is a challenging process. Stool samples contain a variety of bacterial types. The bacterial environment of each individual is determined by several factors. The diet is the most significant factor in stool composition and texture. Taking medicine can also change the balance of bacterial composition in the small and large intestine. Aberrantly methylated and mutated DNA sequences in tumors were detected in the fecal samples of cancer patients. Current studies shows that fecal DNA can be served as colon cancer biomarkers (Azuara, Rodriguez-Moranta et al.; Ito, Kobayashi et al. 2002; Glockner, Dhir et al. 2009). Fecal DNA is now feasible and a panel of DNA markers including mutations in k-ras, APC, p53 and BAT26 shows high sensitivity for colon cancer screening. These markers are highly specific and display the potential to replace current Fecal Occult Blood Test (FOBT) (Atkin 2003). Methylated DNA markers in stool DNA is also used to identify colorectal tumors. Although the sensitivity of methylation detection in stool sample ranges from 69-89%, stool DNA methylation analysis provides a noninvasive and low cost method for colorectal cancer screening (Azuara, Rodriguez-Moranta et al.).

For the DNA methylation HRM analysis, there are two different strategies to design the primers: bisulfite sequencing primers and methylation specific primers (Wojdacz,

Borgbo et al. 2009). Bisulfite sequencing primers do not contain CpG sites within their sequences and are able to unbiasedly amplify both methylated and unmethylated DNA templates. Methylation specific primers contain at least one CpG site within their sequence, and methylation specific PCR preferably amplify the methylated DNA. Because bisulfite sequencing primers can faithfully amplify the both methylated and unmethylated DNA templates, all the HRM primers for methylation detection adopt bisulfite sequencing primers. This property can also be used for semiquantitative measurement of methylation content in melting curve analysis.

4.3.8 Standard Curve Construction for Methylation Detection

The base composition of PCR products derived from sodium bisulfite-modified templates is dependent on the methylation status. Therefore, methylated and unmethylated PCR products show different melting curves when they are subjected to denaturation. The methylation-sensitive high-resolution melting (MS-HRM) is based on the measurement of the melting curves of PCR products from unknown samples, then comparison of the unknown melting curves with standard curves constructed from methylated and unmethylated control DNAs. The 5 ng (before modification) bisulfite-converted DNAs containing 0%, 10%, 50% and 100% methylated DNA were subjected to MS-HRM in triplicate. The standard curves of gene BMP3 and EY4 were present in melting curve profile and derivative plot (Figure 22).

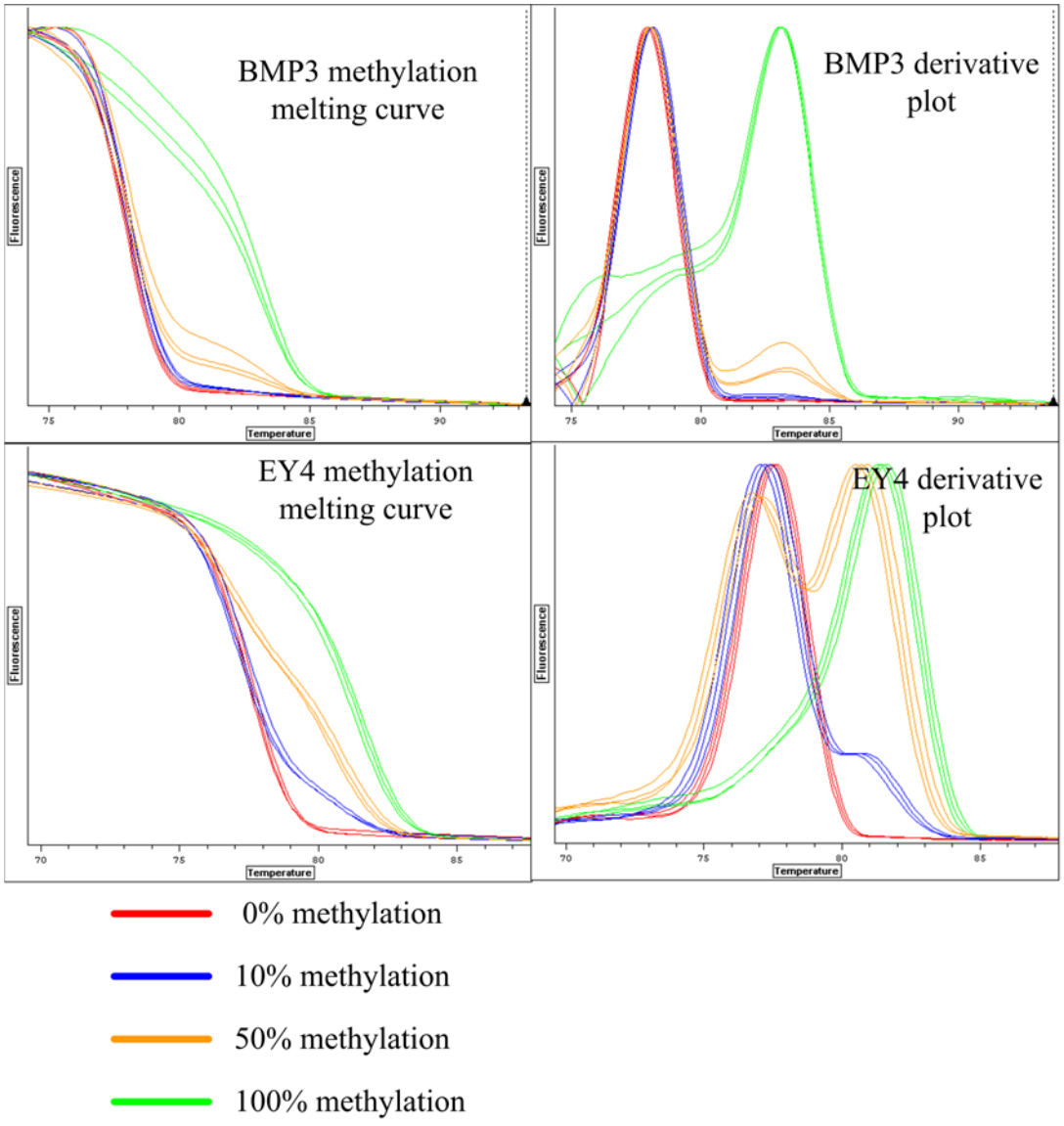


Figure 22 Melting curve of 0%, 10%, 50% and 100% methylation sample. Standard curves of gene BMP3 and EY4 were constructed by serially diluting methylated DNA in unmethylated DNA.

4.3.9 Methylation enriched by PEMM-HRM assay

Probe enrichment technology is a powerful method to increase the sensitivity of mutation and methylation detecting. After successfully applying this technology to mutation enrichment, enrichment of methylation templates was tested as well. The PCR products containing 0%, 10%, 50% and 100% methylation template were subjected to the PEMM-HRM assay. After enrichment, the detection limit greatly extended to less than 5% methylation. The multiplexed feature of PEMM-HRM assay was determined by detecting a methylation sample containing more than one methylation promoter.

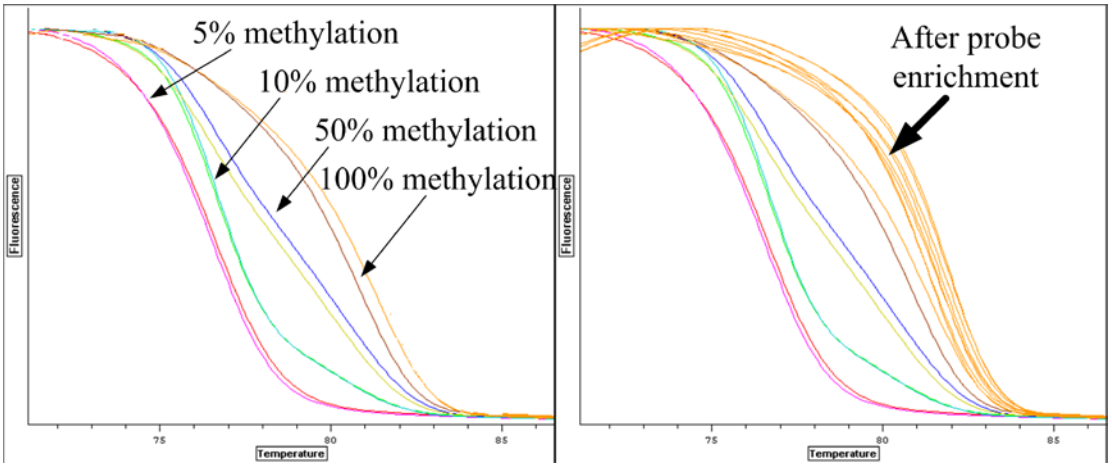


Figure 23 DNA methylation enrichment. 5%, 10%, 50% and 100% methylation can be multiplex enriched by PEMM-HRM assay.

4.3.10 Analysis of methylation profile of stool samples

This study was designed to evaluate methylation frequency of colorectal cancer in clinical stool samples. The PEMM-HRM assay was used to detect methylation frequency in corresponding stools. Samples are comprised of eight stool samples with discovered sporadic colorectal cancer. The whole stool samples were collected in preservative buffer and delivered to our lab within 72 hours and stored in -80ºC refrigerator.

We examined methylation frequency of five genes (BMP3, ALX4, UNC5C, EY4 and GATA5) in eight cancer patient stool samples. Comparing with original HRM analysis, PEMM-HRM assay shows a superior advantage with respect to detection limit. After the probe enrichment process, low concentrations of methylation samples can be detected in the PEMM-HRM assay. Because PEMM-HRM assay shows high sensitivity and specificity, this method should reflect clearly the actual gene methylation status in human body fluids, which is important for study the early stage cancer. Table IX shows the PEMM-HRM assay and original HRM assay show different methylation frequency results.

Table IX List of methylation frequency in 8 stool samples

Sample	BMP3	ALX4	UNC5C	EY4	GATA5
HRM assay	25%	0%	12.5%	25%	12.5%
PEMM-HRM assay	**37.5%**	**25%**	12.5%	**37.5%**	12.5%

4.4 Conclusion

In the present study, we have applied Probe Enrichment Mutation/Methylation -High Resolution Melting analysis to human colorectal cancer. We conclude that HRM melt analysis is a simple and cost effective post PCR technique, which can be used for high throughput mutation scanning, genotyping, and methylation analysis. After the comprehensive tests, we found the HRM analysis perfectly compatible with the mutation/methylation enrichment technology. The study displays that one mutation template in 1000 wild type templates can be readily detected after the mutation targeting probe enrichment. The detecting limits reached to as low as 6 copies of genomic DNA. Moreover, we demonstrated that PEMM-HRM was able to enrich several mutation templates in a multiplex way which makes it a potential high throughput platform. The robustness of traditional PCR method is inherited completely by the HRM method which benefits from a successful rate of stool DNA amplification. This is the key in transferring the proposed technology from bench study to clinical research.

Recently, a new real time PCR instrument is under development. ABI, Roche and Bio-rad all combine 384-well plate compatibility with fully automated robotic loading. ABI and Roche now also offer optional fast real-time PCR capability. With the occurrence of nanoliter qPCR system, the throughput of HRM method may increase dramatically (Dixon, Lubomirski et al. 2009). The nanoliter qPCR system enables up to 3072 nanoliter qPCR assays simultaneously in a high-density array format. PEMM-HRM displayed an intrinsic accurate, large dynamic range, and highly sensitive approach compatible with to high throughputs.

CHAPTER V

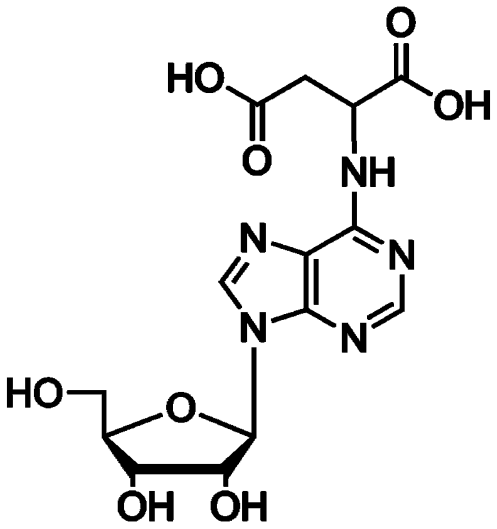
DETECTION OF SA AND SAICAr IN URINE BY A MS METHOD FOR ADENYLOSUCCINATE LYASE DEFICIENCY SCREENING

5.1 Introduction

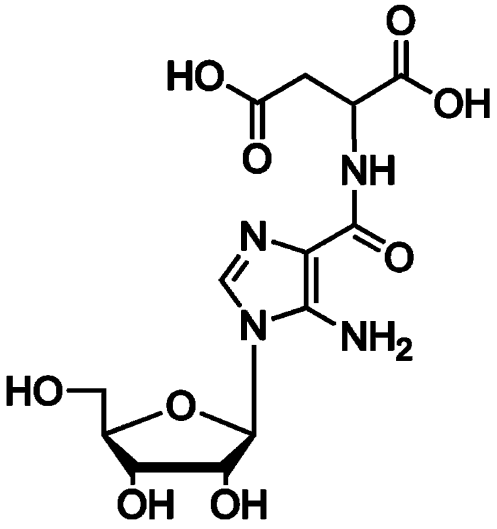
More than 10 specific genetic diseases are associated with autism features, and adenylosuccinate lyase (ADSL, EC 4.3.2.2) deficiency is one of them (Sempere, Arias et al.; Santangelo and Tsatsanis 2005). ADSL deficiency is a defect of purine metabolism manifesting with autism, along with developmental delay and seizures (Spiegel, Colman et al. 2006). In patients with ADSL deficiency, succinyladenosine (SA) and succinylamino

-imidazolecarboxamide riboside (SAICAr) (Figure 24), the two intermediates of the de novo purine biosynthesis pathway, are accumulated in plasma, cerebrospinal fluid (CSF), and urine (Jaeken and Van den Berghe 1984; Race, Marie et al. 2000). These accumulations can serve as reliable biomarkers for screening. There are three distinct clinical phenotypes: neonatal fatal form, type I (severe form) and type II (moderate or mild form) (Jurecka, Zikanova et al. 2008).

Different screening methods for both SA and SAICAr have been established in the past years. The modified Bratton-Marshall test (Laikind, Seegmiller et al. 1986), a urine test for the presence of elevated levels of SAICA riboside, is relatively simple and low cost. However, this method could give a false positive result if the patients take certain medications such as sulfonamide drugs. In theory this test would also give positive results with a number of structurally related purine biosynthetic intermediates. In recent years chromatography has been introduced to detect the two compounds. Thin Layer Chromatography (TLC) (Wadman, de Bree et al. 1986), High Performance Liquid Chromatography (HPLC) (Hartmann, Okun et al. 2006), Capillary Electrophoresis (CE) (Adam, Friedecky et al. 1999; Hornik, Vyskocilova et al. 2007), and Mass Spectrometry (MS) (Krijt, Kmoch et al. 1999; Jeng, Lo et al. 2009) are used to diagnose the ADSL deficiency. In general the disadvantages of these methods are that it is difficult to produce concrete results to identify SA and SAICAr compounds in the body fluids of patients, it is usually time consuming for preparing and analyzing the samples, and they are therefore not suitable for rapid screening of large number of samples. Currently, there are no simple



Succinyladenosine (SA)
Molecular Weight: 383



Succinylamino-imidazolecarboxamide riboside (SAICAr)
Molecular Weight: 374

Figure 24 Structures of accumulated metabolites in ADSL patients

methods that are practical, quantitative, economic, and fast for this disease screening. In this paper, we report a novel Electrospray Ionization Mass Spectrometric (ESI-MS) method to detect and quantify SA and SAICAr in urine, offering a method that can be potentially used to screen ADSL deficiency disease.

5.2 Experimental

5.2.1 Patients and Healthy Volunteers

This study was approved by the DDC Clinic. The urine samples were collected from two patients (P1 and P2), whose ADSL deficiency had been diagnosed clinically. The urine samples of the parents were also collected (T1 and T2 are the parents of P1, and T3 and T4 are the parents of P2, respectively). Four normal urine samples (N1, N2, N3, and N4) collected from normal persons without ADSL deficiency were used as the control.

5.2.2 Urine Sample Preparation

The collected urine samples were stored in -20ºC refrigerator immediately. The disposable Solid Phase Extraction (SPE) column was packed with 100 mg of C18 material (Chromabond C18, 1 mL/100 mg, octadecyl-modified silica, MACHEREY-NAGEL GmbH & Co. Germany) and balanced accordingly with 1 m> of the condition solutions: A, methanol; B, deionized water; C, 50 mM NaH_2PO_4, pH 2.5. The urine sample (0.4 mL, acidified to pH 2.5 with 2 M H_3PO_4) was applied to the column, followed by washing with 1 mL solution C and 0.6 mL solution B. SA and SAICAr were eluted by 0.4 mL of solution D (20% methanol).

5.2.3 Determination of SA and SAICAr by ESI-MS

The SA and SAICAr isolated from urine were then analyzed using an ESI-Ion Trap MS^n System (Bruker Daltonics Inc, Billerica, MA). Briefly, 1 μL of the extracted sample was mixed with 99 μL of an ESI buffer and the sample infusion was performed with a 50 μL syringe at a flow rate of 2 μL /min at room temperature. The mass spectrometer was operated on the positive-ion mode and scanned from 100 to 1000 Da. The ESI buffer consisted of acetonitrile, water, and formic acid (50:50:0.3) (Kamel and Munson 2004).

5.2.4 Quantitation of SA and SAICAr

The commercially available molecule of AICAr was used as an external standard to quantify both SA and SAICAr molecules by ESI-MS. The constant concentration of AICAr was spiked into the urine sample after SPE. The standard calibration curves (I_{SA} /I_{AICA}r vs. C_{SA}/C_{AICAr} or I_{SAICAr}/I_{AICA}r vs. C_{SAICAr}/C_{AICAr}) were constructed for calibration of both molecules.

5.3 Results and discussion

5.3.1 Development of the SPE/ESI-MS analysis

By combining solid phase extraction and mass spectrometric analysis, we were able to successfully detect both SA and SAICAr in urine (Figure 25).

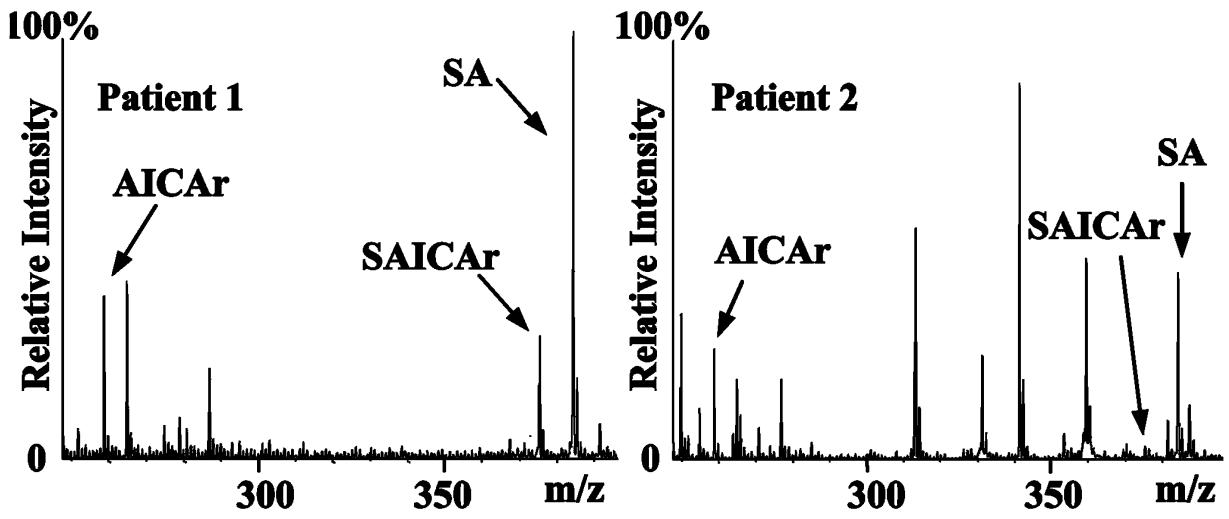


Figure 25 Typical ESI-MS spectra obtained from the urine of Patients Patient 1 and Patient 2. The peaks of 375Da and 384Da show the protonated SAICAr and SA, respectively. The Peak of 259Da corresponds to the external standard molecule of AICAr.

Both urine-soaked filter-paper strips and urine samples were analyzed. With this method, we were able to successfully detect both SA and SAICAr in urine samples. For the urine-soaked filter-paper strips, only SA can be detected. The protonated SA and SAICAr [M+H]+ ions have m/z values of 384 Da and 375 Da, respectively (Figure 25). Collision-induced dissociation of the ions of 384 and 375 Da shown that the resulted daughter ions were generated from loss of a ribose moiety [MH-132]+ (Jeng, Lo et al. 2009), confirming that the ions of 384 Da and 375 Da are the protonated SA and SAICAr, respectively. To the best of our knowledge, this work is the first one that successfully identified both molecules in urine by ESI-MS.

5.3.2 Quantitative Analysis of SA and SAICAr

To determine whether the MS method could accurately quantify these two molecules in urine. We discovered that a commercially available molecule of AICAr is an excellent external standard to quantify both molecules by ESI-MS (the peak of 259 Da in Fig 25). This external standard molecule was spiked into the urine sample after SPE. Because we do not have pure SA and SAICAr samples, we used different quantities of the urine sample collected from Patient P1, while keeping the AICAr concentration constant, to construct the calibration curves. Figure 26 displays the standard calibration curves constructed for calibration of both molecules, in which an excellent linear relationship between the MS ion signal ratio and the concentration ratio was seen for both SA and SAICAr (I_{SA} /I_{AICAr} vs. C_{SA}/C_{AICAr} (Figure 26A) or I_{SAICAr}/I_{AICAr} vs. C_{SAICAr}/C_{AICAr}

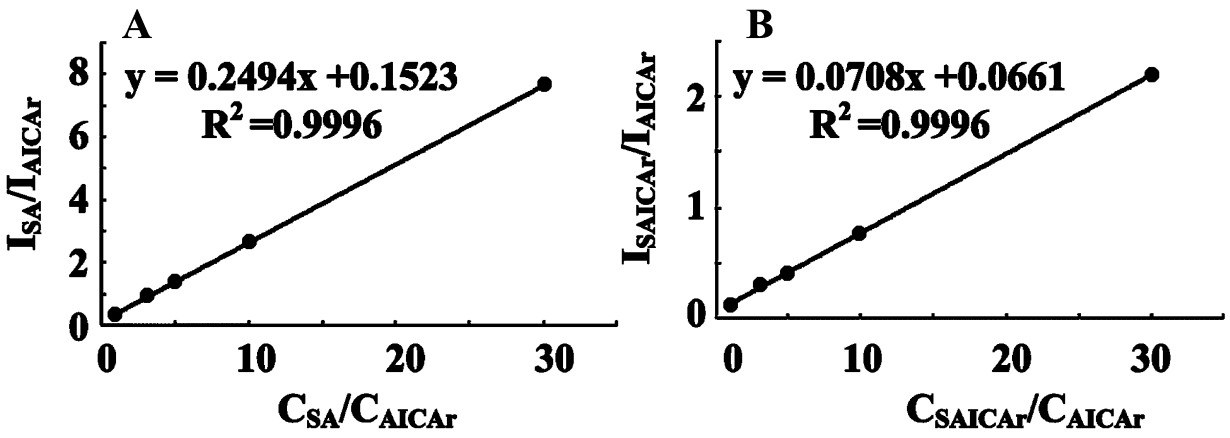


Figure 26 Calibration curves constructed for the quantitative analysis. SA (A) and SAICAr (B) in urine.

(Figure 26 B), indicating that this MS method can be used to quantify both SA and SAICAr in urine.

In addition, because linear calibration curves (Figure 26) can be constructed for both SA and SAICAr molecules with the same external standard of AICAr, the ratio of I_{SA}/I_{SAICAr} measured by MS actually reflects the concentration ratio of C_{SA}/C_{SAICAr}. Since the ratio of C_{SA}/C_{SAICAr} is correlated with the toxicity of the intermediates, particularly neurotoxicity, the ratio of I_{SA}/I_{SAICAr} measured by MS can offer a means to evaluate the severity of ADSL deficiency of a patient as well (Van den Bergh, Vincent et al. 1993).

5.3.3 Analysis of SAICAr

SAICAr is an unstable biomolecule which proved difficult to be analyzed. During the course of this study, we found that compared with SA, SAICAR is highly unstable in urine, leading to large measurement errors if great care was not taken during any step of urine collection, storage, transportation, and solid-phase extraction. In our view, SA is a more practical marker for ADSL deficiency screening. Hence, we only studied SA as a screening marker.

5.3.4 Improvement in diagnosis of ADSL

We measured the ratio of I_{SA}/I_{AICAr} in 10 urine samples and each sample was studied three times. The concentration of the external standard spiked into each of the urine samples was 2 pmol/μL. The ratio of I_{SA}/I_{AICAr} of ADSL patients (P1 and P2) was greater than 1.5, while the ratio is 0 for normal individuals without ADSL deficiency (4 normal controls), indicating that this method can distinguish ADSL deficiency patients from normal individuals. Although a more accurate cut-off ratio of I_{SA}/I_{AICAr} for ADSL

deficiency screening needs to be defined by studying more ADSL deficiency patients, this study clearly establishes that this new MS method can potentially be used for ADSL deficiency screening.

We also found that this new method can identify some ADSL genetic-defect carriers. The ratio of I_{SA}/I_{AICAr} was less than 0.5 for carriers (the parents of P1 and P2), but often larger than 0. This observation is significant as it shows that carriers can also yield observable MS signals, but the ratio of I_{SA}/I_{AICAr} of a carrier is much smaller than the ratio of the ADSL deficiency patients. Previous MS studies reported that ADSL deficiency patients could be simply identified by the appearance of a peak corresponding to SA (Krijt, Kmoch et al. 1999). Clearly, this qualitative approach could falsely identify an ADSL defect carrier as an ADSL patient. In contrast, our quantitative MS method can unambiguously detect ADSL deficiency patients.

5.3.5 Validation of SPE-MS method

Finally, we studied the robustness of this method by examining whether the measured ratio of I_{SA}/I_{AICAr} is susceptible to variation of urine samples, as urine contains many other components and the compositions of urine could vary from one person to another. P1/P2, P1/T3, and P2/T2 urine samples were 50/50 mixed, followed by measuring the

Table X List of the measured and expected ratios of I_{SA} /I_{AICAr} in samples.

I_{SA} /I_{AICAr}	Mixture of P1/P2	Mixture of P1/T3	Mixture of P2/T2
Measured Value	3.203	0.854	2.032
Expected Value	2.926	1.186	1.921

ratio of I_{SA}/I_{AICAr} in each mixture sample. Table 1 lists the measured and expected ratio of I_{SA}/I_{AICAr} in each of the three mixture samples. The expected ratio was derived by averaging the measured ratios of I_{SA}/I_{AICAr} of the two individual urines.

Clearly, the results listed in Table X show that the measured ratio is similar to the expected ratio in all 3 mixture samples, suggesting that the measurement of I_{SA}/I_{AICAr} will not be affected greatly by variations of urine samples and thus this MS method is robust.

5.4 Conclusion

In conclusion, we have developed a novel mass spectrometric method for measurements of SA and SAICAr in urine, which is simple, fast, robust, and cost-effective. As a result, this method can potentially be used in ADSL screening.

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