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Establishing a method for measuring the DNA methylation status of specific human genes

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necessarily to be attributed to the NRF.*

*To trust God is to believe in the fact that God is there
for me and to live in that certainty
(Karl Barth)*

Vir my ouers en Rupert

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LIST OF ABBREVIATIONS

A:

A: Adenine

B:

BER: Base excision repair

bp: Base pair

C:

C: Cytosine

CH₃: Methyl group

cm: Centimetre

cm²: Square centimetre

COBRA: Combined bisulfite restriction analysis

CpG: Cytosine phosphorylated Guanine

D:

ddH₂O: Double distilled water

DIG: Digoxigenin

DMSO: Dimethylsulfoxide

DMT/ DNMT: DNA methyltransferases

DNA: Deoxyribonucleic acid

DOC: Sodium deoxycholate

DSBs: Double strand breaks

E:

E. coli: Escherichia coli

EDTA: Ethylenediaminetetra acetic acid

ER: Estrogen receptor α

Et al: Latin: And others

EtOH: Ethanol

F:

Fe: Iron

G:

g: Gravity (9.8 m.s^{-2})

g: gram

G: Guanine

H:

h: Hours

HCl: Hydrochloric acid

HR: Homologous repair/recombination

K:

kbp: Kilo base pairs

KCl: Potassium chloride

KH_2PO_4 : Potassium dihydrogen orthophosphate

KOH: Potassium hydroxide

L:

l: Litre

LOH: Loss of heterozygosity

M:

M: Molar

MgCl_2 : Magnesium chloride

ml: Millilitre

mM: Millimolar

MMR: Mismatch repair

MSP/ MS-PCR: Methylation-specific PCR

mV: Millivolt

N:

NaCl: Sodium chloride

NaClO_4 : Sodium perchlorate

Na_2HPO_4 : di-Sodium hydrogen orthophosphate

NaOH: Sodium hydroxide

Na₂SO₄:	Sodium sulphate
NER:	Nucleotide excision repair
ng:	Nanogram
NH₄Ac:	Ammonium acetate
NHEJ:	Non-homologous end joining
(NH₄)₂SO₄:	Ammonium sulphate
nm:	Nanometres
O:	
OD:	Optical density
P:	
PBS:	Phosphate-buffered Saline
PCR:	Polymerase chain reaction
pH:	Potential of Hydrogen
PR:	Progesterone receptor
R:	
rpm:	Revolutions per minute
S:	
SAM:	S-adenosylmethionine
SDS:	Sodium dodecylsulphate
SSB:	Single strand break
T:	
T:	Thymine
T_m:	Melting temperature
Tris-Cl:	2-Amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride
U:	
U:	Uracil
U/μl:	Units per microlitre
UV:	Ultra violet

V:

V: Volt

v/v: volume per volume (ml per 100 ml)

W:

w/v: weight per volume (g per 100 ml)

Symbols: α Alpha β Beta λ Lambda $^{\circ}\text{C}$ Degrees Celsius μl Microlitre μM Micromolar

Catalogue number

[] Concentration

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ABSTRACT

DNA methylation is an epigenetic alteration which occurs during the development and lifetime of mammals. It has an essential gene transcription regulation function and has been shown to be a common alteration at the root of several malignancies. Regions with high concentrations of CpG sites are classified as CpG islands which usually lie in the promoter regions. Hypermethylation of these promoter regions represses transcription of tumour repressor genes, which causes gene silencing. Specific methylation profiles can be compiled for different types of cancers. These profiles are of great importance due to their diagnostic, risk assessment and therapeutic potential, in addition to increasing the knowledge and understanding of the pathogenesis of tumorigenesis and oncogenesis.

The aim of this study was to establish and optimize a method to measure the DNA methylation status of specific human genes, i.e. cancer related genes. Methylation-specific PCR (MSP) was used to analyze the methylation status of the *p16* and *RASSF1A* promoter CpG islands following the sodium bisulfite conversion of unmethylated cytosines to uracils since this is a sensitive, efficient and relatively fast method with high-throughput potential.

The MSP assay was established and applied successfully to DNA isolated from whole blood, plasma (free-circulating DNA) and paraffin-embedded tumour tissue. The results obtained showed that for the *p16* promoter sequence, the methylation status in all control individual DNA samples was unmethylated. The patient DNA samples had diverse *p16* promoter methylation levels. The tumour DNA sample displayed both methylated- and unmethylated-specific products. This was also observed for this patient's free-circulating DNA sample, but not for the whole blood DNA sample.

OPSOMMING

DNA-metilering is 'n epigenetiese verandering wat voorkom tydens die ontwikkeling en leeftyd van mense, en speel 'n essensiële rol in die regulering van transkripsie. Daar is aangetoon dat epigenetiese veranderinge algemeen betrokke is by die oorsprong van verskeie kwaadaardighede. DNA-gebiede met hoë konsentrasies CpG-setels word geklassifiseer as CpG-eilande, wat gewoonlik in die promotorgebiede lê en hipermetilering van hierdie promotorgebiede onderdruk transkripsie van tumorsuppressorgene. Spesifieke metileringsprofile kan saamgestel word vir verskillende tipes kanker, en hierdie profile is van groot belang in terme van die diagnostiese, risikobepaling en terapeutiese potensiaal daarvan, asook die vermeerdering van kennis en begrip van die patogenese van tumorigenese en onkogenese.

Die doel van hierdie studie was om 'n metode te vestig en te optimiseer om die DNA-metileringsstatus van spesifieke mensgene, bv. kankerverwante gene, te meet. Metilerings-spesifieke PCR is gebruik om die metileringsstatus van die *p16^{INK4a}*- en *RASSF1A*-promotor CpG-eilande te bepaal na die natriumbisulfit geïnduseerde verandering van ongemetileerde sitosien na urasiel. Hierdie is 'n sensitiewe, doeltreffende en relatief vinnige metode met 'n hoë-deurset potensiaal.

Die MSP-analise is gevestig en suksesvol toegepas op DNA wat uit heelbloed, plasma (vry-sirkulerende DNA) en paraffien-gepreserveerde tumorweefsel geïsoleer is. Die resultate wat verkry is, toon dat vir die *p16*-promotorvolgorde, die metileringsstatus in alle kontrole persone se DNA ongemetileerd was. Die pasiënte se DNA het diverse *p16*-metileringsvlakke getoon. Die tumor DNA het beide gemetileerde- en ongemetileerde-spesifieke produkte bevat. Dit is ook in die pasiënt se vry-sirkulerende DNA waargeneem, maar nie in haar heelbloed DNA nie.

CHAPTER 1

INTRODUCTION

During the development and lifetime of an organism, heritable changes can occur in the expression and activity of genes without changing the specific nucleotide sequence of the genes involved. This can be defined as epigenetic alterations which have various important functions and implications in the genome. One of the most frequent and important of these changes is DNA methylation, which is essential for the viability of individual cells due to its gene transcription regulation function and it has been shown to be a common alteration present in several tumours (Wachsman, 1997; Esteller, 2003; Nephew and Huang, 2003; Egger, *et al.* 2004; Roloff & Nuber, 2005).

In mammals, DNA methylation mainly occurs at CpG sites, and regions with high concentrations of CpG sites are classified as CpG islands which usually lie in the promoter regions (Huang, *et al.*, 1999; Cottrell, 2004; Fazzari and Greally, 2004). Hypermethylation of these promoter regions represses transcription of tumour suppressor genes, which causes gene silencing (An, *et al.* 2002; Esteller, 2005). Regional hypermethylation and global hypomethylation are studied in human cancers to compile specific methylation profiles for different types of cancer. These profiles are of great importance due to its use as a stable mark of identity and, therefore, its potential use as diagnostic markers, in addition to increasing the knowledge and understanding of the pathogenesis of tumorigenesis and oncogenesis. It is, therefore, potentially useful in risk assessment and therapeutic intervention (Nephew and Huang, 2003; Cottrell, 2004; Szyf, *et al.*, 2004; Laird, 2005; Wilson, *et al.*, 2006).

Methylation-specific PCR (MSP) is a rapid and convenient promoter methylation detection assay, which uses two sets of primers to amplify the region of interest in bisulfite modified DNA (Cottrell, 2004; Das and Singal, 2004). This study was designed to establish and optimize the MSP assay, and it was decided to investigate the diagnostic or quantitative potential of the assay via analysis of the methylation status of the CpG islands in the promoter sequences of the *p16* and *RASSF1A*

genes. The MSP assay was established in our laboratory, and applied successfully to determine the methylation status of the *p16* promoter sequence in various control individuals and clinically diagnosed breast cancer patients.

Chapter 2 consists of a literature review describing aspects relating to the integrity of the human genome, DNA methylation and its role in cancer, as well as a brief look at the potential use of methylation signatures as diagnostic markers. This provides a better understanding of the causes of changes in DNA methylation patterns, as well as the effect of these changes on the DNA structure and function. A description of the materials and methodology used to establish appropriate DNA isolation methods for this study, as well as a discussion of the results obtained with the various methods follow in Chapter 3. Chapter 4 describes the establishment of the MSP assay in our laboratory, and the subsequent optimization of the method. The optimized method was applied to DNA isolated from various tissues, including whole blood, free-circulating DNA and paraffin-embedded tumour tissue. The results of these applications are presented in Chapter 5 in the form of a first draft of a research paper prepared for submission to *Cancer Letters*. The results are discussed and summarised, and possible implications of the results are explored in Chapter 6.

CHAPTER 2

LITERATURE REVIEW

2.1. INTRODUCTION

Genetic integrity and genomic stability form the base of normal cellular structure and activity, and damage to the genetic material can induce aberrations such as tumours and mutations (Sekiguchi, 2006). DNA lesions like DNA strand breaks and oxidative damage, as well as DNA repair dysregulation causes genome instability. It is also known that genomic instability is particularly associated with tumour development and progression, since genetic alterations are characteristic of carcinogenesis, and often even a requirement for it (McMurray and Gotschling, 2004; Bindra and Glazer, 2005). Therefore, the repair capability of DNA has great significance in maintaining the integrity of DNA.

2.2. GENOMIC INTEGRITY

The genome is constantly exposed to an overabundance of lesion types due to external and internal stressors (Hoeijmakers, 2001; Allard, *et al.* 2004). This compromises cell survival and fuels tumorigenesis and, therefore, repair of damaged DNA is essential in order to protect against excessive mutation rates and genomic instability (Lisby, *et al.* 2004). In response to the continuous battering DNA endures, genomic stability is maintained by extensive signalling networks in order to maintain homeostasis in an organism. These signals can activate DNA repair, cell-cycle checkpoints and alter various other cellular processes as shown in Figure 2.1 (Shiloh and Lehmann, 2004). A single repair mechanism can, therefore, never deal with the diversity of damage, and for this purpose, specific genes (>130 DNA repair genes) and highly conserved mechanisms to detect and repair DNA degeneration are present in the cell (See Figure 2.2) (Bindra and Glazer, 2005). Different types of damage obviously require different repair mechanisms. For instance, nucleotide deletions require resynthesis, alkylated bases should be removed directly, and single-strand breaks need to be repaired (Bertram, 2001; Bindra and Glazer, 2005).

DNA synthesis is a highly controlled, coordinated process, and cell death frequently occurs due to a damage-induced delay in progression through the S-phase or protein unavailability, both of which usually occurs in carcinogenesis. This might explain why many of the damaged genes in cancer cells are related to cell-cycle checkpoint control (Bertram, 2001). Chromosome instability is defined by Badano *et al.* (2005) as “The increased probability of acquiring chromosomal aberrations owing to defects in processes such as DNA repair, replication or chromosome segregation”, and since defective chromosome replication instigates most of the chromosomal aberrations which occur in cancer, the genome should be free of damage before replication starts, to decrease the occurrence of mutations (Branzei and Foiani, 2005).

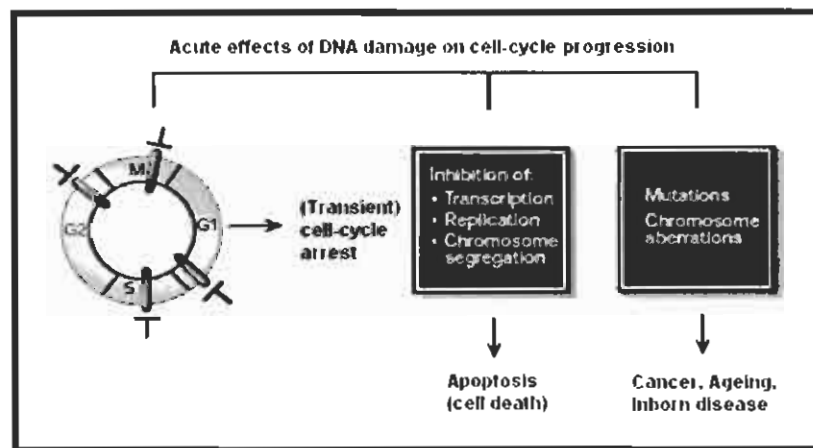


Figure 2.1. The effects of DNA damage on cell-cycle progression. The first two effects, namely transient arrest and DNA metabolism changes, are acute effects of DNA damage, while permanent DNA sequence changes are long-term effects (Adapted from Hoeijmakers, 2001).

During the DNA replication process the cell is most vulnerable to damaging agents and, therefore, when DNA double-strand breaks occur, genomic integrity is threatened or the DNA replication fork collapses, damage is detected and repaired by numerous checkpoint and repair proteins and the cell cycle is resumed (Lisby, *et al.* 2004).

2.3. THREATS TO GENOMIC INTEGRITY

Genomic instability is explained by Shiloh and Lehmann (2004) as numerical and structural chromosomal aberrations, and these alterations in the amount of chromosomes or their normal composition are characteristic of malignant cells.

Since the human genome is constantly subject to internal (e.g. hormones) and/or external (e.g. carcinogens) influences on the genetic material, DNA stability and function is continuously subject to change (Wachsman, 1997; McMurray and Gottschling, 2004). These events include damage through point mutations which could result in amino acid substitutions, frame-shift mutations, stop codon mutations that alters protein product or sequence, additions to or deletions from the genetic sequence, chromosomal imbalance or amplification causing instability, over expression or loss of a gene, chromosomal breakage and rearrangements. All of these may cause chimeric protein and epigenetic alterations of the genome (Wachsman, 1997; Das and Singal, 2004). Eventually these changes accumulate and cause loss of activity like tumour suppressor silencing or proto-oncogene activation in the genome, which is a significant concern in the instigation of cancer and its development (Hoeijmakers, 2001; Nephew and Huang, 2003; McMurray and Gottschling, 2004).

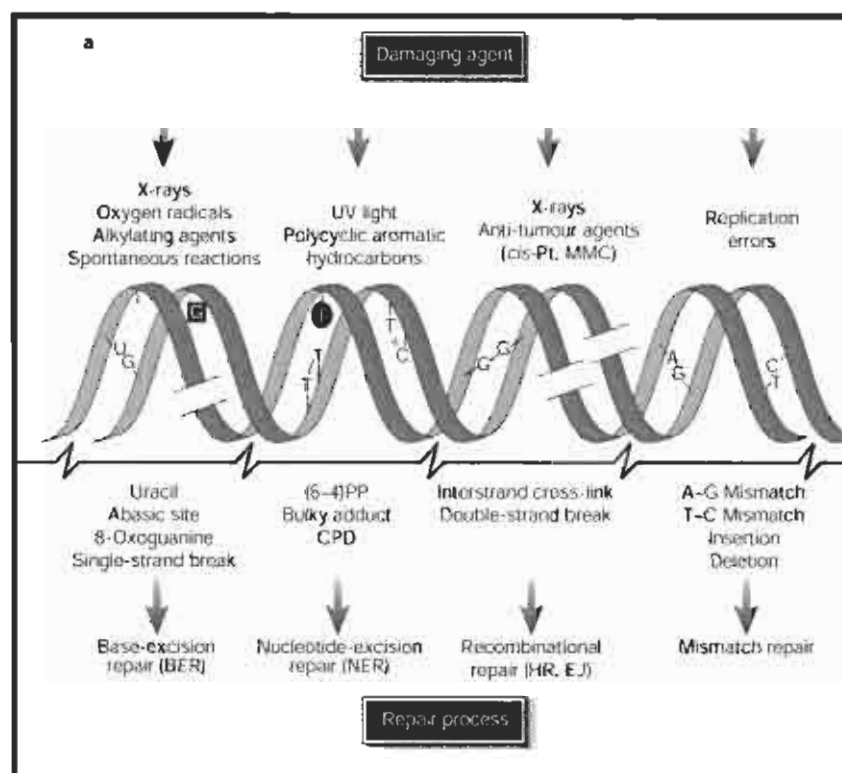


Figure 2.2. DNA damage and its relevant repair mechanisms. Damaging agents induce various aberrations in the DNA and there are specific repair mechanisms to remove or repair each of these (Hoeijmakers, 2001).

The advancing conversion from normal to malignant cells has been suggested to require the sequential gain of mutations or DNA changes which provides a selective

growth benefit to cells (Cottrell, 2004). These mutations and lesions are not inherited, but occur spontaneously because of permanent changes in the genome such as DNA replication errors, intrinsic chemical instability in DNA, free radical damage and interactions with exogenous agents like UV radiation and chemical carcinogens (see Figure 2.2 for examples of these damaging agents and the subsequent lesions they induce). Additional mutations will occur if the mutation inactivates genome integrity maintaining genes (Bertram, 2001; Hoeijmakers, 2001).

2.3.1. Induction of DNA damage by carcinogens

Most carcinogens, applied in sufficient quantities, may damage cells in some way by forming adducts to protein and DNA and most current theories propose that the mechanism responsible is adduct formation via electrophilic derivative interaction (Tardiff, *et al.*, 1994; Bignold, 2003). DNA adduct formation is considered as an action of significant importance in mutation and cancer initiation. Carcinogenic agents can be divided into three classes; physical, chemical and viral carcinogens, although there is not a perfect correlation between the carcinogenic potency of these agents and its mutagenicity. DNA damage caused by exogenous chemical carcinogens is usually through an electrophilic attack on a tissue nucleophile, typically guanine. During DNA replication, these alterations interfere with base-pair recognition. There are, however, some chemicals which are only carcinogenic in conjunction with another chemical (Tardiff, *et al.*, 1994; Bignold, 2003). Physical carcinogens like particulate and photon ionizing radiation and ultraviolet radiation cause DNA damage which could lead to the occurrence of mutations. Ionizing radiation causes single and double strand DNA breaks and it can induce free radical formation from water which damages the DNA, while UV radiation is absorbed by bases in the DNA sequence and induces chemical reactions which disrupts normal base pairing and is obtrusive to DNA polymerase activities. Cancer patients treated with carcinogenic cancer chemotherapeutic agents are at an increased risk of iatrogenic cancer. These agents include alkylating agents like cyclophosphamide which chemically reacts with DNA similar to carcinogens, and antibiotics like Doxorubicin (which induces free-radical damage through non-covalent interaction with the genome) (Bertram, 2001).

2.3.2. Epigenetic alterations

During the lifetime of an organism, and especially during its development, meiotically and mitotically heritable changes in gene function or expression can occur without changing the specific nucleotide sequence of the genes involved (Wachsman, 1997; Nephew and Huang, 2003; Egger, *et al.* 2004; Roloff & Nuber, 2005). These stable, reversible changes can be defined as epigenetic alterations and they play a very important part in several fields of study, including gene expression regulation and cancer biology (Esteller, 2005). It, therefore, refers to varying phenotypic conditions that are not founded in dissimilarity in genotype (Laird, 2005). The literal meaning of epigenetics is 'outside conventional genetics', and these processes have functional roles during development and differentiation (Jaenisch and Bird, 2003), although Roloff and Nuber (2005) have proposed a modified definition of epigenetics: "...the study of changes in gene transcription that is based on chromatin and cannot be explained by changes in DNA sequence". As shown in Figure 2.3, epigenetic silencing is activated and maintained by DNA methylation, histone modification and RNA-associated silencing. Two of the most frequent molecular modifications in human neoplasia are chromatin adjustments and DNA methylation status alterations (Verma and Manne, 2006). Histone alterations block transcriptional initiation and cause chromatin condensation, but can also instigate cytosine methylation through DNA methyltransferases. The methylation in turn can increase silencing. RNA possibly interferes with heterochromatic state formation and silencing (Egger, *et al.* 2004).

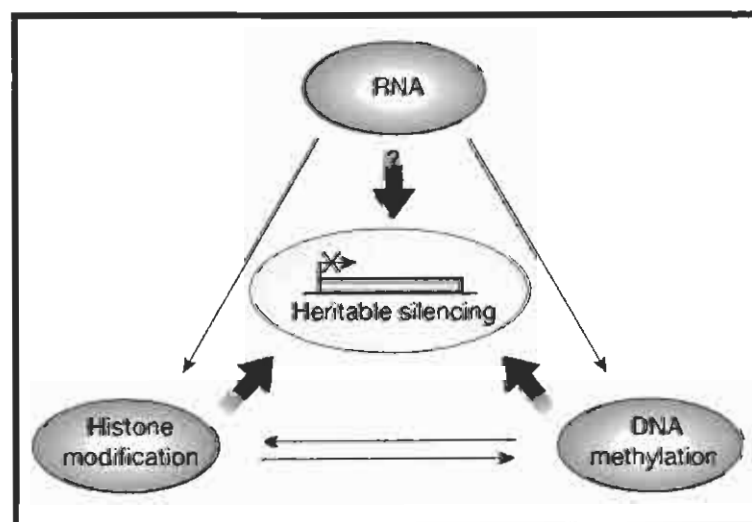


Figure 2.3. The systems involved in epigenetic silencing (Egger, *et al.* 2004).

As in the case of genetic alterations, epigenetic alterations in mature organisms can be induced by endogenous and exogenous factors and although the mechanisms involved are not clear yet, it has been proposed that post-synthetic modifications of DNA or DNA-associated proteins are involved (Wachsman, 1997; Jaenisch and Bird, 2003). Genetic and epigenetic changes can sequentially influence each other significantly, as well as gene expression, development and carcinogenesis. Epigenetic modifications, unlike genetic changes, do however occur gradually, like the global methylation reduction during the aging of cells (Egger, *et al.* 2004). This, as well as the fact that only 15% of cancers have a familial connection, shows that genomic alterations in cancers can not solely be viewed as a result of genetic aberrations, but that epigenetic alterations greatly influence the establishment of a malignant phenotype (Plass, 2002; Verma and Manne, 2006). The numerous changes which may occur in malignant cells, i.e. chromosomal instability, activation of proto-oncogenes, silencing of tumour suppressor genes and DNA repair system inactivation, is apparently not only caused by genetic aberrations, but also by epigenetic abnormalities (Jaenisch and Bird, 2003). These modifications alter the ideal epigenetic balance in normal cells significantly and might lead to adverse expression or silencing of genes, leading to "epigenetic diseases" (Esteller, 2003; Egger, *et al.* 2004).

A well-studied epigenetic alteration present in genomic DNA is DNA methylation which normally primarily sustains normal gene expression and genome stability (Esteller, 2003). Changes in the normal methylation profile influences cancer related gene transcription, resulting in genomic instability, predisposition of cells into precancerous stage and abnormal cell cycle regulation (Yu, *et al.* 2004; Verma and Manne, 2006), and Strathdee and Brown (2002) stated that: "DNA methylation is the only commonly occurring modification of human DNA". DNA methylation is also the most accessible epigenetic alteration to study when trying to identify epigenetic signatures, because of its stability in the DNA (Murell, *et al.*, 2005). The variable DNA methylation profiles can, therefore, be used as molecular markers for both classification and detection in cancer diagnostics.

Another epigenetic modification of note is histone modification. Histones are post-translationally modified by acetylation and methylation of lysine residues on the

amino-terminal tail domains. Acetylation of histones normally marks regions in chromosomes which are active and transcriptionally competent, while methylation of histones acts as an active and inactive chromatin region marker (Egger, *et al.* 2004).

2.4. DNA METHYLATION

DNA methylation is a “molecular mechanism underlying the heritability of gene expression throughout cell divisions” (Roloff and Nuber, 2005). In the mammalian genome DNA methylation is a tightly regulated, frequent and important post-replication modification (Plass, 2002; Jaenisch and Bird, 2003). During cleavage of the blastocyst cells, demethylation increases until implantation, followed by total genomic *de novo* methylation (Robertson and Wolffe, 2000; Jaenisch and Bird, 2003). It is a product of the activity of several components, including the four DNA methyltransferase enzymes (DNMTs), demethylases, methylation trigger centres and methylation protection centres (Das and Singal, 2004; Toyooka and Shimizu, 2004). Although it is not clear yet, regulation of DNA methylation may be influenced by chromatin modifications (Plass, 2002).

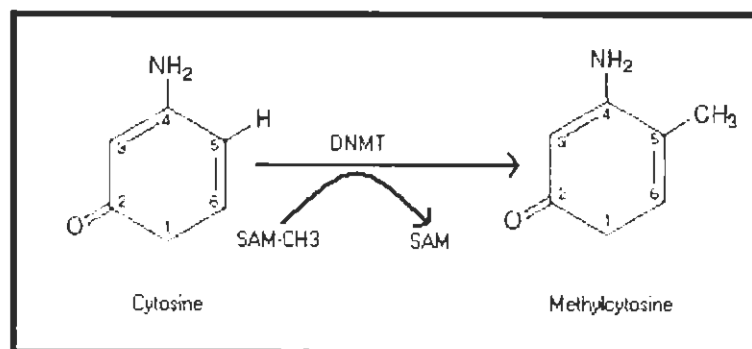


Figure 2.4. The DNA methylation mechanism (Adapted from Strathee & Brown, 2002).

As shown in Figure 2.4, DNA methylation occurs at the 5-position of the cytosine ring through the addition of a methyl residue ($-\text{CH}_3$) from a donor, S-adenosylmethionine (SAM), to DNA by the DNA methyltransferases (Cottrell, 2004; Szyf, *et al.* 2004). It is, however, a reversible change. In mammals, this covalent addition usually happens at a CpG dinucleotide sequence (Huang, *et al.*, 1999; Fazzari and Greally, 2004).

The genome is not methylated uniformly because the CpG sites are not evenly distributed along the genetic sequence, since these sites tend to be clustered in specific regions, which are called CpG islands (Cottrell, 2004). CpG islands are recurrent throughout the genome, but usually lie in the promoter regions or near genes (See Figure 2.5), and it has been estimated that there are approximately 29,000 CpG islands in the human genome (Nephew and Huang, 2003; Brena, *et al.* 2006). Approximately 40% of these CpG islands are found in promoter regions (Egger, *et al.* 2004). Some of the CpG islands can be found in the first exons and introns, or near the 5' regulatory regions of numerous genes (Silva, *et al.* 1999; Esteller, 2003).

CpG islands normally have a number of important properties: i) They are unmethylated in normal cells (See Figure 2.5) except on the inactive female X-chromosome, ii) they are GC rich, and iii) they have a CpG to GpC ratio of minimum 0.6 (Cottrell, 2004; Das and Singal, 2004; Miyamoto and Ushijima, 2005). The CpG islands also influence DNA-protein associations directly and, therefore, methylation of these islands can alter these interactions and subsequently also histone modifications (Yu, *et al.* 2004). Up to 90% of the CG dinucleotides are found in repetitive DNA sequences like satellite repeat DNA, retrotransposons (mobile genetic elements) and parasitic DNA elements in the genome and they are usually methylated (Früwald and Plass, 2002; Egger, *et al.* 2004; Brena, *et al.* 2006).

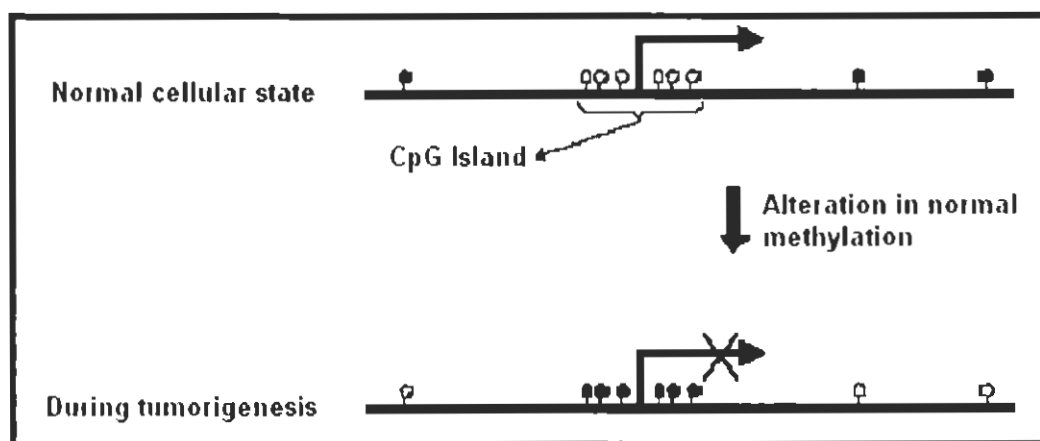


Figure 2.5. The methylation status of CpG sites and CpG islands normally, and during tumorigenesis. The dark dots indicate methylated CpG sites, while the clear dots indicate unmethylated CpG sites. The arrows to the right indicate gene transcription (Adapted from Cottrell, 2004).

Two types of methylation occur to bring about the various methylation patterns. *De novo methylation* occurs when the CpG sites in both DNA strands are unmethylated or a site which was not previously methylated becomes methylated by DNMT3A and DNMT3B (Laird, 2003). This usually occurs during development after implantation, but rarely during post-gastrulation growth. It is, however, a frequent occurrence in cancer (Jaenisch and Bird, 2003). *Maintenance methylation* occurs when the CpG sites in only one strand/parental strands are methylated and the daughter strand is then methylated by DNMT1 to sustain methylation patterns during replication (Miyamoto and Ushijima, 2005). According to Miyamoto and Ushijima (2005), hemi-methylated CpG sites are recognized by these maintenance methylases at the replication fork, which then methylate the relevant sites. This classic model assumes that the parental DNA's methylation pattern exclusively determines DNA methylation, and that the pattern is permanent (Das and Singal, 2004; Szyf *et al.*, 2004). There are, however, several studies which suggest that other factors influence the replication and maintenance of methylation patterns. It suggests that an active equilibrium between methylation and demethylation sustains somatic cell DNA methylation, and that this stability is determined by the "state of activity of the chromatin structure" (Szyf, *et al.* 2004).

2.5. THE SIGNIFICANCE OF DNA METHYLATION

It has been shown that gene expression and genomic stability are altered by aberrant DNA methylation, and these abnormalities are maintained through cell divisions (Yu, *et al.* 2004; Miyamoto and Ushijima, 2005).

2.5.1. Protection of the genome and its structural integrity

Several DNA repair pathways are adversely affected by DNA methylation (Esteller, 2003). Therefore, any abnormalities in the normal methylation patterns will have severe consequences for the normal repair of DNA damage. These may include microsatellite instability, mutations and global gene-expression changes (Kouidou, *et al.*, 2006).

It is possible that methylation may inhibit homologous recombination between large areas of repetitive DNA, which increases genome stability. It is, however, not clear yet how homologous recombination is decreased by methylation. Possible mechanisms include recombination initiation-site masking, recombination intermediate destabilization and interference with recombination machinery assembly (Robertson and Wolffe, 2000; Wilson, *et al.*, 2006).

DNA methylation might be a defensive system to silence expression of CpG-rich parasitic DNA elements or retrotransposons to inhibit their spreading to the rest of the genome, since these elements may be a great threat to the structural integrity of a genome. This is possible as transcription of the retrotransposon's promoter is silenced by methylation. Due to these parasitic and repetitive DNA sequences, which are usually methylated quite extensively, tumour cells show lowered global methylation but have a region-specific methylation increase at normally unmethylated CpG islands. Transcription patterns may be altered because of strong promoter reactivation by changing the amount of transcription factors present. It could change the growth-regulatory genes in which they lie as well (Robertson and Wolffe, 2000; Soppe, *et al.*, 2002).

2.5.2. Regulation of gene expression

Regulation of gene expression, which is a very complex and rigidly controlled process, and the consequent transcription of RNA are greatly influenced by DNA methylation. For instance, promoter methylation is associated with a condensed chromatin structure at that area, and gene expression is reduced or stopped, but different changes occur in expression when the transcribed region is methylated (see Figure 2.5), since genes can only be transcribed if: i) the correct transcription factors can bind to their specific targets, ii) histones are acetylated and unmethylated and iii) CpG islands are unmethylated (Cottrell, 2004; Das and Singal, 2004). Therefore, DNA methylation and the associated repressive heterochromatin assembly are among the most important mechanisms for stable gene inactivation in a heritable form (Robertson and Wolffe, 2000; Esteller, 2003; Yan, *et al.*, 2003). DNA methylation is also one of the six principal chromatin state determining mechanisms. The other five are chromatin protein related mechanisms (Roloff & Nuber, 2005).

Widschwendter and colleagues, however, suggested that this correlation between gene expression and methylation may be more than a linear relationship, and that so called threshold effects might even occur. They came to this conclusion because they did not observe a perfect inverse relationship between the level of DNA methylation and changes in gene expression (Widschwendter, *et al.* 2004). The specific mechanisms of transcriptional repression by methylation have not been identified yet, but several possible mechanisms have been proposed. These mechanisms include i) interference with specific transcription factor binding with regulatory sequences in promoters by local cytosine methylation, ii) binding of specific transcriptional repressors to methylated DNA through a methyl CpG binding domain or a zinc finger motif (Toyooka and Shimizu, 2004; Miyamoto and Ushijima, 2005).

Gene expression is also influenced by histone alterations and changes in the chromatin structure, both of which can be affected by DNA methylation seeing as histone deacetylation is guided by the cytosine methylation patterns (Miyamoto and Ushijima, 2005). According to Das and Singal (2004), recent studies suggest that histone alterations can induce the methylation process on its own. This is in contrast to earlier beliefs that histone modification was secondary to DNA methylation. Many transcriptional machinery components can be prevented from binding to nucleosomal DNA by DNA-histone interaction(s) (Robertson and Wolffe, 2000). It has been proposed by Esteller (2003) that methylation may be a factor in the tissue-specific expression of some genes, as well as acting as a defence mechanism for protecting human DNA against insertion of parasitic DNA sequences. This might present one explanation for the function of methylation outside the CpG islands. Gene expression is completely dependant on the non-imprinted allele of the gene. It has been shown that regulation areas co-localize with sequences with allele-specific patterns of DNA methylation (Esteller, 2003).

Another important role that DNA methylation plays in the human body is X-inactivation which causes the transcriptional silencing of one of the female's two X-chromosomes. This is brought on by several epigenetic factors like methylation, histone deacetylation, replication timing, compartmentalization and establishment of inactive chromatin (Früwald and Plass, 2002). Genomic imprinting is, therefore, influenced by DNA methylation.

2.6. DNA METHYLATION AND CANCER

Cancer is a “genetic, epigenetic and cytogenetic disease” according to Verma and Manne (2006), which occurs when the genome is affected by disease or other adverse factors and is caused by the occurrence of a genetic defect in oncogenes (proliferation stimulating genes) and tumour suppressors (proliferation inhibiting genes). It also involves numerous and profound changes in gene expression programming (Früwald and Plass, 2002; Brena, *et al.* 2006). When several cancer-associated genes have defects which accumulate, a process called tumorigenesis occurs, although these gene function alterations are very complex for each cancer (Plass, 2002).

In cancer there are several assessments necessary for each case, which includes early detection, diagnosis, treatment, prognosis, risk assessment and recurrence. For each of these there is a constant search for better, more accurate, faster and more definitive methods to determine the disease state of each patient, and to monitor the recurrence during treatment (Laird, 2005). Biomarkers are often used in these methods, and can be, among others, genetic or epigenetic of origin (Verma and Manne, 2006). Some genetic markers in cancer include nucleotide excision repair (NER), loss of heterozygosity (LOH), microsatellite instability (MIS), mitochondrial DNA mutations and circulating DNA. DNA methylation modification and chromatin alterations are the most common epigenetic biomarkers currently investigated, since DNA methylation markers have great disease detection potential, maybe even more than the existing markers for disease classification (Laird, 2005). Biomarkers have tremendous potential and exceptional sensitivity, and although there are some challenges such as specificity, identification and application of biomarkers in cancer is an important research area.

Several studies have shown the connection between the abnormal cellular development of cancer and abnormal tumour suppressor gene methylation (Nephew and Huang, 2003; Cottrell, 2004). Methylation may, therefore, have a fundamental part in the accumulation of genetic change.

Genetic defects which cause cancer and have been shown to be steered by DNA methylation are classified into defects of:

1. Classic tumour suppressor genes ("gatekeepers"). Mutations could directly play a role in the occurrence of carcinogenesis.
2. Cancer susceptibility genes. They contribute to tumorigenesis indirectly.
3. Landscaper defects. Provide a tumour development supportive environment.

Two categories of methylation patterns are involved in the aberrant epigenetic profiles of cancer: Global genomic hypomethylation and transcriptional silencing by gene-specific hypermethylation (See Figure 2.6) (Esteller, 2003).

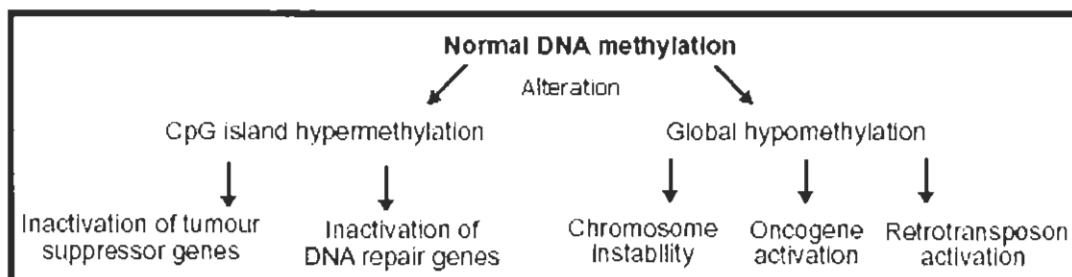


Figure 2.6. Possible roles of increased CpG island and decreased global DNA methylation in tumour development (Adapted from Strathdee & Brown, 2002).

Each of these two categories of DNA methylation will subsequently be discussed briefly.

2.6.1. Hypermethylation

Healthy cells in general have unmethylated promoter regions of certain tumour suppressor genes, but several human carcinomas show concentrated gene-associated hypermethylation which leads to the epigenetically mediated silencing of these genes (See Table 2.1) (An, *et al.* 2002; Esteller, 2005). All human cancers studied show abnormal hypermethylation to some extent, but promoter regions of tumour-suppressor genes, including *p16*, *BRCA 1* and *E-cadherin*, shows a direct correlation between inactivation in some cancers and CpG island hypermethylation (Miyamoto and Ushijima, 2005). Hypermethylation is significantly more frequent than hypomethylation in cancer, although there are a number of mechanisms like active

transcription, active demethylation, replicon timing and local chromatin structure, to protect the CpG islands against hypermethylation. Hypermethylation does, however, only occur in CpG islands (Esteller, 2003; Das and Singal, 2004).

In a variety of cancers, there are specific genes that are commonly methylated, but some genes appear to have a specific tissue of origin in specific cancers, and the malignant capability increases with the number of genes showing hypermethylation. The responsible mechanisms for this gene targeting are still undetermined, although DNA methyltransferases might be recruited by certain proteins (Das and Singal, 2004; Esteller, 2005).

Table 2.1. Selected CpG hypermethylation-silenced genes in human cancer

Gene	Function	Location	Tumour profile
BRCA 1	DNA repair, transcription	17q21	Breast, ovary
p16 ^{INK4a}	Cyclin-dependent kinase inhibitor	9p21	Multiple types
ER	Oestrogen receptor	6q25.1	Breast
PR	Progesterone receptor	11q22	Breast
RASSF1A	Ras effector homologue	3p21.3	Multiple types
CDH1	E-cadherin, cell adhesion	16q22.1	Breast, stomach, leukaemia
CDH13	H-cadherin, cell adhesion	16q24	Breast, lung

(Adapted from Esteller, 2005)

In this study, only two of the genes will be investigated for hypermethylation, namely p16^{INK4a} and RASSF1A. These two potential markers were chosen because of their well-described use and hypermethylated status in various cancers.

2.6.1.1. p16^{INK4a} tumour suppressor gene

The p16 protein is a D-type cyclin dependent kinase 4 (cdk4) inhibitor and is involved in cell growth and differentiation regulation. The p16^{INK4a} gene is also frequently inactivated in cancer (Silva, *et al.* 1999; Widschwendter and Jones, 2002). Cells are stimulated by the cyclin D1-cdk4 complex to enter the S phase of the cell cycle and, therefore, cell proliferation can be regulated by p16 inhibition of cdk4.

Homozygous deletions always inactivates *p16*, and this inactivation is supported by methylation since numerous cancers show aberrant *p16* methylation, and breast tumours display *de novo* methylation of $\pm$ 23-31% (Silva, *et al.* 1999; Ferguson and Nass, 2000; Tong and Lo, 2006). *p16* is generally associated with higher-stage disease and limitless replicative potential of cells.

2.6.1.2. *RASSF1A*

The Ras association domain family 1A gene is a recognized tumour suppressor gene, present on locus 3p21.3 (Dammann, *et al.* 2001). This transcript is expressed in all normal tissues, although the gene has been shown to be hypermethylated in some cancers, including primary lung tumours, ovarian tumours and some breast cancers (Dammann, *et al.* 2000, 2001; Tong and Lo, 2006).

Dammann, *et al.* (2001) also showed that CpG island hypermethylation in the *RASSF1A* promoter sequence has been associated with loss of expression of this gene. Some studies also associated *RASSF1A* methylation with tumour size, cell death and a good response to treatment (Tong and Lo, 2006).

2.6.2. Hypomethylation

Hypomethylation of genomic DNA is usually a global defect, mainly affecting repetitive sequences, following CpG island hypermethylation, occurring mainly in solid tumour cells like prostate tumours, cervical cancer, etc. This leads to genomic instability, tumour formation, abnormal gene expression, reactivation of transposable elements and loss of imprinting (as shown in Figure 2.6 and 2.7) (Esteller, 2003; Miyamoto and Ushijima, 2005).

A decrease of 20-60% CpG methylation can occur in malignant cells mainly affecting the coding regions, introns and repetitive sequences. Although, and in contrast to this, it has been reported that in several types of cancer, such as breast, cervical and brain cancer, there is an increase in global DNA hypomethylation associated with the grade of malignancy (Das and Singal, 2004). Das and Singal (2004) also suggested

that hypomethylation activates oncogenes and latent retrotransposons, or causes chromosome instability, and so contributes to oncogenesis.

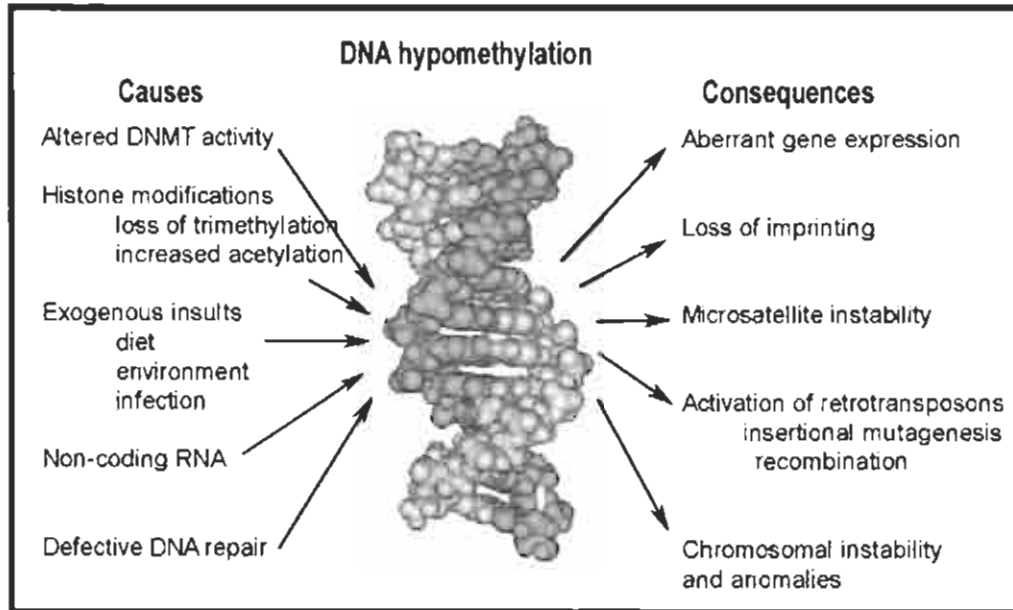


Figure 2.7. DNA hypomethylation: causes and effects. A summary of the possible causes of DNA demethylation and the subsequent effects on the genome (Wilson, *et al.*, 2006).

It has been shown in several studies that the DNA obtained from certain carcinomas is less methylated than normal tissue. The *H-ras* oncogene, for instance, has been observed to be hypomethylated in stomach, colon and lung carcinomas. It may also be possible that viruses and retroviral elements, which are normally controlled transcriptionally by DNA methylation, are reactivated by DNA hypomethylation. CG-rich areas that are hypomethylated may also contribute to chromosomal instability (e.g. the mutated *DNMT3B* in human ICF syndrome) (Früwald and Plass, 2002). We can, therefore, assume that stabilization of specific heterochromatin patterns and overall chromosomal integrity are dependent on regulated DNA methylation.

2.6.2.1. Satellite repeat DNA

Tandem repeat sequences, called satellite DNA, are usually located near pericentromeric and/or telomeric heterochromatin (Ugarkovic and Plohl, 2002.). It is known that global hypomethylation increases chromosomal instability, and in breast cancer cell lines chromosomal instability has been linked with the degree of hypomethylation (Szyf, *et al.* 2004). Satellite 2 is situated next to the centromeres of chromosome 1 and 16, and forms a large part of the chromosome 1 juxtacentromeric

heterochromatin (Jackson, *et al.* 2004). Its demethylation has been shown to correlate with global DNA hypomethylation. It is also known that in breast cancer these regions frequently show rearrangement, which could enhance tumorigenesis. According to Schueler, *et al.* (2001) Satellite α DNA consists of a 171bp motif in a head-to-tail tandem repeat manner. It is present near centromeres of all chromosomes and has been shown to be hypomethylated in Wilms tumours, although Jackson, *et al.* (2004) states that Sat α does not display the same significant methylation alteration in cancer as Sat 2 DNA. Studies have shown that somatic adult tissues in normal adults disclose different α -satellite DNA methylation profiles in different chromosomes, while normal fetal tissues show homogenous hypomethylation of α -satellite sequences (Miniou, *et al.* 1997). Furthermore, these studies have indicated that hypomethylation of highly repetitive satellite sequences are related to chromosome modifications.

2.6.3. Mutations induced by methylation

Mutations induced by methylation seem to affect some tumour suppressor genes and CpG dinucleotides contribute to about 30% of all germ line point mutations (Robertson and Wolffe, 2000). The methylated cytosines show a very high mutability, leading to C:G to T:A transitions due to easy spontaneous deamination of the methylated cytosine to thymine (Wachsman, 1997). This mutation is not recognized and repaired frequently since it is a normal DNA base and T:G mismatch repair is error prone, leading to increased C:T transitions (Robertson and Jones, 2000). This causes CpG methyl acceptor site suppression (Egger, *et al.* 2004). It has also been shown that methylation increases DNA adduct formation significantly and that 5mC flanked guanines are the preferential adduct formation sites. The genome is unable to repair these defects. The significance of mutations occurring because of abnormal DNA methylation is thus related to differences in repair efficiency, enhanced spontaneous deamination, rate of cell division and increased methylated CpG dinucleotide affinity for DNA-reactive carcinogens (Robertson and Jones, 2000; Fr  wald and Plass, 2002). CpG dinucleotides in tumour suppressor gene coding regions are also "hotspots for acquired somatic mutations leading to cancer", e.g. *p53* (Robertson and Jones, 2000).

2.6.4. An example: DNA methylation in breast cancer

Breast cancer is the most common cancer in South African women with a prevalence of 16.6%, according to the National Cancer Registry (Sitas, *et al.* 1998)¹. It is, therefore, one of the most important and relevant research areas to investigate DNA methylation profiles.

Distinguishing DNA methylation patterns are present for primary breast cancer and metastatic breast cancer since different gene expression pathways are involved (see Figure 2.8). In breast cancer there are two methylation profile transformations which arise, namely global hypomethylation and regional hypermethylation. This contrast suggests that the different types of methylation are caused by individual and diverse methods (Szyf, *et al.* 2004).

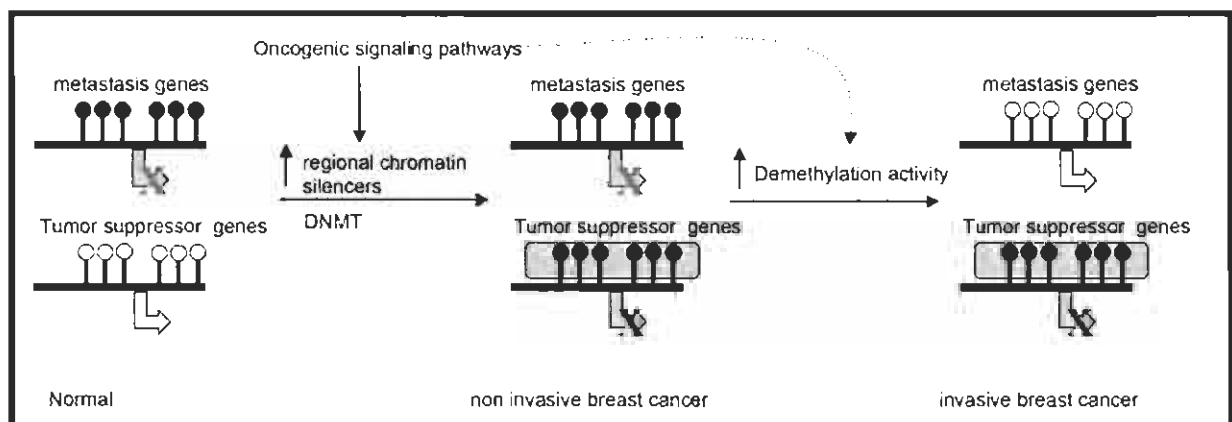


Figure 2.8. Hypermethylation and hypomethylation in breast cancer. Transcription is indicated by a horizontal arrow, open circles indicate unmethylated CGs and closed circles indicate methylated CGs (Szyf, *et al.* 2004).

As is shown in Figure 2.8, tumour suppressor genes are usually active in epithelial breast cells and are unmethylated, while metastatic genes are inactive and usually methylated. Interaction of specific repressors with certain tumour suppressors are initiated by the activation of oncogenic pathways, which leads to DNA methylation and subsequent chromatin inactivation. These oncogenic pathways, or additional pathways, can induce demethylase activity at a more advanced stage which may lead to global demethylation. It might also activate metastasis genes which will result in an exceedingly metastatic phenotype (Szyf, *et al.* 2004; Wilson, *et al.*, 2006).

¹ The NCR compiles all information on cancer morbidity in South Africa from pathology laboratories and, therefore, these results are underestimated and outdated (Sitas, *et al.* 1998).

A unique combination of methylated CpG islands in cancer cells, called methylation signatures, has been associated with stages in breast cancer and might, therefore, be used as a diagnostic indicator for breast cancer cells. These methylation signatures were identified through the establishment of a list of hypermethylated genes. Hypermethylation is definitely of great importance, but global hypomethylation of repetitive and satellite DNA sequences also plays a big role. An aberrant increase in demethylation activity due to defective DNA methylation routes in cancer cells might be responsible for this global hypomethylation. It has been suggested that CG hypomethylation may be associated with the cancer's histological grade and malignancy (Szyf, *et al.* 2004; Wilson, *et al.*, 2006).

2.7. NUTRIENTS AND DNA METHYLATION

Methyl donors and cofactors necessary for S-adenosylmethionine (SAM) are obtained from the diet. Since SAM is necessary for CpG methylation, nutrition during the early embryonic methylation programming is very important and may influence the adult phenotype (Vercelli, 2004). The effect of the interaction of nutrients with genetic polymorphism and modulation of DNA methylation on gene expression has been shown to be very important. This is clear as disturbances like essential micronutrient deficiencies in the homeostasis of the vitamin-dependent, methyl-group metabolism, increases cancer, heart disease, etc. These nutrients are folate and vitamin B12 (cobalamin). Insufficient folate intake causes hypo- and hyper-gene specific methylation (Das and Singal, 2004).

2.8. DNA METHYLATION AS DIAGNOSTIC TOOL IN CANCER

Different cancer types show different profiles of methylated tumour-related genes. To understand the cancer pathogenesis and have a fixed representation of the complete genome, it is, therefore, very important to have a gene expression signature of each cancer, and there are exclusive stable methylation profiles or methylotypes emerging for multiple cancers (Esteller, 2003; Nephew and Huang, 2003; Das and Singal,

2004; Szyf, *et al.* 2004). These profiles are needed for diagnostic method development, risk assessment and deciding on a therapeutic treatment targeted at the underlying cause of the cancer (Robertson and Wolffe, 2000). Detection of gene-specific hypermethylation and global genome hypomethylation, therefore, has promising prognostic value for each of these steps, and since somatic epigenetic changes seemingly reflect environmental and dietary exposures for the life duration, these epigenetic markers should be proficient for risk assessment (Früwald and Plass, 2002; Laird, 2005). Since physicians often decide on a specific course of treatment based on pathology which classifies tumours into subtypes, it is becoming clear that because of the wide diversity of genetic abnormalities in cancer, genotyping of individual tumours with standard pathological procedures are becoming a necessity (Bertram, 2001; Cottrell, 2004). By using molecular markers, tumours can be subclassified, and by using the methylation profiles, tumour types, subtypes and possibly the response to chemotherapeutic agents and survival may be distinguished from each other. Phenotype prediction of prognosis, etc. is, however, only possible if abnormal CpG-island methylation shows correlations with disease phenotype (Miyamoto and Ushijima, 2005). Early sensitive and specific tumour detection and even prediction of cancer or tumour development might, therefore, be possible due to the methylation events which sometimes precede malignant changes and can be detected in body fluids a long time before the patient is clinically diagnosed (Esteller, 2003; Cottrell, 2004; Brena, *et al.* 2006). This is possible if the relevant changes in normal tissue can be shown to be a cancer related risk factor (Miyamoto and Ushijima, 2005). After treatment of a diagnosed patient is complete, these biomarkers can also be utilized to confirm elimination of the cancer, as well as continuous monitoring of the patient (Cottrell, 2004).

There are multiple genes known to be silenced by CpG island hypermethylation in cancer which may be potentially useful as diagnostic markers of cancer cells and the establishment of unique methylation profiles/signatures for the different cancer types (Table 2.1). Methylation-based diagnostic markers have several benefits: i) It is frequently a binary signal, therefore, the presence of malignant cells can be indicated by the presence of DNA methylation. This situation requires no quantification which eliminates sample heterogeneity problems. ii) If low concentrations of markers need

to be detected, the signal can be amplified. iii) DNA strands and their methylation are much more stable than RNA (Cottrell, 2004).

These potential markers do have certain requirements. For example, the unmethylated state of the markers in non-cancerous cells. If specific CpG-island methylation abnormalities can be correlated with cancer cells; tissue biopsies and cell-free DNA released by cancer cells may also have diagnostic value (Esteller, 2003; Miyamoto and Ushijima, 2005). Hypermethylation at different locations appears to be unrelated and, therefore, a combination of several markers could be informative about the patient's specific methylation profile (Esteller, 2005).

Tumour behaviour might be predictable due to the hypermethylation status of specific genes, although this is not exclusively negative! According to Esteller (2005), both poor and positive prognoses can be determined from CpG island hypermethylation. Some genes also have potential to be predictive for treatment response, although this will have to be investigated for each individual gene. The methylation status of certain cellular receptors like Estrogen receptor and Progesterone receptor genes might explain why some tumours lose their hormone response in some prostate, breast cancers, etc. which results in inefficient responses to therapy (Esteller, 2003; Esteller, 2005). There are several experimental models available to study DNA methylation profiles, including *in vitro* models, *in vivo* models and *in silico* models (Toyooka and Shimizu, 2004). In this study *in vitro* models will be used.

2.9. PLASMA NUCLEIC ACIDS IN CANCER

Several aberrations present in tumour-derived DNA and RNA, can also be identified in DNA and RNA isolated from plasma and serum (Tong and Lo, 2006). Small amounts of this free circulating DNA can be detected in plasma and serum from healthy people, although patients with cancer, rheumatoid arthritis, etc., show higher concentrations of this free DNA (Shaw, *et al.*, 2000). The presence of these nucleic acids in plasma and serum has long been recognized, but its neoplastic characteristics have only recently received attention. It has also been shown in several publications that epigenetic aberrations can be detected in plasma with

Methylation-specific PCR, although some studies observed that disease stage has significant influence on detection (Johnson and Lo, 2002). Shaw, *et al.* (2000) stated that they found that tumour DNA can promptly be detected in plasma of primary and metastatic breast cancer patients. They also stated that the DNA aberrations correlated with the alterations found in the tumour itself.

Free circulating DNA will be isolated from plasma of healthy individuals, as well as from diagnosed breast cancer patients as described by Van der Vaart (2006). The DNA will subsequently be used in this study to investigate DNA methylation alterations in various sources of DNA.

2.10. METHYLATION DETECTION ASSAYS

Methylation changes and patterns which are tumour-specific have been identified in different genes. In the last couple of years, some detection techniques with great potential have been developed (Cottrell, 2004; Das and Singal, 2004). These techniques include:

- Sodium bisulfite conversion
 - Methylation-specific PCR (MSP)
 - ConLight-MSP
 - MethyLight
 - Combined bisulfite restriction analyses (COBRA)
 - Methylation-sensitive single nucleotide primer extension
 - Methylation-sensitive single-strand conformational polymorphism.
- cDNA microarray
- Oligonucleotide microarrays
- Restriction landmark genomic scanning
- CpG island microarrays
- Southern Blot assay with methylation-sensitive restriction enzymes
- Expression array analysis of genes reactivated by methylation inhibitors
- Methyl CpG binding domain column, subtractive hybridization (ICEAMP)
- Etc.

To be useful in clinical diagnosis, the assay applied needs to be standardized and proven useful (Das and Singal, 2004). The assay should rapidly produce reliable results in large sample sets, while still providing quantitative information (Brena, *et al.* 2006). Sensitive detection does, however, have specific requirements which differ from classification assays. Detection of abnormal DNA methylation against normal background methylation requires high signal-to-noise ratios and these sensitive assays usually incorporate sodium bisulfite-based assays like Methylation-specific PCR, which produces high signal-to noise ratios (Laird, 2005).

Techniques focused on the identification of genetic alterations throughout the genome are currently attracting much attention in the scientific community, for instance, genome scanning via array-based techniques enables multiple biomarker analysis simultaneously and generating vast amounts of information (Plass, 2002). Much work has been done using gene expression pattern microarray analysis to identify possible gene signatures. This has improved diagnosis and risk assessment in various cancers (Chang, *et al.* 2005). This technology, however, is not widely available or accessible, and the high cost of these analyses makes it ineffective for routine work. Therefore, this study will only focus on a limited number of these biomarkers as previously identified by microarray analysis in the literature.

Methylation-specific PCR (and its variation, COBRA) were chosen for this study because it is most commonly used internationally to investigate DNA methylation (Cottrell, 2004), and for the large amount of information the method produces. The technique also has numerous applications and adaptations to increase use and functionality and according to Das and Singal: "To be useful as a routine diagnostic tool, the actual methylation detection method has to be sensitive, quick, easy, and reproducible. Of the various techniques available, methylation-specific polymerase chain reaction seems to be most useful at present" (Das and Singal, 2004). The proposed method will be discussed in more detail in Chapter 4.

2.11. THE AIM AND APPROACH OF THE STUDY

The aim of this study is to establish and optimize a method for measuring gene-specific DNA methylation in breast cancer patients. The aim is to standardize the Methylation-specific PCR assay by determining the methylation status of the CpG islands in the promoter sequences of the *p16* and *RASSF1A* genes.

The following approach was formulated for this study.

1. Various methods to isolate DNA from different tissues have been identified, and will be evaluated to establish which methods are most suitable to isolate pure DNA for further application in this study.
2. Conditions for the MSP assay will be optimized, e.g. bisulfite treatment, PCR amplification, etc.
3. DNA will be isolated from various tissue types, including blood and paraffin-embedded tumour tissue samples, obtained from control individuals and diagnosed breast cancer patients after written informed consent was obtained.
4. The optimized MSP assay will be applied to these samples to investigate the methylation status of the *p16* and *RASSF1A* genes.

CHAPTER 3

DNA ISOLATION AND QUANTIFICATION

The method used in this study to detect DNA methylation was chosen on the grounds that it is frequently described in publications and because it produces sensitive, relevant, valid and applicable information about the DNA methylation profile of a person (Laird, 2003; Cottrell, 2004). This method is called the methylation-specific PCR assay (MSP) which is a PCR-based assay and will be discussed in more detail in chapter 4.

For this method to work properly, pure, concentrated DNA must first be isolated from the relevant sources, being whole blood, tumour tissue or cultured cells. In this chapter various DNA isolation methods from a variety of sources and for specific applications, were optimized and evaluated for their suitability in MSP.

3.1. ETHICAL APPROVAL

Ethical approval for this study was obtained with the title *DNA methylation and breast cancer: a case control study of prognostic outcome measurements*, from the Ethical Committee of the North-West University (No. 05M12). Patient material was supplied by our clinical co-workers after written informed consent was obtained from the patients. All control individuals also gave written informed consent before blood samples were acquired. This study was done in collaboration with Dr S.J. Fourie and Dr A. Oosthuizen, oncologists associated with the Wilmed Hospital in Klerksdorp.

3.2. GENOMIC DNA ISOLATION FROM WHOLE BLOOD – MINIPREP METHOD

3.2.1. Principle of the method

High molecular weight genomic DNA can be isolated from small volumes of whole blood by collecting fresh blood in tubes containing an anticoagulant like EDTA or heparin. In the purification process the first step uses a cell lysis solution to induce lysis of the red blood cells. This is followed by the addition of nuclei lysis solution which induces lysis of the white blood cells and their nuclei to release the enclosed DNA. The addition of RNase digests all RNA present in the lysate. All cellular proteins must be removed from the lysate as well to ensure the isolation of pure DNA, and this is done via selective salt precipitation. Isopropanol is subsequently used to desalt and precipitate the DNA to yield pure, concentrated genomic DNA ideal for manipulations like restriction enzyme digestion, amplification or membrane hybridization (Promega Technical manual, # TM050).

3.2.2. Materials used

- Wizard® Genomic DNA purification kit (Promega)
- Isopropanol
- 70 % ethanol

3.2.3. Method

1. Collect fresh whole blood in heparin or EDTA blood collection tubes.
2. Pipette 900 µl cell lysis solution into a sterile 1.5 ml microcentrifuge tube.
3. Mix the collected blood thoroughly and add 300 µl of the whole blood to the cell lysis solution. Invert the tube 6 - 10 times to ensure proper mixing.
4. Incubate the tube at room temperature for 10 minutes, inverting the tube 4 - 6 times during incubation.
5. Centrifuge the tube for 40 seconds at 13 000 - 16 000 x g.
6. Decant the supernatant, leaving approximately 10 µl without disturbing the white pellet which has formed.

7. Vortex vigorously to resuspend the white blood cell pellet.
8. Add 300 μ l nuclei lysis solution and pipette the solution continuously to break any clots present. Incubate the tube at 37°C for 10 minutes, or until all clots are dissolved.
9. Add 1.5 μ l RNase solution and invert to mix.
10. Incubate 15 minutes at 37°C and allow cooling to room temperature.
11. Add 100 μ l protein precipitation solution and vortex for 10 - 20 seconds.
12. Centrifuge for 3 minutes at 13 000 - 16 000 x g.
13. Carefully remove the supernatant and add it to 300 μ l isopropanol in a sterile 1.5 ml microcentrifuge tube.
14. Invert several times to precipitate DNA until a white mass is formed.
15. Centrifuge for 1 minute at 13 000 – 16 000 x g.
16. Remove the supernatant and add 600 μ l 70 % ethanol to the pellet, mix well.
17. Centrifuge for 1 minute at 13 000 – 16 000 x g.
18. Remove the ethanol and air-dry the tube inverted on absorbent paper for 10 - 15 minutes.
19. Add 50 - 100 μ l DNA rehydration solution to the tube and rehydrate the DNA for 1 hour at 65°C or overnight at 4°C.

3.3. GENOMIC DNA ISOLATION FROM WHOLE BLOOD – MAXIPREP METHOD

3.3.1. Principle of the method

Larger quantities of high molecular weight genomic DNA can be isolated with a method using a large volume of whole blood collected in tubes containing the anticoagulant EDTA. We used the method as described by Irma Möhr in her M.Sc. dissertation (1989) and it is currently used as a standard method in this laboratory.

In the purification process the first step uses a lysis buffer to induce lysis of the red blood cells and the subsequent centrifugation to collect the white blood cells from the blood. This is followed by treatment with SDS and NaClO₄ to induce lysis of the white blood cells and their nuclei. All cellular proteins are removed via repeated

organic extraction with chloroform:isoamylalcohol (24:1) and cold ethanol is used to precipitate the DNA which produces pure, concentrated genomic DNA ideal for restriction enzyme digestion, amplification or membrane hybridization.

3.3.2. Materials used

- Lysis buffer¹
- TNE buffer¹
- 10 g % (m/v) SDS
- 5 M NaClO₄
- Chloroform:isoamylalcohol (24:1)
- 100 % ethanol
- TE buffer¹

3.3.3. Method

1. Collect 3 - 20 ml fresh whole blood in EDTA blood tubes.
2. Mix collected blood thoroughly with an equal volume of lysis buffer.
3. Centrifuge the tube for 30 minutes at 9 800 x g at 4°C.
4. Remove the supernatant, not disturbing the white pellet which has formed.
5. Add 5 - 10 ml TNE buffer and vortex vigorously to resuspend the white blood cell pellet. Transfer the suspension to a 50 ml polypropylene tube.
6. Add 10 % (w/v) SDS to a final concentration of 1 g %, followed by 5 M NaClO₄ to a final concentration of 1 M. Mix well.
7. Add an equal volume chloroform:isoamylalcohol (24:1) and shake gently for 15 minutes.
8. Centrifuge for 10 minutes at 250 x g at room temperature.
9. Carefully remove the top aqueous phase with a blunt end pipette and place it in a clean 50 ml polypropylene tube.
10. Repeat the extraction two more times.
11. Place the final extract in a glass beaker and pour two volumes cold ethanol on top of the aqueous phase to precipitate the DNA.

¹ See Appendix H.

12. Carefully wind the DNA around a Pasteur pipette, break off the tip of the pipette and place it in a 1.5 ml microcentrifuge tube.
13. Dry the tube for 20 minutes in the Speedvac® at the medium heat setting.
14. Resuspend the DNA overnight in 100 - 500 µl TE buffer.

3.4. DNA ISOLATION FROM RAT LIVER AND BRAIN TISSUE WITH PHENOL: CHLOROFORM EXTRACTION

3.4.1. Principle of the method

Equal parts phenol and chloroform are frequently used with nucleic acid preparations to remove proteins and other impurities. This is done, after tissue homogenisation and cell lysis, by protein denaturation by the chloroform, which also facilitates aqueous and organic phase separation producing pure genomic DNA. The nucleic acids are suspended in the aqueous phase, while the proteins and impurities are removed with the lower organic phase. After this purification the nucleic acids can be precipitated and washed with ethanol, resuspended in TE buffer or water and stored at 4°C/-20°C.

3.4.2. Materials used

- TNE buffer¹
- 10 g % (w/v) SDS
- Phenol:chloroform (1:1)
- 1 mg/ml RNase solution
- 2 M NaCl
- Ethanol (Cold)
- TE buffer¹

¹ See Appendix H.

3.4.3. Method

1. Homogenise the entire liver/brain in 10 ml TNE-buffer in a sterile 50 ml polypropylene tube. Mix well.
2. Add 10 % SDS to a final concentration of 1 % and mix well, but carefully.
3. Incubate overnight at 37°C.
4. Add an equal volume of phenol:chloroform to the cell suspension and mix for 10 - 15 minutes.
5. Centrifuge the tube for 10 minutes at 250 x g to separate the phases.
6. Transfer the top aqueous phase to a new 50 ml polypropylene tube with a blunt end pipette, and repeat the extraction twice (second repeat with only chloroform).
7. Add 5 µl RNase solution to the final aqueous phase and invert to mix.
8. Incubate 15 minutes at 37°C and allow cooling to room temperature.
9. Increase the NaCl concentration to 250 mM.
10. Mix well and pour into a 250 ml glass beaker. Carefully pour 2.5 volumes of cold ethanol on top of the suspension.
11. Carefully wind the precipitated DNA onto the tip of a Pasteur pipette and place in a sterile 15 ml polypropylene tube.
12. Add 1 ml 70 % ethanol to the DNA, mix well and centrifuge for 1 minute at 13 000 x g.
13. Remove supernatant and cover the tube with filter paper. Dry the tube for 20 minutes in the Speedvac[®] at the medium heat setting.
14. Add 1 ml TE buffer to the tube to resuspend DNA and store at 4°C.

3.5. DNA ISOLATION FROM PARRAFIN-EMBEDDED TUMOUR TISSUE

3.5.1. Principle of the method

The E.Z.N.A.[®] Tissue DNA mini kit is a genomic DNA purification system using a HiBind[®] silica-based matrix to bind DNA smaller than 60 kbp reversibly. Following removal of the paraffin used to embed the tissue sample with xylol, lysis of the tissue is done at 55°C and RNA is digested with RNase A. The DNA is then collected on the

HiBind[®] silica-based matrix and several washing steps are used to remove all contaminants. The purified DNA can then be eluted from the column with elution buffer. (PEQLAB Biotechnologie, E.Z.N.A.[®] Tissue DNA mini kit – Instruction manual)

3.5.2. Materials used

3.5.2.1. DNA isolation

- E.Z.N.A.[®] Tissue DNA mini kit (PEQLAB Biotechnologie GmbH)
- Xylol/xylene
- RNase A (10 mg/ml in 10 mM Tris-Cl, pH 8.0)
- 100 % ethanol

3.5.2.2. DNA purification

- Wizard[®] DNA Clean-Up system (Promega)
- Vac-Man[®] Laboratory Vacuum manifold (Promega)
- 80 % isopropanol

3.5.2.3. PCR amplification

- Mastercycler[®] ep (Eppendorf)
- PCR primers (Inqaba Biotech)
- *Taq* Polymerase (Promega)
- *Taq* MgCl₂-free buffer (Provided with *Taq* polymerase)
- 25 mM MgCl₂ (Provided with *Taq* polymerase)
- dNTP mix (New England Biolabs)
- DMSO
- Agarose
- 1 x TAE-buffer¹
- Syngene ChemiGenius Bio-Imaging System (Genesnap 6.04)

3.5.2.4. Gel extraction

- Wizard[®] SV gel and PCR Clean-Up system (Promega)

¹ See Appendix H

3.5.3. Method

3.5.3.1. E.Z.N.A.[®] Tissue DNA mini kit

The kit was used according to the manufacturer's instructions.

1. Place < 30 mg of paraffin-embedded tissue in a 2 ml microcentrifuge tube.
2. Add 1 ml xylol and vortex well.
3. Centrifuge for 10 minutes at 10 000 x g and discard the supernatant carefully.
4. Repeat step 2 and 3 twice.
5. Wash the pellet with 1 ml ethanol and centrifuge another 5 minutes at 10 000 x g. Discard the supernatant and repeat the washing step twice.
6. Air-dry the pellet at 37°C for 15 - 25 minutes.
7. Add 200 - 400 µl TL buffer and 25 µl OB[™] Protease to the pellet.
8. Vortex until the pellet is resuspended.
9. Incubate at 55°C in a shaking water bath for 3 hours – overnight.
10. (Optional) Digest any RNA present by adding 5 µl RNase A per 30 mg tissue and incubate at 37°C for 10 minutes.
11. Add 220 µl BL buffer per 200 µl TL buffer and vortex well.
12. Incubate at 70°C for 10 minutes.
13. Add 220 µl ethanol per 200 µl TL buffer and vortex.
14. Place a HiBind[®] DNA column in a 2 ml collection tube and load up to 650 µl of the preparation.
15. Centrifuge the column at 8 000 x g for 1 minute and discard flow-through.
16. Repeat until entire preparation is loaded.
17. Discard the flow-through and collection tube.
18. Place the column in a clean 2 ml collection tube. Add 600 µl DNA wash buffer and centrifuge at 8 000 x g for 1 minute.
19. Discard the flow-through and repeat the wash step with 600 µl wash buffer.
20. Discard the flow-through and centrifuge again at maximum for 2 minutes.
21. Place the column in a clean 1.5 ml microcentrifuge tube.
22. Add 100 µl elution buffer (70°C) and incubate at room temperature or 70°C for 3 minutes.
23. Centrifuge at 8 000 x g for 1 minute and repeat the elution step.

3.5.3.2. Wizard® DNA Clean-Up system

The kit was used according to the manufacturer's instructions.

1. Attach one minicolumn to the provided syringe barrel and the vacuum manifold.
2. Mix the Clean-Up resin well and add 1 ml to the sample in a 1.5 ml microcentrifuge tube and vortex.
3. Transfer the sample to the syringe barrel and apply vacuum to the minicolumn until the solution is drawn through the column.
4. Wash with 1 ml of 80 % isopropanol and re-apply vacuum until the solution is drawn through the column, and repeat the wash step.
5. Apply an additional 30 seconds of vacuum to dry the column.
6. Transfer the minicolumn to a 1.5 ml microcentrifuge tube and centrifuge for 2 minutes at maximum speed.
7. Transfer the minicolumn to a new 1.5 ml microcentrifuge tube and add 50 µl of prewarmed (70°C) water to the column.
8. Incubate 1 minute at room temperature and centrifuge the minicolumn for 20 seconds at maximum speed to elute the DNA.
9. Store the DNA at 4°C.

2.5.3.3. PCR amplification

p16 promoter CpG island:

Previously published primers for the wild type sequence (Herman, *et al.* 1996) were purchased from Inqaba Biotech:

P16WF: 5'- CAGAGGGTGGGGCGGACCGC -3'

p16WR: 5'- CGGGCCGCGGCCGTGG -3'

Table 3.1 contains the specific composition for the PCR amplification of the *p16* promoter sequence.

Table 3.1. *p16* promoter DNA sequence PCR reaction mixture

Reagent	Volume	Final concentration
H ₂ O	x µl	
10x PCR buffer (Promega)	5 µl	1 x
MgCl ₂ [25 mM]	8 µl	4 mM
DNA	100 ng	
dNTPs (10 mM)	6.25 µl	1.25 mM
Forward primer (50 ng/µl)	5 µl	5 ng/µl
Reverse primer (50 ng/µl)	5 µl	5 ng/µl
DMSO	2.5 µl	5 %
<i>Taq</i> polymerase (5 U/µl) (Promega)	0.5 µl	2.5 U
Total volume	50 µl	

1. Start DNA denaturation at 95°C for 5 minutes.
2. Add the *Taq* polymerase.
3. Amplify for 35 cycles (30 seconds at 95°C, 30 seconds at 65°C, and 30 seconds at 72°C)
4. Incubate for 4 minutes at 72°C for final extension.
5. Load 15 µl of each PCR product and 5 µl loading dye onto a 1.5 % agarose gel containing ethidium bromide for electrophoresis at 40 mV for 1.5 hours.
6. Visualize under UV illumination.

RASSF1A promoter CpG island:

Previously published primers for the wild type sequence (Dammann, *et al.* 2000) were purchased from Inqaba Biotech:

RAWF: 5'- CCCCACAGTCCCTGCACCCAGGT -3'

RAWR: 5'- GCTCCCGCAGCTCAATGAGCTCAGGCTCCCC -3'

Table 3.2. contains the specific composition for the PCR amplification of the *RASSF1A* promoter sequence.

Table 3.2. *RASSF1A* promoter DNA sequence PCR reaction mixture

Reagent	Volume	Final concentration
H ₂ O	37.4 µl	
10x PCR buffer (Promega)	5 µl	1 x
MgCl ₂ [25mM]	1 µl	0.5 mM
DNA	100 ng	
dNTPs (10 mM)	0.5 µl	0.1 mM
Forward primer (50 ng/µl)	1.8 µl	18 ng/µl
Reverse primer (50 ng/µl)	1.8 µl	18 ng/µl
Taq polymerase (5 U/µl) (Promega)	0.5 µl	2.5 U
Total volume	50 µl	

1. Start the reactions at 95°C for 5 minutes.
2. Amplify for 35 cycles (1 minute at 95°C, 1 minute at 55°C and 1 minute at 74°C)
3. Incubate for 4 minutes at 72°C for final extension.
4. Load 15 µl of each PCR product and 5 µl loading dye onto a 1.5 % agarose gel containing ethidium bromide for electrophoresis at 40 mV for 1.5 hours.
5. Visualize under UV illumination.

3.5.3.4. Wizard® SV gel and PCR Clean-Up system

The kit was used according to the manufacturer's instructions.

1. Excise the DNA fragment of interest with a clean scalpel and transfer the slice to a weighed 1.5 ml microcentrifuge tube. Record the weight of the slice.
2. Add membrane binding solution at a ratio of 10 µl per 10 mg agarose gel slice.
3. Vortex well and incubate the mixture at 65°C for 10 minutes until the gel is completely dissolved.
4. Place one SV minicolumn in a collection tube and transfer the prepared solution to the minicolumn.
5. Centrifuge the assembly for 1 minute at 16 000 x g and discard the flow-through.
6. Wash with 700 µl membrane wash solution and centrifuge for 1 minute at 16 000 x g. Remove the flow-through.

7. Repeat the wash step with 500 μ l membrane wash solution and centrifuge for 5 minutes at 16 000 x g.
8. Discard the flow-through and centrifuge the assembly for 1 minute.
9. Transfer the minicolumn to a 1.5 ml microcentrifuge tube and centrifuge for 2 minutes at maximum speed.
10. Transfer the minicolumn to a new 1.5 ml microcentrifuge tube and add 50 μ l of nuclease-free water to the column.
11. Incubate 1 minute at room temperature and centrifuge the minicolumn for 1 minute at maximum speed to elute the DNA.
12. Store the DNA at 4°C.

3.6. DNA ISOLATION FROM CULTURED CELLS

3.6.1. Principle of the method

The basis of this method lies in the lysis of cells after a PBS washing step to remove all residual trypsin in the cell pellet. Lysis of the cells and their nuclei releases the high molecular weight genomic DNA which can then be isolated. All RNA present in the lysate is removed by the addition of RNase. Salt precipitation is used to remove all cellular proteins from the lysate to ensure the isolation of pure DNA. Isopropanol is subsequently used to desalt and precipitate the DNA to yield pure, concentrated genomic DNA ideal for manipulations like restriction enzyme digestion, amplification or membrane hybridization (Promega Technical manual, # TM050).

3.6.2. Materials used

- Wizard[®] Genomic DNA purification kit (Promega)
- PBS¹
- Isopropanol
- 70% ethanol

¹ See Appendix H

3.6.3. Method

1. Place 25 cm² trypsinised cells in a 1.5 ml microcentrifuge tube.
2. Centrifuge the tube at 13 000 – 16 000 x g for 10 seconds.
3. Remove the supernatant, leaving 10 - 50 µl of the supernatant without disturbing the cell pellet.
4. Add 200 µl PBS to wash the cells and centrifuge another 10 seconds at 13 000 – 16 000 x g.
5. Remove the PBS (leaving 10-20 µl) and vortex the tube to resuspend the cells.
6. Add 600 µl nuclei lysis solution and pipette the solution to break any clots present. Incubate the tube at 37°C for 10 minutes, or until all clots are dissolved.
7. Add 3 µl RNase solution and invert to mix.
8. Incubate the lysate for 25 minutes at 37°C and let cool to room temperature for 5 minutes.
9. Add 200 µl protein precipitation solution and vortex immediately for 10 - 20 seconds.
10. Keep the tube on ice for 5 minutes, centrifuge 4 minutes at 13 000 - 16 000 x g.
11. Carefully remove supernatant containing the DNA and add it to 600 µl isopropanol in a clean 1.5 ml microcentrifuge tube.
12. Invert the tube several times to precipitate DNA until a white mass is formed.
13. Centrifuge for 1 minute at 13 000 – 16 000 x g.
14. Remove supernatant and add 600 µl 70 % ethanol to the pellet, mixing well.
15. Centrifuge for 1 minute at 13 000 – 16 000 x g.
16. Remove supernatant and air-dry the tube inverted on absorbent paper for 10 - 15 minutes.
17. Add 50 µl DNA rehydration solution to the tube and rehydrate DNA overnight at 4°C or at 65°C for 1 hour.
18. Store the rehydrated DNA at 4°C until use.

3.7. DNA CONCENTRATION VERIFICATION

3.7.1. Principle of the method

The NanoDrop® ND-1000 is a spectrophotometer able to accurately measure concentration and purity of DNA in a 1 µl sample with high reproducibility. It is a full-spectrum spectrophotometer with a range of 220 – 750 nm. DNA (1 - 2 µl) is pipetted onto the receiving fibre which consists of a fibre optic cable. After the source fibre makes contact with the sample, it forms a controlled bridge between the optic ends through which pulsed xenon flash light passes. The light is then analysed by a linear CCD array. The sample concentration is calculated by the software in ng/µl. This is done with a modification of the Beer-Lambert equation for nucleic acids. This modification uses an extinction coefficient with units of ng-cm/ml (NanoDrop® ND-1000 User's Manual).

$$c = (A \times e)/b$$

Where c = nucleic acid concentration (ng/µl)

A = absorbance (AU)

e = wavelength-dependent extinction coefficient (ng-cm/µl)

b = path length (cm)

Generally the extinction coefficient for nucleic acids is accepted as:

$$\text{Double-stranded DNA} = 50 \text{ ng-cm/ml}$$

3.7.2. Materials used

- NanoDrop® ND-1000 Full-spectrum UV/Vis Spectrophotometer

3.7.3. Method

1. Place 2 μ l ddH₂O onto the lower measurement pedestal and close the sampling arm to initiate the apparatus.
2. Open the sampling arm and wipe the sample from the pedestals.
3. Place 2 μ l ddH₂O or the rehydration buffer onto the lower measurement pedestal to blank the machine.
4. Open the sampling arm and wipe the sample from the pedestals.
5. Place 2 μ l of the DNA sample in the NanoDrop[®] after mixing the sample well.
6. Initiate the measuring of the DNA concentration.
7. Repeat the measurement twice and determine the average.

3.8. RESULTS AND DISCUSSION

3.8.1. Genomic DNA isolation from whole blood – Miniprep method

DNA was isolated from 300 μ l whole blood of both control subjects and clinically diagnosed breast cancer patients with the Wizard® Genomic DNA purification kit (Promega) as previously described. Table 3.3 shows some of the DNA concentrations and purity as determined with the NanoDrop® spectrophotometer.

Table 3.3. Properties of DNA isolated from whole blood with the Wizard® Genomic DNA purification kit

Control subject/patient	[DNA] ng/ μ l	Purity (OD ₂₆₀ /OD ₂₈₀)
AJF	315	1.9
DJY	65	1.87
MSJ	40	1.85
AES	605	1.86
AJ	151	1.88
MMB	520	1.87
C3	34	1.8
AMK 003	16	1.6
231105_01	88	1.8
231105_02	77	1.8
231105_03	48	1.8
231105_05	47	1.8
231105_07	108	1.8

From these results it is clear that the Wizard® Genomic DNA purification kit is very suitable for genomic DNA isolation from small volumes of fresh whole blood because of an expected yield of 5-15 μ g DNA per 300 μ l whole blood. The isolated DNA contained very low protein content and the marked variation in the DNA yield can be ascribed to individual variation between different people.

As shown in Figure 3.1, this method evidently produces large quantities of high molecular mass genomic DNA because the major bulk of the band did not move far from the wells. It is, therefore, clearly a very suitable method for genomic DNA isolation from whole blood in a few quick, simple steps which also makes it very suitable for a relatively high-throughput laboratory.

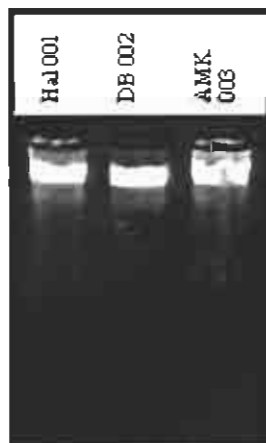


Figure 3.1. Gel electrophoresis of human genomic DNA isolated with the Wizard® Genomic purification kit. Each lane contains genomic DNA from a different breast cancer patient. 1% agarose gel.

3.8.2. Genomic DNA isolation from whole blood – Maxiprep method

Whole blood of both control subjects and clinically diagnosed breast cancer patients was collected in EDTA tubes and DNA was isolated as previously described. Examples of the DNA concentrations and purities achieved are presented in Table 3.4.

These results indicate that the method is useful to isolate highly concentrated preparations of DNA from large volumes of blood, although the yield is not as high as would be expected from the volume used. The purity of the DNA is high and, therefore, suggests that the DNA is suitable for use in further applications such as PCR or restriction enzyme digestion.

Table 3.4. Properties of DNA isolated from whole blood with the Maxiprep method

Control subject/patient	[DNA] ng/ μ l	Purity (OD ₂₆₀ /OD ₂₈₀)
AJF	1158	1.87
DJY	74.5	1.80
MSJ	41.6	1.75
AES	1506	1.88
AJ	616	1.85
MMB	2676	1.86
200706_01	58	1.89
270705_03	132	1.89

3.8.3. DNA isolation from rat liver and brain tissue with phenol:chloroform extraction

Brain and liver tissue from a young adult male rat was obtained from the Experimental Animal Centre, Potchefstroom Campus, North-West University, and DNA was subsequently isolated with phenol:chloroform extraction.

Before the phenol:chloroform extraction, a small amount of tissue was first used with variations of the Wizard[®] kit (Promega). The result was unsatisfactory and had a very low yield and, therefore, isolation with the phenol:chloroform extraction was considered. The isolation was very successful, and large amounts of relatively pure DNA were isolated as shown in Table 3.5.

The larger amount of DNA isolated in comparison with the genomic DNA isolated with the Wizard[®] kit, may be due to the large amount of tissue which was used for the extraction.

The results given in Table 3.5 confirm that this tried and tested method of DNA isolation can still be used with confidence in spite of the hazardous reagents used in this method.

Table 3.5. Properties of DNA isolated from rat tissue with phenol:chloroform extraction

Tissue	[DNA] ng/ μ l	Purity (OD ₂₆₀ /OD ₂₈₀)
Brain tissue	228.59	1.85
Liver tissue	243.18	1.85

This method is, however, most suitable for isolation of DNA from fresh tissue samples, and is not standardized for samples that have been embedded in paraffin.

3.8.4. DNA isolation from paraffin-embedded tumour tissue

Only one paraffin-embedded tumour tissue sample was available for DNA isolation. DNA was isolated following the protocol as described in section 3.5.2. The isolated DNA concentration was determined with the NanoDrop® as 31.5 ng/ μ l (purity of 1.75), and the DNA was subsequently purified with a Wizard® Clean-Up column (Promega) to remove any remaining impurities. The DNA concentration was then determined as 38 ng/ μ l, although the purity remained unchanged.

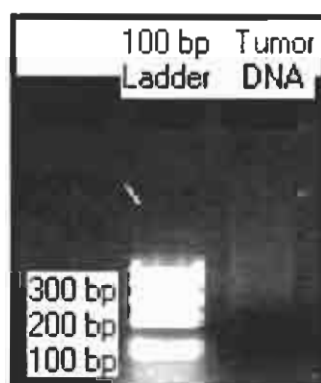


Figure 3.2. Gel electrophoresis of DNA isolated from breast cancer tumour tissue. A 100 bp ladder was used as molecular marker, with the DNA isolated from the tumour tissue in lane 3. 1% agarose gel.

As shown in Figure 3.2, the DNA is present as a very light smear. This is to be expected, since the DNA can be degraded during the fixation of the tissue, and

during the isolation procedure. The presence of DNA was subsequently confirmed with PCR amplification of the *p16* and *RASSF1A* promoter sequences (Figures 3.3 and 3.4).



Figure 3.3. *p16* promoter amplification of DNA isolated from paraffin-embedded breast cancer tumour tissue. A 100 bp ladder was used as molecular marker, with genomic DNA isolated from blood in lane 2, HepG2 DNA in lane 3 and the DNA isolated from the tumour tissue in lane 4. The last lane contains a Control with no DNA.

Amplification of the 140 bp *p16* promoter sequence was successful (see Figure 3.3), and the amplicons were consistent for the various DNA samples. This confirms the successful isolation of DNA from the paraffin-embedded tumour sample, as well as its suitability for subsequent analysis.

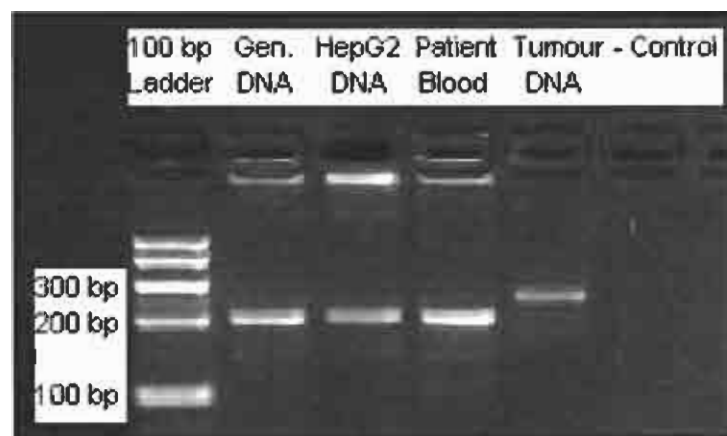


Figure 3.4. *RASSF1A* promoter amplification of DNA isolated from paraffin-embedded breast cancer tumour tissue. A 100 bp ladder was used as molecular marker, with genomic DNA isolated from blood in lane 2, HepG2 DNA in lane 3, DNA isolated from whole blood from the patient in lane 4 and the DNA isolated from the tumour tissue in lane 5. The last lane contains a Control with no DNA.

The amplicon obtained for the tumour tissue with the wild-type *RASSF1A* promoter sequence primers was visibly larger than the 204 bp product obtained for the other DNA samples. This PCR amplification was repeated to verify this result, and this

product, as well as the product obtained for the genomic DNA isolated from the patient's whole blood, was subsequently excised and gel-extracted with the Wizard® SV gel and PCR Clean-up system (Promega). These products were sequenced by Inqaba Biotech¹.

```
TCCCCACAGT CCCTGCACCC AGGTTTCCAT TGC GCGGCTC TCCTCAGCTC CTTCCCGCCG
CCCAGTCTGG ATCCTGGGGG AGGCGCTGAA GTCGGGGCCC GCCCTGTGGC CCCGCCCGGC
CCGCGCTTGC TAGCGCCCAA AGCCAGCGAA GCNCGGGCCC AACCGGGCAT GTCGGGGGAG
CCTGAGCTCA TTGAGCTGCG GGAGAA
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Figure 3.5. *RASSF1A* promoter sequence of patient AES as amplified from genomic DNA isolated from whole blood.

This sequence was obtained by forward and reverse sequencing of the PCR amplicon. These two sequences aligned very well (Appendix C) and the sequence subsequently obtained was submitted for a BLAST search (www.ncbi.nlm.nih.gov/BLAST/). The result showed a very specific alignment ($2e-103$) with the *Homo sapiens* *RASSF1* gene, promoter and partial cds, as well as the human cosmid clone LUCA12 from 3p21.3 which was used to design the primers for the wild type *RASSF1A* amplification (Appendix D). This confirms that the *RASSF1A* promoter region was amplified, and was present unaltered in the patient's genome.

```
GGAAGGNAGN NTANTCCTGT TTCCAGGAAC TGAAACCTAA CACCATGAAC CTGTCCTCTA
TCACATGGAG AAAGTGGGCC CCCTGACGAA CGCCCAGGAA TCTTCCTACC ATTGGCAGGT
GCAGGGAACc TAGGACCTAA TATCCTCGTG ATCCTTGGGA G
```

Figure 3.6. The sequence obtained from patient AES as amplified from DNA isolated from paraffin-embedded tumour tissue with the *RASSF1A* promoter primers.

The sequence presented in Figure 3.6 was also obtained via forward and reverse sequencing of the PCR product. These two sequences were also aligned to obtain the sequence given above (Appendix E). As with the previous sequence, the sequence for the tumour tissue DNA was submitted for a BLAST search (www.ncbi.nlm.nih.gov/BLAST/). The result unexpectedly did not report an alignment with the *RASSF1A* promoter gene or the human cosmid clone LUCA12 sequence (Appendix F), but aligned with a human DNA sequence from clone RP5-1061H20 on

¹ Inqaba Biotech uses a Genetic analysis system, SCE2410, with 24 capillaries (SpectruMedix LLC - Pennsylvania, USA) to sequence DNA. For the analysis, they use BaseSpectrum V2.1.1 software (SpectruMedix) and the BigDye (version 3.1) dye terminator cycle sequencing kit (Applied Biosystems).

chromosome 1 (Contains three novel genes, a novel pseudogene, the 5' end of the RAB4A gene for RAB4A, a member of the RAS oncogene family and a CpG island, complete sequence). This lack of more specific alignment might be due to the incomplete sequencing of the tumour DNA, as neither the forward, nor the reverse sequencing could be completed (after several attempts) for the entire length of the amplicon and, therefore, only approximately 60 % of the PCR product was sequenced. The tumour tissue DNA sequence could also not be aligned with the blood DNA sequence (result not shown), as there are no corresponding regions and we, therefore, propose that the tumour tissue DNA contains a tumour and/or tissue specific isoform of the *RASSF1A* gene, or a mutated form as it is a malignant growth.

The DNA isolated from the tumour tissue was subsequently treated with bisulfite and used for MSP as described in chapter 4 and 5.

3.8.5. DNA isolation from cultured cells

DNA was isolated from various cultured cell lines, including fibroblast cells, HeLa cells and HepG2 cells with the Wizard[®] Genomic DNA purification kit (Promega) as previously described. The DNA concentrations and purity were determined using the NanoDrop[®] spectrophotometer, and are given in Table 3.6.

Table 3.6. Properties of DNA isolated from various cultured cells with the Wizard[®] Genomic purification kit

Cell line	[DNA] ng/μl	Purity (OD ₂₆₀ /OD ₂₈₀)
Fibroblast cell culture	27.2	1.62
HepG2 cell culture	412	1.83
HeLa cell culture	61	1.75

DNA isolation from cultured cells was very successful, although there were some fluctuations in the concentration of DNA isolated. This might occur as a result of variation in the amount of cells present in the culture. The method is clearly suitable for the isolation of DNA from various types of cell cultures, producing high concentrations of pure DNA.

3.9. SUMMARY

In this study, different methods to isolate DNA were performed, since DNA would have to be isolated from various tissue types. Table 3.7 combines the results of the different methods used. Although only a small number of samples were used for each application, it was sufficient to draw the necessary conclusions for this study.

Table 3.7. Average properties of DNA isolated with various methods or kits

Method/ Kit	Average [DNA] ng/ μ l	Average Purity (OD ₂₆₀ /OD ₂₈₀)	Suitable application
Wizard [®] Genomic DNA purification kit	162.62/ 166.73	1.82/ 1.73	Genomic DNA isolation from small volumes whole blood or cell cultures
Möhr method (maxiprep method)	782.76	1.85	Genomic DNA isolation from large volumes whole blood
Phenol:chloroform extraction	235.89	1.85	Genomic DNA isolation from whole blood or fresh tissue samples
E.Z.N.A. [®] Tissue DNA mini kit	31.5	1.75	DNA isolation from paraffin- embedded tissue samples and cell cultures

Although the Möhr method produces a very high yield and purity from large volumes of blood and is very cost-efficient, it takes much longer to isolate the DNA than with the Wizard[®] Genomic DNA purification kit. The higher yield is because of the larger amount of starting material used for the isolation. It also incorporates much harsher chemicals and the purity of the DNA is not always on par with the Wizard[®] kit. The Wizard[®] kit is very quick and easy, and restricts exposure to hazardous chemicals. It produces high concentrations pure DNA from very small sample volumes and the DNA can be used for various applications immediately with no additional purification steps. It can, therefore, be concluded that the Wizard[®] Genomic DNA purification kit is the best method to isolate genomic DNA from whole blood samples, except in the event of the need for larger amounts of DNA, where the Möhr method is most probably more suitable, although we would recommend a subsequent purification step to ensure purity.

The standard phenol:chloroform extraction method is a well-established method in our laboratories, and produces a high yield of pure DNA from tissue samples and blood, but it is a time consuming method using very hazardous materials and the method is only applicable for fresh tissue samples. Since all the tumour tissue samples from patients are only available as paraffin-embedded samples, the phenol:chloroform extraction is not suitable and, therefore, the E.Z.N.A.[®] Tissue DNA mini kit is better suited for DNA isolation from the paraffin-embedded tumour tissue samples in this study.

Isolation of DNA from various cell cultures was done with the Wizard[®] Genomic DNA purification kit because of its availability in our laboratory and because it is a quick and easy method for DNA isolation. The method, just as with the whole blood samples, produced high quantities pure DNA from the various cell cultures used, and it was, therefore, decided to continue the DNA isolations from the cell cultures with this kit, although there are other kits and methods available for this extraction.

Several methods were used to isolate genomic DNA from various sample types to determine which method is most suitable for each tissue. This is very important since yield and purity can influence the subsequent applications of the isolated DNA, although aspects like time and cost effectiveness must be taken into consideration when choosing a method for further use.

Using these methods as described above, sufficient amounts of high-purity genomic DNA was isolated and was used for the subsequent MSP assay as described in Chapters 4 and 5.

CHAPTER 4

METHYLATION-SPECIFIC PCR AND COBRA ASSAY

4.1. METHYLATION-SPECIFIC PCR

4.1.1. Principle of the method

This assay is based on the sodium-bisulfite treatment induced differences between methylated and unmethylated DNA. This treatment converts unmethylated cytosines to uracils, while methylated cytosines remain unchanged (Figure 4.1).

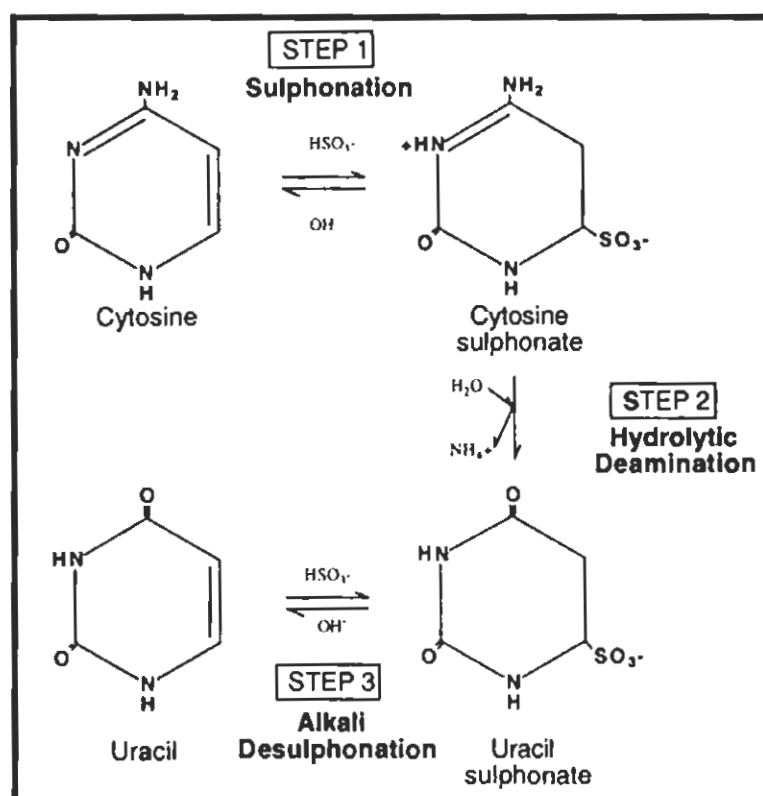


Figure 4.1. The bisulfite mediated conversion reaction (Clark, *et al.* 1994).

The bisulfite-treated DNA is then used as a template in PCR amplification using two different sets of primers specific for methylated versus unmethylated DNA as illustrated in Figure 4.2 (Herman, *et al.* 1996; Toyooka and Shimizu, 2004). There is also an alternative application of the bisulfite conversion treatment, namely the COBRA assay, which will be discussed in section 4.2.

MSP can be used with small amounts of DNA such as DNA extracted from paraffin-embedded tissue samples or free-circulating DNA in plasma or serum.

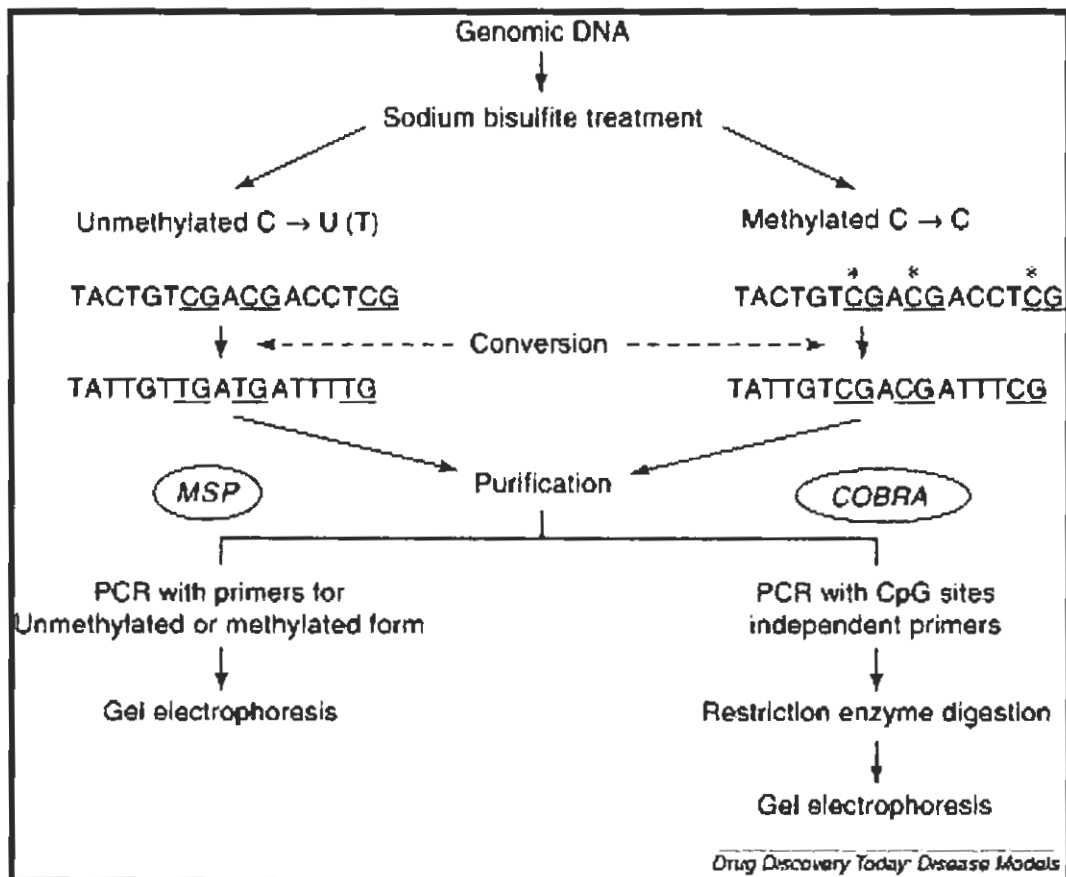


Figure 4.2. MSP and COBRA as methylation detection assays (Toyooka & Shimizu, 2004).

The MSP assay is noticeably more sensitive than the Southern blot assay as it can detect abnormal methylation of a small DNA sequence surrounded by normal methylated DNA to a sensitivity of 0.1 % methylated alleles of a specified CpG island locus (Herman, *et al.* 1996; Miyamoto and Ushijima, 2005). It is, therefore, able to eliminate false positive results frequent in restriction enzyme based assays and it is sensitive and specific for methylation of almost any CpG block in a CpG island. It can also examine all CpG sites because it is not restricted by the sequence recognized by the methylation-sensitive restriction enzymes. This increases the number of sites that can be mapped. Another advantage of MSP is the ability to detect methylated and unmethylated products simultaneously in one sample. This confirms the DNA integrity as a PCR template. Thus, it can be concluded that MSP is a quick, sensitive and specific method, able to examine the methylation status of almost any CpG-rich sequence (Herman, *et al.* 1996).

An example of expected results

Herman, *et al.* (1996) designed primers for the *E-cadherin* promoter region and performed MSP on this promoter. *E-cadherin* is an invasion/metastasis suppressor gene which is often silenced by abnormal 5' promoter methylation in breast and other carcinomas.

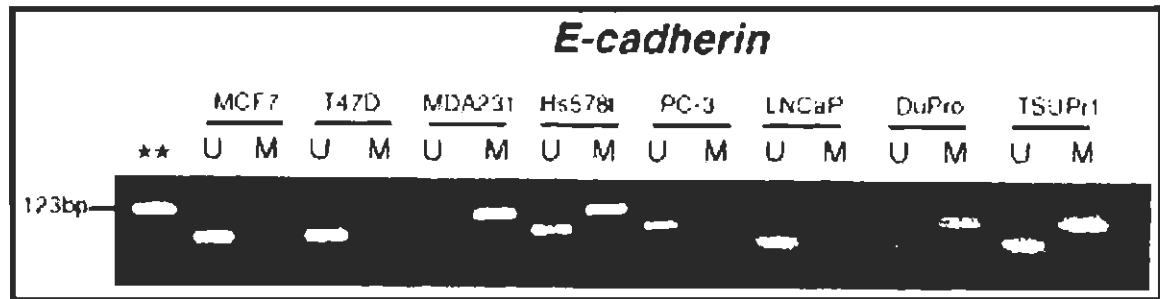


Figure 4.3. An example of methylation detection of *E-cadherin* promoter region by MSP assay. U = unmethylated; M = methylated; ** = 123-bp molecular weight marker (Herman, *et al.* 1996)

As shown in Figure 4.3, the presence or absence of an amplicon for the different primer sets clearly indicates the methylation status of the original DNA sample. For example, samples MCF7 and T47D are unmistakably unmethylated because there are very clear products present for the unmethylated-specific primers, but no product for the methylation-specific primers. Sample MDA231, however, is definitely methylated because of the methylated product. The presence of amplicons for both sets of primers (e.g. sample Hs578t) indicates the presence of both methylated and unmethylated CpG sites in the sample DNA, and the DNA is, therefore, not completely unmethylated or methylated.

4.1.2. Materials used

Bisulfite treatment:

- 3 M NaOH
- 3.6 M sodium bisulfite (MP Biomedicals)
- 0.1 M hydroquinone (MP Biomedicals)
- 7.5 M NH₄Ac
- 70 % ethanol
- 100 % ethanol

- Wizard® DNA clean-up system (Promega)
- Vac-Man® Laboratory Vacuum manifold (Promega)
- 80 % isopropanol
- 2 mg/ml glycogen

PCR:

- Mastercycler® ep (Eppendorf)
- MSP primers – HPLC purified (Whitehead Scientific Pty Ltd.)
- *Taq* Polymerase (Promega)
- 10 x MgCl₂-free PCR buffer (supplied with *Taq* polymerase)
- 25 mM MgCl₂ (supplied with *Taq* polymerase)
- 10 x PCR sulphate buffer¹
- Biotools DNA polymerase (Biotools) [1U/μl]
- 10 x Standard buffer (Biotools – supplied with DNA polymerase)
- Phusion™ High-fidelity DNA polymerase (Finnzymes)
- 5 x Phusion™ GC buffer (Finnzymes – supplied with DNA polymerase)
- dNTP mix (New England Biolabs)
- Formamide
- Agarose
- 0.1 x TBE-buffer¹
- Syngene ChemiGenius Bio-Imaging System (Genesnap 6.04)

Standards

- Universal Methylated Human DNA Standard (Zymo Research)
- Fibroblast cell line²
- HeLa cell line²
- HepG2 cell line²
- Herring sperm DNA

¹ See Appendix H.

² Supplied by the laboratory cell culture bank. Unknown passage.

4.1.3. Method – Bisulfite treatment

The following reagents (excluding the NH_4Ac) should always be prepared fresh, just before use:

3 M NaOH

1. Dissolve 1.2 g NaOH in 10 ml H_2O .

3.6 M Sodium Bisulfite (pH 5.5)¹

1. Dissolve 3.8 g sodium bisulfite in 8 ml H_2O .
2. Add 2 ml freshly prepared 3 M NaOH.

0.1 M Hydroquinone¹

1. Dissolve 0.11 g hydroquinone in 10 ml H_2O .

7.5 M Ammonium Acetate

1. Dissolve 28.91 g NH_4Ac in 50 ml H_2O .

4.1.3.1. Bisulfite modification

1. Place $\pm 1.5 \mu\text{g}^2$ DNA suspended in TE-buffer or H_2O in a 1.5 ml microcentrifuge tube.
2. Add dd H_2O to a final volume of 18 μl .
3. Add 2 μl NaOH (3 M) and vortex, followed by a short centrifugation step.
4. Incubate at 37°C for 15 minutes.
5. Add 12 μl freshly prepared hydroquinone (0.1 M) (mixture turns yellow) and 208 μl freshly prepared sodium bisulfite (3.6 M), and mix gently.
6. Incubate the samples at 55°C for 5 - 16 hours, depending on the fractionated nature of the DNA³.

¹ Sodium bisulfite and hydroquinone are easily oxidised and should, therefore, be mixed gently.

² Use a maximum volume of 18 μl DNA if the DNA concentration requires a larger volume.

³ Whole genomic DNA is preferably incubated for the maximum time, 16 hours, while free-circulating DNA and DNA isolated from paraffin-embedded tissue samples are only incubated for the minimum time, 5 hours.

4.1.3.2. Purification of modified DNA

The Wizard® DNA Clean-Up system (Promega) was used according to the manufacturer's instructions.

1. Attach one minicolumn to the provided syringe barrel and the vacuum manifold.
2. Mix the Clean-Up resin well and add 1 ml to the sample in a 1.5 ml microcentrifuge tube and vortex.
3. Transfer the sample to the syringe barrel and apply vacuum to the minicolumn until the solution is drawn through the column.
4. Wash with 1 ml of 80 % isopropanol and re-apply vacuum until the solution is drawn through the column. Repeat the wash step.
5. Apply an additional 30 seconds of vacuum to dry the column.
6. Transfer the minicolumn to a 1.5 ml microcentrifuge tube and centrifuge for 2 minutes at maximum speed (16 000 x g).
7. Transfer the minicolumn to a new 1.5 ml microcentrifuge tube and apply 25 µl of prewarmed (70°C) water to the column.
8. Incubate 1 minute at room temperature and centrifuge the minicolumn for 20 seconds at maximum speed (16 000 x g) to elute the DNA.
9. Repeat step 11 and 12.
10. Store the DNA at 4°C.

4.1.3.3. Completion of modification

1. Add 5 µl 3 M NaOH and incubate at 37°C for 15 minutes.
2. Add 55 µl 7.5 M NH₄Ac and 2 µl glycogen. Mix well.
3. Add 275 µl 100 % ethanol and mix very well.
4. Incubate for 10 minutes to overnight at -20°C.
5. Centrifuge for 10 minutes at 13 000 – 16 000 x g.
6. Remove supernatant and wash the pellet with 200 µl 75 % ethanol.
7. Centrifuge for 5 minute at 13 000 – 16 000 x g.
8. Remove supernatant and air-dry the pellet with the tube inverted on absorbent paper for 10 - 15 minutes.
9. Resuspend the DNA in 50 µl H₂O.

4.1.4. PCR amplification of target sequences

The different methylated-specific and unmethylated-specific primer sequences used for the methylation-specific PCR of *p16* and *RASSF1A* are described in Table 4.1.

Table 4.1. Primers used for Methylation Specific PCR as previously published

		Forward sequence (5' - 3')	Reverse sequence (5' - 3')	Pro- duct (bp)	T _m (°C)	Ref.
p 16	M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65	Herman, <i>et al.</i> 1996
	U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	62	Herman, <i>et al.</i> 1996
RASSF1A	M	GTGTTAACGCGTTGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	93	67	Lo, <i>et al.</i> 2001
	U	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACTAACTAAAAACAA	105	62	Lo, <i>et al.</i> 2001

M=methylated; U=unmethylated

In this study two promoter CpG islands were investigated, namely that of genes *p16* and *RASSF1A*, and each will subsequently be described briefly as well as their specific PCR conditions. These two potential markers were chosen because of their well-described use and hypermethylated status in various cancers, including breast cancer (see below).

4.1.4.1. *p16* tumour suppressor gene

The *p16* protein is involved in cell growth and regulation of differentiation. In cancer the *p16^{INK4a}* gene has been noted to be regularly inactivated (Silva, *et al.* 1999; Widschwendter and Jones, 2002). This inactivation is generally instigated by homozygous deletion which is supported by methylation, since numerous cancers show aberrant *p16* methylation, and breast tumours display *de novo* methylation of $\pm 23 - 31\%$ (Silva, *et al.* 1999; Ferguson and Nass, 2000; Tong and Lo, 2006). *p16* is generally associated with higher-stage disease and unlimited Replicative Potential for cells, and a direct correlation between methylation status of *p16* and survival has been established (Cottrell, 2004). The PCR reaction mixture and amplification

conditions are different for each gene examined. The following reaction conditions were optimized for the *p16* promoter CpG island (Table 4.2).

Table 4.2. PCR reaction mixture for MSP amplification of *p16*

Reagent	Volume	Final concentration
10 x MgCl ₂ -free PCR buffer (Promega)	2.5 µl	1 x
MgCl ₂ (25 mM)	3 µl	3 mM
dNTPs (10 mM each)	2 µl	0.8 mM each
Forward primer (10 pmol/µl)	1 µl	0.4 pmol/µl
Reverse primer (10 pmol/µl)	1 µl	0.4 pmol/µl
Formamide	1 µl	4 %
Bisulfite-modified DNA	10 µl	Depend on start concentration
Total volume	20.5 µl	
Taq mix		
H ₂ O	4 µl	
Taq polymerase (5 U/µl) (Promega)	0.5 µl	2.5 U
Total volume	4.5 µl	

1. Start the reactions at 68°C for 2 minutes.
2. Add 4.5 µl *Taq* polymerase mix.
3. Amplify for 40 cycles (30 seconds at 92°C, 30 seconds at T_m (62°C for unmethylated-specific primers and 65°C for methylated-specific primers), and 30 seconds at 72°C).
4. Incubate for 4 minutes at 72°C for final extension.
5. Load 10 µl of each PCR product onto a 2.5 % agarose and 0.1 x TBE-buffer gel containing ethidium bromide for electrophoresis.
6. Visualize under UV illumination.

4.1.4.2. *RASSF1A*

The *RASSF1A* gene is a recognized tumour suppressor gene, and is expressed in all normal tissues. The gene has been shown to be hypermethylated in multiple cancers, including primary lung tumours, ovarian tumours and some breast cancers (Dammann, *et al.* 2000, 2001; Tong and Lo, 2006). Dammann, *et al.* (2001) also showed that CpG island hypermethylation in the *RASSF1A* promoter sequence has been associated with loss of expression for this gene. Some studies also associated *RASSF1A* methylation with tumour size, cell death and a good response to treatment, and hypermethylation of this gene has been found in plasma of patients receiving chemotherapy (Tong and Lo, 2006). The following PCR conditions (Table 4.3) were optimized for the amplification of the *RASSF1A* promoter CpG island.

Table 4.3. PCR reaction mixture for MSP amplification of *RASSF1A*

Reagent	Volume	Final concentration
10 x PCR buffer (Promega)	2.5 µl	1 x
MgCl ₂ (25 mM)	4 µl	4 mM
dNTPs (10 mM each)	2 µl	0.8 mM each
Forward primer (10 pmol/µl)	1 µl	0.4 pmol/µl
Reverse primer (10 pmol/µl)	1 µl	0.4 pmol/µl
Formamide	0.5 µl	2 %
Bisulfite-modified DNA	10 µl	Depend on start concentration
H ₂ O	3.5 µl	
Taq polymerase (Promega) (5 U/µl)	0.5 µl	2.5 U
Total volume	25 µl	

1. Start the reactions at 95°C for 2 minutes.
2. Amplify for 34 cycles (30 seconds at 95°C, 30 seconds at T_m (62°C for unmethylated-specific primers and 67°C for methylated-specific primers), and 30 seconds at 72°C).
3. Incubate for 2 minutes at 60°C.
4. Incubate for 4 minutes at 72°C for the final extension step.

5. Load 10 μ l of each PCR product onto a 2.5 % agarose and 0.1 x TBE-buffer gel containing ethidium bromide for electrophoresis.
6. Visualize under UV illumination.

4.2. COBRA ASSAY

(COMBINED BISULFITE RESTRICTION ANALYSIS)

4.2.1. Principle of the method

The COBRA assay is a technique which also involves the bisulfite-conversion of unmethylated cytosines, followed by PCR amplification with primers with no CpG sites and subsequent restriction enzyme treatment. It is a relatively simple technique, based on the loss of a restriction enzyme site or the creation of such a site by the bisulfite induced conversion (Figure 4.4). In this study *Taq* I was used. This restriction enzyme recognizes the T*CGA sequence.

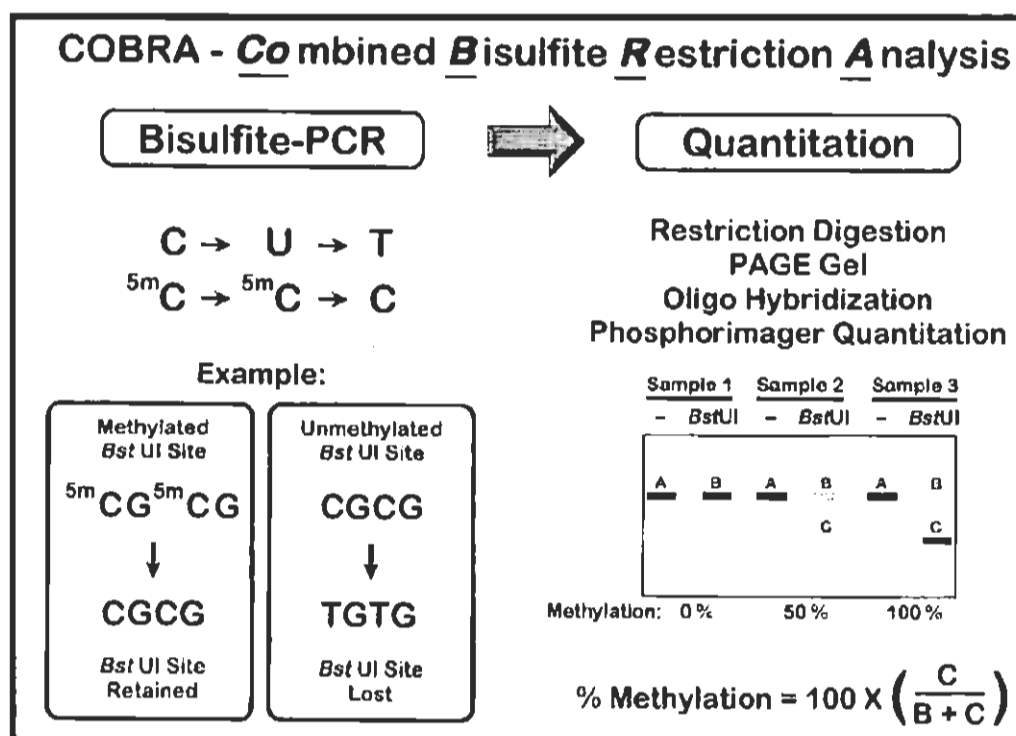


Figure 4.4. The COBRA assay. DNA is treated with sodium bisulfite to induce conversion of unmethylated cytosines to thymine, resulting in a sequence alteration, followed by PCR amplification of the region of interest. The product is then digested with specific restriction enzymes sensitive for the sequence variations, and the products of the digestion can then be analysed via electrophoresis (Xiong and Laird, 1997).

In the original paper the authors stated that the COBRA assay is quantitative, compatible with paraffin sections and applicable to large sample sets (Xiong and Laird, 1997). The assay is, however, restricted to the specific restriction sites available in a region, but it is possible to analyse multiple regions in one reaction (Brena, *et al.* 2006).

4.2.2. Materials used

Since this method was performed in Germany, the following reagents were purchased from European suppliers.

- Eppendorf PCR machine
- MSP primers (sequences are confidential)
- 10x MgCl₂-free PCR buffer
- 25 mM MgCl₂
- *Taq* Polymerase
- dNTP mix
- Formamide
- Agarose
- 0.5 x TBE-buffer¹
- Bisulfite treated DNA (as for MSP)
- *Taq* I restriction enzyme (Roche)

4.2.3. Method

4.2.3.1. PCR amplification of Satellite 2 DNA

The following PCR conditions in Table 4.4 were optimized for the amplification of the Sat 2 sequence.

1. Start the reactions at 90°C for 2 minutes before adding the *Taq* polymerase.
2. Amplify for 40 cycles (30 seconds at 90°C, 45 seconds at 54°C, and 30 seconds at 72°C)

¹ See Appendix H.

3. 2 minutes at 54°C, and 4 minutes at 72°C for final extension.
4. Load 5 - 10 µl of each PCR product onto a 2 % agarose and 0.5 x TBE-buffer gel containing ethidium bromide for electrophoresis.
5. Visualize under UV illumination to confirm the amplification of each sample.

Table 4.4. PCR reaction mixture for amplification of Sat 2

Reagent	Volume	Final concentration
10 x MgCl ₂ -free PCR buffer	2.5 µl	1 x
MgCl ₂ (25 mM)	1.5 µl	1.5 mM
dNTPs (10 mM)	2 µl	0.8 mM each
Forward primer (Sat2USB2)	1 µl	Concentration unknown*
Reverse primer (Sat2LSB2)	1 µl	Concentration unknown*
Formamide	0.5 µl	Concentration unknown*
Bisulfite-modified DNA	3 µl	Depend on start concentration
Total volume	11.5 µl	
Taq mix		
H ₂ O	13 µl	
Taq polymerase	0.5 µl	2.5 U
Total volume	13.5 µl	

* - performed in Germany and, therefore, the final concentration is unknown.

4.2.3.2. Restriction enzyme digestion

1. Place 5 µl of each PCR product in two 1.5 ml microcentrifuge tubes.
2. Add 1 µl buffer B, 1 µl Taq I and 3 µl ddH₂O to one of the two tubes.
3. Add 1 µl buffer B and 4 µl ddH₂O to the other tube.
4. Incubate at 60°C for 2 hours.
5. Load 10 µl of each reaction on a 2 % agarose and TBE-buffer gel, and do electrophoreses with 0.5 x TBE buffer.
6. Visualize under UV illumination.

4.3. RESULTS AND DISCUSSION

4.3.1. Methylation-specific PCR

Methylation-specific PCR was performed according to the original method described by Herman, *et al.* (1996). The DNA was denatured in the presence of NaOH, followed by the bisulfite modification with sodium bisulfite. After a 16 hour incubation period, the DNA was cleaned with the Wizard[®] SV gel and PCR clean-up system (Promega) and modification was completed in the presence of NaOH, followed by ethanol precipitation. The DNA was resuspended in TE buffer and used as template for MSP. The PCR reaction was compiled and the amplification was performed as described in the original method.

The method as described by Herman, *et al.* (1996) did not produce suitable results, and due to the multiple-step nature of this method, speculation on the reason for obtaining this result is difficult. However, a feasible explanation could be that the DNA might have been lost during the ethanol precipitation step after the bisulfite treatment because this treatment breaks the DNA in short fragments (Grunau, *et al.* 2001). This can be prevented with the use of a carrier like glycogen or carrier DNA in the precipitation step.

An opportunity arose for me to visit the laboratory of Dr. Reinhard Dammann at the Institute for Human Genetics and Medical Biology, Martin-Luther-Universität Halle-Wittenberg, Germany. Several papers were published by his group describing their expertise with the MSP assay. They mainly used DNA extracted from paraffin-embedded normal and tumour tissue as substrate for sodium bisulfite treatment and MSP assay of the *p16* promoter region, as well as the CpG island in the *RASSF1A* promoter sequence.

There I performed bisulfite treatment and MSP for both *p16* and *RASSF1A* using their reagents, PCR machine, standardized protocol and DNA isolated from paraffin-embedded tissue. I also had the opportunity to learn the COBRA assay. Back in our laboratory, I had to apply their protocol in our own laboratory.

4.3.1.1. *p16* promoter sequence

In fig 4.5 the results are given for *p16* MSP as obtained by me in Germany. The PCR product of the different methylated-specific and unmethylated-specific primer sets of each patient sample are shown next to each other, and the normal and tumour tissues of each patient are presented next to each other, except for patient KH, where no tumour tissue was available.

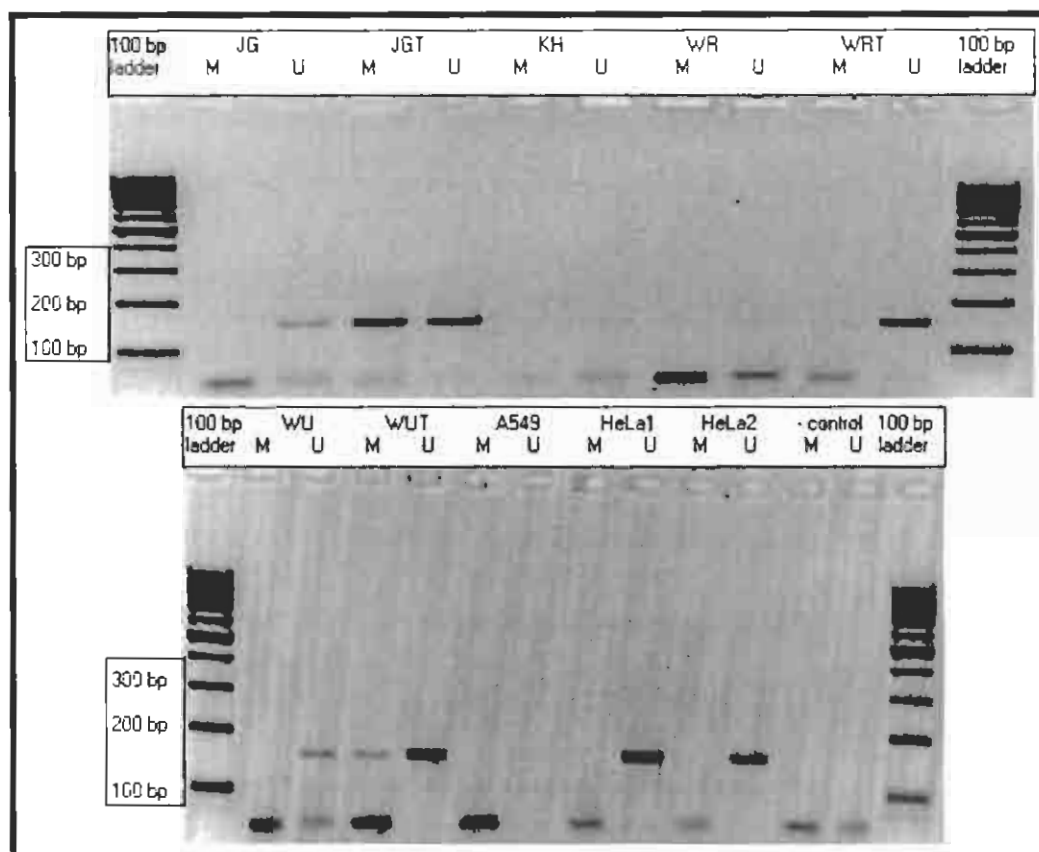


Figure 4.5: Methylation-specific PCR for the *p16* promoter sequence as obtained in Germany. A 100 bp DNA molecular weight marker, a negative DNA control and unmethylated controls (HeLa) were included. The small bands at the bottom are primer dimers, and A549 is a sample irrelevant to this study. Each patient's normal tissue is left (e.g. JG) and the tumour tissue on the right (e.g. JGT).

The fragments expected to amplify with the primers for methylated DNA were 150 bp and 151 bp for the unmethylated sequences. As is clearly visible in the results of patients JG and WU, the normal tissue is unmethylated (as indicated by the absence of any amplicons with the methylated-specific primers and the presence of product with the unmethylated-specific primers). In contrast with the normal tissue, the tumour tissue presented amplicons for both the methylated and unmethylated

primers. The difference in amplicon intensity raises a few questions, though. It could indicate different amounts of DNA being recovered after the bisulfite treatment, or it could be an indication of the level of methylation present in the sequence being investigated. This is discussed further in Chapter 5. The presence of large amounts of primer dimers is a frequent and “accepted” feature for MSP, and, therefore, additional optimization to remove it is unnecessary.

I repeated this experiment in our laboratory. However, since it was done with local reagents and a different PCR machine, various adjustments were necessary to obtain similar results to those presented in Figure 4.5. To determine which parameters could be adjusted to standardize the method in our laboratory, I reviewed some published papers and protocols on this method.

The method as described by Herman, *et al.* (1996) was the original method, but several other methods have been published since. These methods were based on the original method with some modifications, for instance a recent modification includes the use of real-time PCR of the bisulfite treated DNA. In Table 4.5 a comparison is given of several of these published techniques. These multiple variations shown in Table 4.5 are possibly due to different laboratory conditions, reagents and PCR machines, and we may conclude from this that the method is most probably sensitive to alterations in virtually every step, e.g. temperature, denaturation conditions, etc.

Various possible alterations were identified and implemented, including

- Use of dimethylformamide instead of formamide
- Use of DMSO instead of formamide
- Increasing the volume of bisulfite-treated DNA in the PCR reaction
- Type of PCR buffer
- Temperature
- MgCl_2 concentration

Table 4.5. A comparison of different published bisulfite modification methods

	Herman, <i>et al.</i> (1996)	Frommer, <i>et al.</i> (1992)	Clark, <i>et al.</i> (1994)	Protocol online (www.protocol-online.org)	MDA-Cancer center (www.mdanderson.org)
Amount of DNA	1 µg in 50 µl. (ng DNA + 1 µg herring sperm DNA)	10 µg DNA (genomic + plasmid DNA)	200 pg – 50 µg DNA in 20 – 200 µl	1-2 µg DNA in 50 µl	< 2 µg DNA in 50 µl
Shearing/ enzyme digestion		Restriction enzyme digestion with <i>EcoR</i> I or shear		Restriction enzyme digestion outside sequence of interest or shear, followed by a phenol:chloroform extraction.	
NaOH denaturation	Final concen- tration of 0.2 M at 37°C for 10 min.	Alkali denaturation, followed by neutralization and precipitation	Final concentration of 0.3 M at 37°C for 15 min.	5 µl 3 M NaOH at 37 - 42°C for 15 -30 min.	5.5 µl 2M NaOH at 37°C for 10 min.
Bisulfite treatment	30 µl 10 mM hydroquinone, 520 µl 3 M sodium bisulfite at 50°C for 16 hours.	0.5 mM hydroquinone, 1.2 ml 3.1 M sodium bisulfite at 50°C for 16 - 40 hours.	10 mM hydroquinone, 3.6 M sodium bisulfite at 55°C for 16 hours	30 µl 10 mM hydroquinone, 510 µl 40.5 % sodium bisulfite to a final concen- tration of 3.3 M at 55°C for 8 – 16 h.	30 µl 10 mM hydroquinone, 520 µl 3 M sodium bisulfite at 50°C for 16 hours
Purification	Wizard [®] DNA purification.	Dialysis.	Dialysis or desalting column	Geneclean [®] II kit	DNA wizard [®] cleanup and Isopropanol wash.
Completion of modification	NaOH to a final concentration of 0.3 M for 5 minutes at room temp.	NaOH to a final concentration of 0.3 M for 10 minutes at room temp.	NaOH to a final concentration of 0.3 M for 15 minutes at 37°C.	NaOH for 15 minutes at 37°C.	5.5 µl 3M NaOH for 5 minutes at room temperature.

Table 4.5. (Continue)

Neutralization		NH ₄ Ac to a final concentration of 3 M. Precipitate.	NH ₄ OAc to a final concentration of 3 M. ethanol precipitate.	NH ₄ Ac to a final concentration of ethanol precipitate.	1 μ l glycogen, 33 μ l 10 mM NH ₄ Ac, 3 volumes ethanol.
Resuspension	In water.	100 μ l TE buffer.	100 μ l DNA buffer.	Resuspend in 50 μ l TE buffer.	Resuspend in 20 ml water.

In Figure 4.6 the results are given for a *p16* MSP performed at various temperatures with the unmethylated-specific primers.

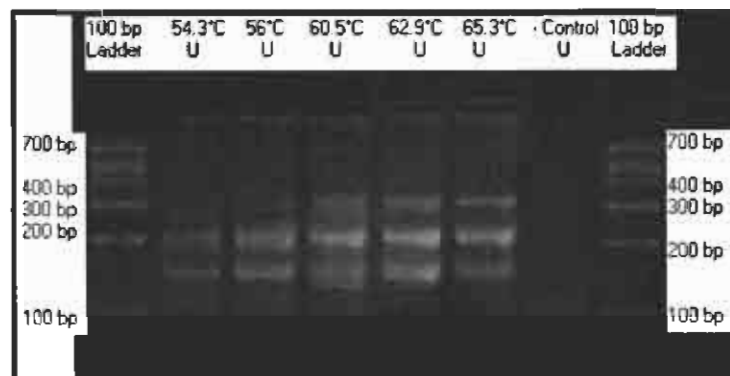


Figure 4.6. Gel electrophoresis of a *p16* temperature gradient MSP for the unmethylated-specific primers. Lane 1 and 8: 100 bp Ladder; lane 2 to 6: amplicons obtained at various temperatures; lane 7: the DNA negative control. The various temperatures are shown at the top.

After several temperature gradient PCRs, the optimal PCR temperature for the *p16* unmethylated-specific primers was decided on 62.9°C, since the expected amplicon of 151 bp was most visible at this temperature. The optimal temperature for the methylated-specific *p16* primers was chosen as 65.3°C. Methylated DNA as template should give an amplicon of 150 bp. Since the PCR was not yet optimal, the temperature gradient experiment was followed by *p16* MSP with a MgCl₂ concentration series.

MSP was executed at various MgCl₂ concentrations, with concentrations ranging between 0.67 and 4 mM. In Figure 4.7 the results are given for a *p16* MSP performed at various MgCl₂ concentrations. The bisulfite-treated DNA was collected

from an apparently healthy person, and should, therefore, theoretically be unmethylated. The optimal concentration for *p16* MSP was, therefore, chosen as 3 mM MgCl_2 , because amplification of the expected fragment was obtained with PCR using the primers for unmethylated DNA, while no non-specific amplification occurred with the methylation-specific primers.

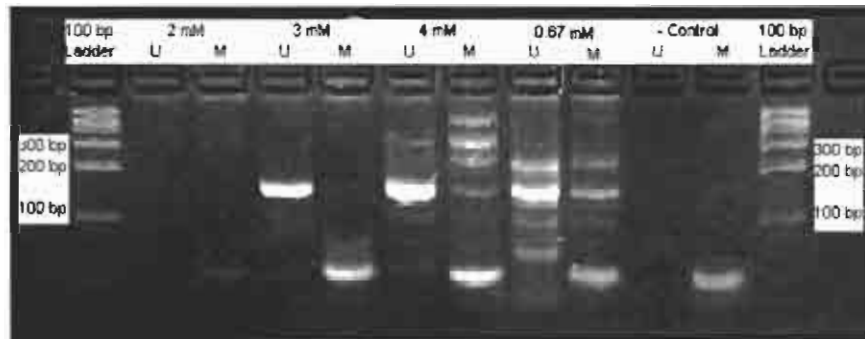


Figure 4.7. Gel electrophoresis of a *p16* MgCl_2 gradient MSP. Lane 1 and 12: 100 bp Ladder; lane 2 to 9: amplicons obtained with a temperature MgCl_2 gradient; lane 10 and 11: the DNA negative controls. The various MgCl_2 concentrations are shown at the top.

These conditions were, therefore, determined as the optimal PCR composition and conditions for amplification of methylated and unmethylated *p16* promoter CpG island sequences. These optimized conditions were subsequently applied to commercially available methylated control DNA (Zymo Research), as well as a herring sperm DNA sample and fibroblast DNA to confirm the conditions (Figure 4.8). Herring sperm DNA, as well as fibroblast DNA have been noted to be unmethylated for *p16*.



Figure 4.8. Optimized *p16* MSP applied to various standards. Lane 1: a 100 bp Ladder; lane 2 and 3: Methylated Standard (U/M); lane 4 and 5: Herring sperm DNA (U/M); lane 6 and 7: Fibroblast cell DNA (U/M). The small bands at the bottom are primer dimers.

As shown in Figure 4.8, the methylated standard was only amplified by the methylation-specific primers, while the unmethylated fibroblast DNA was only amplified by the unmethylated-specific primer set. The lack of amplification of the herring sperm sample could be a result of the sonicated state of the DNA before the bisulfite treatment. This PCR undeniably shows that the reaction conditions as standardized are optimal and applicable to differentiate between methylated and unmethylated DNA in different samples (see Chapter 5).

4.3.1.2. *RASSF1A* promoter sequence

The results for *RASSF1A* MSP as obtained by me in the laboratory of Dr Dammann are given in Figure 4.9. The PCR products obtained with the methylated-specific primers and the unmethylated-specific primers for the *RASSF1A* promoter of one patient is shown next to each other, as well as the results obtained for normal and tumour tissue of the same patient.

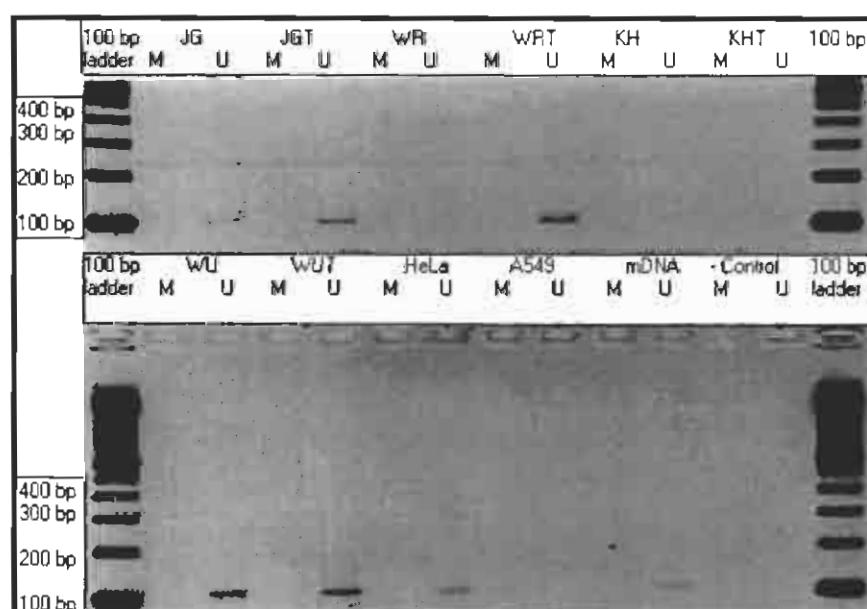


Figure 4.9. Gel electrophoresis of Methylation-specific PCR for the *RASSF1A* promoter sequence as performed in Germany. A 100 bp DNA molecular weight marker, a negative DNA control and unmethylated controls (HeLa) were included. A549 and mDNA are samples irrelevant to this study. Each patient's normal tissue is left (e.g. JG) and the tumour tissue on the right (e.g. JGT).

The amplicons expected for the methylated DNA are 93 bp and for the unmethylated DNA, 105 bp. Comparing the results given in Figure 4.9, it is evident that the DNA isolated from all the normal tissues, as well as the tumour tissue, were unmethylated.

As was the case for the *p16* promoter, the optimal reaction conditions for our laboratory were established by performing various temperature gradients for each set of primers, as well as experiments to determine the optimal $MgCl_2$ concentration for these primer pairs. The optimal annealing temperatures for the different primer sets were subsequently determined as 62°C for the unmethylated-specific primers and 67°C for the methylated-specific primers.

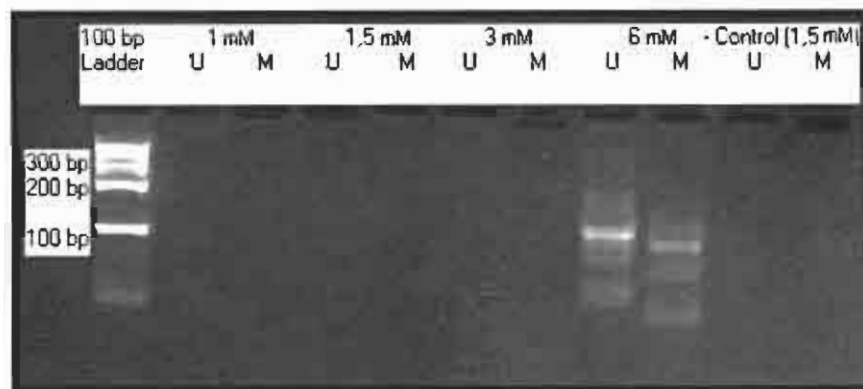


Figure 4.10. Gel electrophoresis of *RASSF1A* Methylation-specific PCR with various $MgCl_2$ concentrations (1). Lane 1: 100 bp Molecular weight marker; lane 2 and 3: 1 mM $MgCl_2$ (U/M); 4 and 5: 1.5 mM $MgCl_2$ (U/M); 6 and 7: 3 mM $MgCl_2$ (U/M); 8 and 9: 6 mM $MgCl_2$ (U/M); and lane 10 and 11: negative controls at 1.5 mM $MgCl_2$ (U/M).

Although there were amplicons of the expected size produced at 6 mM $MgCl_2$, there was a lot of non-specific amplification present (Figure 4.10). This could be a result of annealing temperatures being too high or too low, suboptimal $MgCl_2$ concentrations or too many amplification cycles. An increase in $MgCl_2$ concentration did not increase specificity of the amplification process, and I, therefore, repeated the $MgCl_2$ concentration gradient at lower concentrations.

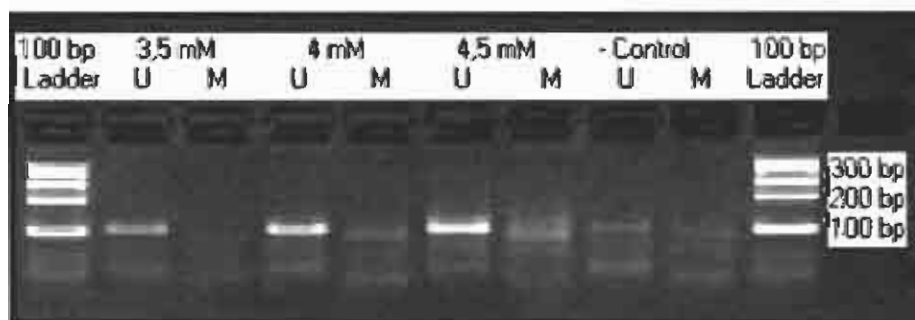


Figure 4.11. Gel electrophoresis of *RASSF1A* Methylation-specific PCR with various $MgCl_2$ concentrations (2). Lane 1 and 10: 100 bp Molecular weight marker. Lane 2 and 3: 3.5 mM $MgCl_2$ (U/M); 4 and 5: 4 mM $MgCl_2$ (U/M); 6 and 7: 4.5 mM $MgCl_2$ (U/M); and lane 8 and 9: negative controls with 4 mM $MgCl_2$ (U/M).

In Figure 4.11 the results are given for *RASSF1A* MSP with lower MgCl_2 concentrations. It is clear that 4 mM MgCl_2 is the optimal concentration for amplification of the unmethylated-specific primers. There was, however, no MgCl_2 concentration where a specific, clear amplicon for the methylated-specific primers was produced, although the best result was obtained at 4 mM. Different formamide concentrations were subsequently used to increase specificity. Although the addition of 1 μl formamide instead of 0.5 μl resulted in no amplification in the presence of either the methylated-specific or the unmethylated-specific primers, it was concluded that 0.5 μl formamide is the optimal volume of formamide per 25 μl reaction.

The only remaining reagents that could be altered to amplify the methylated-specific amplicon were the DNA polymerase and its buffer. Therefore, two other polymerases, PhusionTM High-fidelity DNA polymerase (Finnzymes) and Biotools DNA polymerase (Biotools) were used at the optimal conditions determined with the Promega *Taq* polymerase. The Biotools polymerase is supplied with a 10 x PCR buffer containing ammonium sulphate.

The PhusionTM DNA polymerase did not produce any amplification, and although the Biotools DNA polymerase amplified the unmethylated-specific amplicon as efficient as the Promega *Taq* polymerase, it showed almost no visible amplification of the methylated-specific amplicon.

The results obtained with the various modifications to the *RASSF1A* MSP amplification method suggest that it is a very sensitive method. Although very good results were obtained for the unmethylated-specific primer set, however, specificity and yield with the methylated-specific primer set can still be improved by trying different reagents, methods and conditions. It was, therefore, decided to continue with *p16* in various applications (see Chapter 5).

4.3.2. COBRA assay

I performed bisulfite treatment and the subsequent COBRA assay for Satellite 2 DNA in the laboratory of Dr Dammann, using their reagents, PCR machine and standardized protocol (Figure 4.12). The primer sequences used for this assay were designed by him, and are confidential. In Figure 4.12 the results are given for the Sat 2 PCR amplification as obtained by me in Germany.

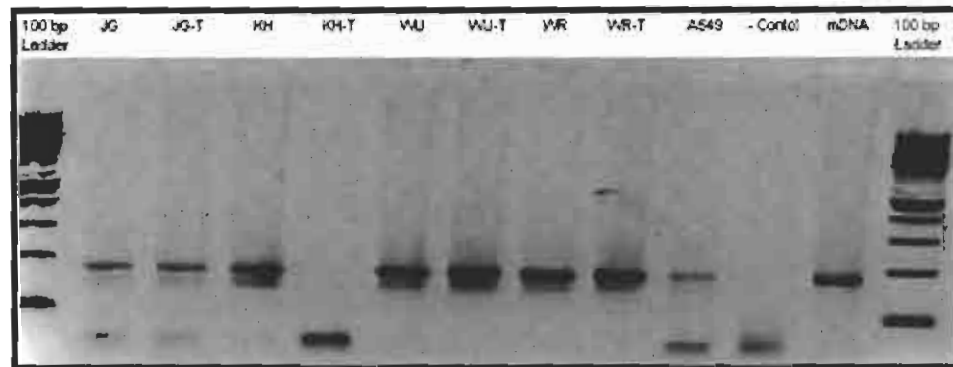


Figure 4.12. Gel electrophoresis of the PCR amplification of the Sat 2 DNA sequence. Lane 1 and 13: 100 bp Molecular weight marker; lane 2 to 8: Patient samples, normal and tumour tissue; lane 9 and 11: methylated controls; lane 10: a DNA negative control. Each patient's normal tissue is left (e.g. JG) and the tumour tissue on the right (e.g. JGT).

After the amplification of the relevant DNA sequence, the success of the PCR was first verified with gel electrophoresis. The absence of a product for sample KH-T could be a result of extremely low template DNA concentration. This amplified DNA sequences were subsequently digested with a specific restriction enzyme, in this case *Taq* I.

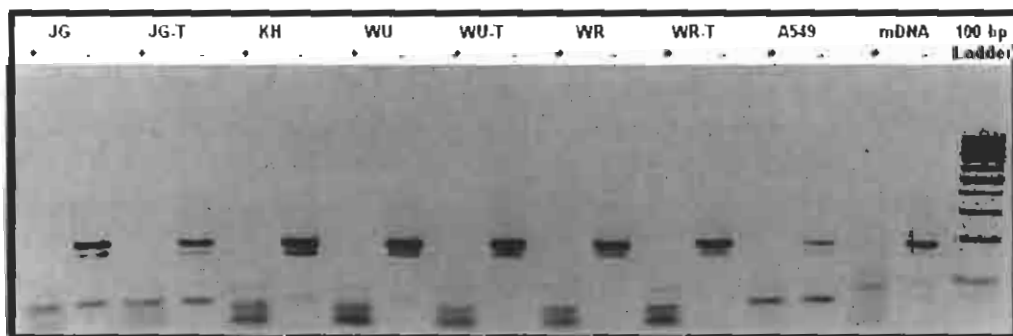


Figure 4.13. *Taq* I digested PCR amplicons of the Sat 2 DNA sequence. Lane 1 to 14: Patient samples, normal and tumour tissue (+ is the sample digested with *Taq* I, - is the undigested control sample). Lane 15 to 18: methylated controls; lane 19: 100 bp Molecular weight marker. Each patient's normal tissue is left (e.g. JG) and the tumour tissue on the right (e.g. JGT).

The absence of any residual DNA after digestion with the enzyme (Figure 4.13) confirms that all the samples were methylated in their Sat 2 sequence. If there were any remaining amplicons after the restriction enzyme digestion, it would indicate that the restriction site had been removed, and would, therefore, indicate an unmethylated state.

The presence of methylated CpG sites in the Sat 2 sequence indicates that there was no hypomethylation of the satellite sequence 2 present. If there were unmethylated sites present, it may have indicated the hypomethylated state of the Sat 2 DNA, which is often associated with genomic instability and loss of heterozygosity (see section 2.5.2). The COBRA assay has significant potential for the evaluation of the methylation status of specific CpG sites (methylation variable positions or MVPs), although there are several restrictions which inhibits the assay.

In this study it was decided to focus on the MSP assay instead of the COBRA assay, since it provides more information about a specified sequence, and because it has fewer limitations, e.g. no specific restriction enzyme sites has to be present, more than one specific CpG site can be analysed with one assay, etc.

4.4. SUMMARY

The aim of this study was to establish and optimize the bisulfite treatment of DNA and the subsequent methylation-specific PCR assay. I used the *p16* and *RASSF1A* promoter CpG islands as template due to their well-established use in various laboratories in several countries, and their noted significance in several malignant growths (See section 2.5.1).

This experiment was performed successfully by me in Germany; however, it did not immediately provide satisfactory results in our laboratory. This may have been due to different laboratory conditions, instruments or template used. Following several alterations and adaptations, the method was, however, established and optimized with satisfactory results for the *p16* promoter CpG island sequence and could, therefore, be subsequently used for various applications. The *RASSF1A* MSP assay was established and results were obtained with the unmethylated-specific primer set, although further optimization is necessary for the methylated-specific primer set due to an unknown factor.

The MSP method has tremendous potential, and can easily be used for high throughput analysis once it is established and optimized in a laboratory.

CHAPTER 5

DRAFT OF ARTICLE

The optimized MSP assay was applied to DNA isolated from various tissues, i.e. whole blood, plasma and paraffin-embedded tumour tissue. The results of these applications are presented in the form of the first draft of a research paper prepared for submission to *Cancer Letters*^{*} (*Elsevier journals*).

This research paper was prepared according to the "Guide for authors" - available online at <http://intl.elsevierhealth.com/journals/cale/>.

^{*} The first author was responsible for all contributions to this article, except for the isolation of the free-circulating DNA from the plasma (section 2.9).

DNA methylation status of the *p16* promoter sequence in blood and tumour tissue

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Abstract

Aberrant methylation of CpG islands located in tumour-suppressor gene promoters causes gene silencing, and contributes to tumorigenesis. In this study the authors analysed the *p16* methylation status of 13 control individuals and 10 diagnosed breast cancer patients with the methylation-specific PCR assay using DNA isolated from whole blood, plasma and paraffin-embedded tumour tissue. In the control individuals all the *p16* promoters were unmethylated for both the genomic DNA and the free-circulating DNA. The *p16* promoter methylation status of the patient samples was diverse, and both methylated- and unmethylated-specific products were observed for the tumour DNA sample.

Key words: DNA methylation; *p16* promoter; methylation-specific PCR; free-circulating DNA

1. Introduction

During the development and lifetime of an organism, epigenetic alterations occur, resulting in meiotically and mitotically heritable changes in gene expression or function, without changing the specific nucleotide sequence of the genes involved [1-4].

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Epigenetic modifications, unlike genetic changes, occur gradually, e.g. global methylation reduction during aging of cells [3]. This, as well as the fact that only 15 % of cancers have a familial connection, implies that genomic alterations in cancers can not solely be viewed as a result of genetic aberrations, but that epigenetic alterations greatly influence the establishment of a malignant phenotype [5,6]. DNA methylation is a well-studied epigenetic alteration which primarily sustains normal gene expression and genome stability [7]. Changes in the normal methylation profile influences cancer related gene transcription, resulting in genomic instability, predisposition of cells into precancerous stage and abnormal cell cycle regulation [6,8]. CpG islands are clustered CpG sites, and it has been estimated that approximately 40 % are found in promoter regions [2,3,9]. Cancer occurs when the genome is affected by disease or other adverse factors and it also involves numerous and profound changes in gene expression programming [9,10].

There is a constant search for better, more accurate, faster and more definitive methods to determine the disease state of each patient, and to monitor the recurrence during treatment. Biomarkers are often used in these methods and can be, among others, genetic or epigenetic of origin, since DNA methylation markers have great disease detection potential, maybe even more than the existing markers for disease classification [6,11]. Several human cancers show clustered gene-associated hypermethylation, which leads to the epigenetically mediated silencing of these genes. In some cases there is a direct correlation between promoter inactivation and CpG island hypermethylation [12-14]. The p16 protein is a D-type cyclin dependent kinase 4 (cdk4) inhibitor and is involved in cell growth and differentiation regulation. The *p16^{INK4a}* gene is also frequently inactivated in cancer [15,16]. Homozygous deletion generally inactivates *p16*, and this inactivation is supported by methylation, since numerous cancers show aberrant *p16*

methylation and breast tumours display *de novo* methylation in about 23 to 31 % of cases [15,17,18]. *p16* inactivation is generally associated with higher-stage disease and limitless replicative potential for cells.

In this study, we analysed the methylation profile of the *p16* promoter CpG island in control individuals and in diagnosed breast cancer patients in order to determine the relationship of methylation profiles between DNA isolated from whole blood, plasma and paraffin-embedded tumour tissue.

2. Materials and methods

2.1. DNA isolation

Ethical approval for this study was obtained from the Ethical Committee of the North-West University (# 05M12). Patient material was obtained after written informed consent was given by the patients. All control individuals gave written informed consent before blood samples were obtained. Genomic DNA was isolated from whole blood with the Wizard Genomic DNA purification kit (Promega # A1120). Free-circulating DNA was isolated from plasma with the QIAamp DNA Blood Mini Kit (QIAGEN, # 51104), and DNA was isolated from ± 25 slices of paraffin-embedded tumour tissue with the E.Z.N.A.[®] Tissue DNA mini kit (PEQLAB Biotechnologie GmbH # 12-3396-01).

2.2. Methylation-specific PCR (MSP)

Approximately 1.5 μ g genomic DNA (18 μ l free-circulating DNA) was treated with sodium bisulfite for 16 hours (5 hours for free-circulating DNA) as published by Dammann, *et al.* [19], and was resuspended in 50 μ l ddH₂O. 10 μ l bisulfite-modified DNA was subsequently used for PCR with 1 x MgCl₂-free PCR buffer, 3 mM MgCl₂, 0.8 mM dNTPs,

10 pM of each primer [20], 1 µl Formamide and 2.5 U *Taq* polymerase (Promega) in a 25 µl hot-start reaction. The annealing temperatures for the unmethylated-specific primer set was determined at 62°C and for the methylated-specific primer set at 65°C with 40 cycles. In this assay a commercially available methylated standard (Zymo research) was used as a positive control and DNA isolated from a cultured HepG2 cell line as an unmethylated control [21].

2.3. Quantification*

Following analysis of the PCR product with agarose gel electrophoresis, relative quantification of the levels of DNA methylation was performed. Using the Syngene ChemiGenius Bio-Imaging System (Genetools 3.06.04 software), various electrophoresis lanes was specified and specific values was allocated to specific fragments as reference values for subsequent quantification. Once a specific fragment was assigned the calibrated quantity, specific peaks were selected to quantify specific amplicons relative to the calibrated peak. These peaks were measured to have a constant base-width of 5 mm. The same area was defined for every sample, whether a visible amplicon was present or not, in order to determine the methylation score of every DNA sample and minimize background variations. Once every peak was defined, all the relative quantities of those peaks were used to calculate the methylation scores of the samples.

2.4. Methylation score

The methylation score of each DNA sample was calculated as follow:

$$[1 - (U/M)] \quad (1)$$

* A more detailed explanation of this quantification method is available in Appendix G.

Equation (1) calculated the relative methylation status of each sample, and the result was either positive (more methylated) or negative (more unmethylated). The methylation score is more of a qualitative indication of the level of methylation of a DNA fragment rather than a quantitative measure, because although all samples start with the same amount of DNA (1.5 µg), the amount of DNA after bisulfite treatment may vary [22]. This is evident from several publications, where a similar degree of variation in the intensities of the amplification products is evident [23]. This calculation is, therefore, useful in samples where amplicons are obtained with both sets of primers. Similar calculations, although based on real-time PCR, have been used to determine the methylation status of DNA sequences [24].

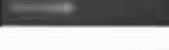
3. Results

3.1. Methylation analysis of the p16 promoter sequence in control individuals

The methylation status of the *p16* promoter sequence was ascertained by MSP using DNA isolated from whole blood as well as free-circulating DNA obtained from 13 control individuals. The expected DNA fragment of 151 bp was amplified with the unmethylated-specific primer set from all the whole blood DNA samples obtained from the control subjects. No DNA was amplified with the methylated-specific primer set from these samples. The methylation score calculated for all of these samples provided negative values, which correspond with the value obtained for the DNA isolated from HepG2 cells. This means the *p16* promoter sequence is not methylated in all of these samples. A similar result was obtained for the free-circulating DNA samples subjected to MSP analysis. In all the cases where both the whole blood and free-circulating samples were available for the same individual, the *p16* promoter sequence was unmethylated in both samples. However, for most of the free-circulating DNA samples slightly lower values

were obtained than for the whole blood DNA samples, although this may be explained by the lower DNA concentrations of the free-circulating DNA. Even so, it indicates that the *p16* promoter sequence in the free circulating DNA is unmethylated.

Table 1
MSP analysis of control individuals: DNA from whole blood and plasma

Control			<i>p16</i>					
Samples	Gender	Age	Whole blood			Free circulating DNA		
			U	M	Meth. Score	U	M	Meth. Score
Methylated std.*					0.43			n.a.
HepG2*					-2.25			n.a.
120406_03	F	20		n.a.				-0.08
231105_01	F	21		n.a.				-0.53
120406_01	F	21		n.a.				-0.24
270705_04	F	22			-0.35			n.a.
231105_07	F	22			-0.54			-0.40
080206_03	F	22			-0.79			n.a.
231105_03	F	23			-2.58			n.a.
270705_03	F	49			-2.65			n.a.
231105_02	M	21			-1.86			-0.15
220705_01	M	22			-0.85			n.a.
231105_05	M	23			-2.27			n.a.
200706_01	M	55			-2.38			n.a.
231105_04	M	59			-1.50			-0.34

n.a. – Not available

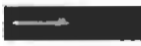
* These samples are included in this table as an illustration of the methylated and unmethylated standards used in this study.

3.2. Methylation analysis of the *p16* promoter sequence in clinically diagnosed breast cancer patients

The methylation status of the *p16* promoter sequence in 10 clinically diagnosed breast cancer patients was determined in DNA isolated from whole blood, plasma and a paraffin-embedded tumour tissue sample.

Table 2

MSP analysis of breast cancer patients: DNA from whole blood, plasma and paraffin-embedded tumour tissue

Patients				p16							
Samples	Gender	Age	Receptor status	Whole blood			Free circulating DNA			Paraffin-embedded tumour tissue	
				U	M	Meth. Score	U	M	Meth. Score		Meth. Score
EV	F	40	ER+,PR+,HER-		n.a.				-1.76		n.a.
MSJ	F	41	ER+++,PR+,HER+			-0.69			0.04		n.a.
ESDV	F	41	ER++,PR-,HER-		n.a.				-1.82		n.a.
EJP	F	50	ER++,PR++,HER-		n.a.				-9.26		n.a.
DJY	F	55	ER+/-,PR-,HER-			0.53			-0.57		n.a.
SJM	F	62	ER+++,PR++,HER-		n.a.				-2.28		n.a.
AES	F	64	ER+,PR+			-0.91			0.31		-0.26
MMB	F	69	ER+,PR-			-3.22			-2.90		n.a.
AJJ	F	71	ER+++,PR+++,HER-			-1.89			-5.33		n.a.
AJF	F	74	ER-,PR-			0.11			-4.44		n.a.

n.a. – Not available; ER – Estrogen receptor; PR – Progesterone receptor; HER – Her2/neu receptor

From Table 2 it is evident that a positive methylation score was calculated for only two of the six whole blood samples. These values are relatively small, which means that the *p16* gene promoter was only slightly more methylated than unmethylated. This sequence was either slightly more unmethylated, or completely unmethylated for the other four whole

blood samples as is evident from the presence of either an amplicon for the unmethylated-specific primer set or the presence of amplicons for both primer sets. The methylation score was relatively small for two of the ten free-circulating DNA samples while for the other eight samples relatively large negative values were calculated. In the case of the only available tumour tissue sample a relatively small negative methylation score is evident in both the genomic DNA and the DNA isolated from the waxed tissue. A small positive value was calculated for the free circulating DNA obtained from this patient.

4. Discussion

The methylation status of the *p16* promoter sequence for 13 control individuals and 10 clinically diagnosed breast cancer patients was analysed using the MSP assay. Among the 16 control and patient whole blood DNA samples, 14 were unmethylated to some extent, and of the 16 free-circulating control and patient DNA samples, the *p16* promoter sequence of only two were methylated. It is to be expected that all of the *p16* promoter sequences from the normal individuals should be unmethylated, or only slightly methylated since it is evident from the literature that this sequence is hypermethylated only in malignant tissues, e.g. breast, liver, colon, stomach and lung cancer [13,14]. In line with this, it would be expected that more of the patient DNA samples should be methylated to some degree, since aberrant *p16* methylation has been noted at a frequency of 23 to 30 % in DNA isolated from breast cancer tissue [15,17,18]. This is evident from the results provided in Table 2, since the appropriate DNA fragment was amplified in several cases when DNA was isolated from whole blood. Since this was not the case for any of the control samples, it means that, at least in these cases, the methylation status of the *p16* promoter sequence is reflected in the genomic DNA. Obviously, this observation needs to be confirmed for many more samples.

The results in Table 1 suggest that free-circulating DNA and genomic DNA may have the same methylation status, although the level of the methylation scores varied. However, when the results provided in Table 2 are taken into account, this conclusion is challenged. The methylation status of the *p16* promoter sequence differed between the tissues in four of the six samples. This may suggest an alteration in the methylation status of either the genomic DNA or the free-circulating DNA of patients, which was not reflected in the other tissues. This observation may indicate that the free-circulating DNA represents the methylation status of the *p16* promoter in the tumour tissue, which is to be expected if the hypothesis that free-circulating DNA is derived from the tumour is accurate [6]. If the free-circulating DNA is indeed an accurate reflection of the methylation status of this gene in the tumour tissue, it confirms the phenomenon of tissue specific methylation aberrations in malignancies [25].

However, when the methylation status of *p16* in the tumour tissue sample is taken into account, this argument is challenged, seeing that the methylation status, as determined in the genomic DNA, reflects the methylation status of the tumour tissue DNA, while the free-circulating DNA does not. In light of the hypothesis that free-circulating DNA is derived from the tumour, this observation is unexpected. It is, however, possible that the free-circulating DNA was derived from both normal and tumour cells, or that the tumour is heterogeneous with genetically different subclones [25]. This hypothesis is supported by the presence of PCR products with both sets of primers for the free-circulating DNA sample of patient AES, and since the whole blood DNA sample of patient AES also has a methylation score of only -0.91, while the unmethylated standard has a score of -2.25, it is presumable that there may be a degree of hypermethylation present, although not visible as an amplicon. Four of the six patient whole blood DNA samples presented

amplicons for both sets of primers, which would indeed support the findings of other publications that there is a degree of hypermethylation of the *p16* promoter sequence in breast cancer patients, since none of the control individuals presented this result.

As illustrated in fig. 1, the majority of calculated methylation scores, for both the genomic DNA samples, and the free-circulating DNA samples, were observed between 0 and -1. There is, however, some concern about the cut off point between significant and insignificant methylation scores, for example patient MSJ in Table 2, where a methylation score of 0.035 was observed for the free-circulating DNA sample. For this sample, there was no visible amplicon for either the methylation-specific primer set, or the unmethylated-specific primer set, which questions the significance of this sample's methylation score. However, for the free-circulating DNA sample of control individual 120406_03 in Table 1, a methylation score of -0.078 was calculated and there was a visible amplicon for the unmethylated-specific primer set. It is, therefore, proposed that potential borderline values of <-0.05 and >0.05 may be useful to indicate the significance of a specific score. This suggestion is based on a small number of samples, and it is advisable to use a greater number of samples to determine these parameters.

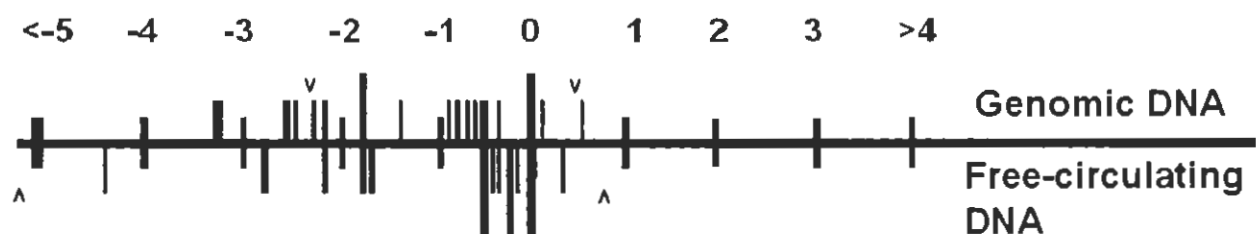


Fig. 1. Illustration of the distribution of the methylation scores. The shortest lines are the two samples lower than 0.09, the medium length lines are one sample of that score, and the longest lines indicates the presence of two samples of that specific score. The arrowheads indicate the methylation score of the methylated and unmethylated standards.

From the patients' clinicopathological features, it was observed that patients with a negative methylation score for the whole blood DNA, but a positive methylation score for the free-circulating DNA, also had a positive Estrogen Receptor and Progesterone Receptor status. Patients with a positive methylation score for the whole blood DNA, and a negative score for the free-circulating DNA, however, had a negative ER and PR status, while patients with a negative score for both blood samples had a positive ER and positive or negative PR status. Although a limited number of patient samples were used for this study, the result obtained indicate much the same observations as those found in the literature, for instance, if the average age of the patients with methylated whole blood DNA samples is calculated, a mean of 64 years is observed. The average age calculated for the patients with unmethylated whole blood DNA samples was 61 years. This correlates with published findings of 63 and 59 years, respectively [23]. 20 % of the patient free-circulating DNA samples were methylated, which also correlates with various published findings, which varies between 10 to 14 % [25]. However, due to the limited number of samples available, no definitive conclusion can be made from these observations, and more samples are needed to investigate possible significance to this observation, and to determine the association between all the relevant parameters in the South African context.

The results of our study confirm the applicability of the methylation-specific PCR assay to measure the methylation status of the *p16* promoter sequence in various sample types, including genomic DNA, free-circulating DNA and DNA isolated from paraffin-embedded tumour tissue, and the different associations of these tissues have several potential applications, although further studies with more samples are necessary to resolve the remaining questions.

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CHAPTER 6

SUMMARY AND CONCLUSION

Alterations in the human genome are related to changes in gene expression and the onset of cancer. DNA methylation is one of the most frequent and important epigenetic changes in the human genome (Früwald and Plass, 2002; Das and Singal, 2004; Toyooka and Shimizu, 2004). CpG islands are regions with high concentrations of normally unmethylated CpG sites, which have numerous important qualities and functions, and are frequently present in promoter sequences of genes (Robertson and Wolffe, 2000). DNA methylation influences the regulation of gene expression to a great extent and the associated repressive heterochromatin assembly are among the most important mechanisms for stable gene inactivation in a heritable form (Robertson and Wolffe, 2000). DNA methylation may also increase genome stability by inhibiting homologous recombination between large areas of repetitive DNA and it is associated with X-inactivation in females (Früwald and Plass, 2002).

Abnormal hypermethylation to some extent has been identified in all studied human cancers. It has been stated that genomic alterations in cancers can not solely be viewed as a result of genetic aberrations, but that epigenetic alterations greatly influence the establishment of a malignant phenotype (Plass, 2002; Verma and Manne, 2006). It appears that malignant capability increases with the number of genes showing hypermethylation, although the responsible mechanisms for this gene targeting are still unresolved. DNA methyltransferases may, however, be recruited by certain proteins (Esteller, 2005). In breast cancer there are two methylation profile transformations which arise: 1) global hypomethylation and 2) regional hypermethylation of certain genes. Although there are a number of mechanisms to protect the CpG islands against hypermethylation, hypermethylation is much more frequent than hypomethylation in cancer (Das and Singal, 2004; Szyf, *et al.* 2004).

In the constant search for better, faster and more definitive methods to determine and monitor the disease state of each patient, biomarkers are often used and can be,

among others, genetic or epigenetic of origin (Laird, 2005; Verma and Manne, 2006). Different cancer types show different profiles of methylated tumour-related genes, and these methylation signatures have been associated with different stages in breast cancer (Szyf, *et al.*, 2004). Because of the strong association between methylation and gene expression regulation, it is important to have a gene expression signature of each cancer to understand the cancer pathogenesis, and have a fixed representation of the transformed genome in these cancers as a potential biomarker. Characteristic stable methylation profiles or methylotypes are also emerging for various cancers, and DNA methylation is the most accessible epigenetic alteration to study when trying to identify epigenetic signatures, because of its stability in the DNA (Esteller, 2003; Nephew and Huang, 2003; Das and Singal, 2004; Szyf, *et al.* 2004; Murell, *et al.*, 2005). DNA methylation profiles can, therefore, be used as molecular markers for both classification and detection in cancer diagnostics (Laird, 2005).

Genotyping of individual tumours with standard pathological procedures is becoming a necessity due to the wide diversity of genetic abnormalities in cancer (Bertram, 2001). By using molecular markers and methylation profiles, tumours can be subclassified, and tumour types, subtypes and possibly the response to chemotherapeutic agents and survival can be distinguished from each other. Early detection and even prediction of cancer or tumour development may be possible due to changes in methylation patterns, which sometimes precede malignant changes (Früwald and Plass, 2002; Das and Singal, 2004).

The aim of the study was to establish and optimize a method in our laboratory to measure the DNA methylation status of specific human genes. Methylation-specific PCR was used to determine the methylation status of the *p16* and *RASSF1A* promoter CpG islands (detailed in section 2.5.1) since this is a sensitive, efficient and relatively fast method with high-throughput potential and the ability to examine almost any CpG-rich sequence's methylation status (Herman, *et al.* 1996, section 4.1.1). This method is frequently used worldwide to identify aberrant methylation in numerous genes and in various tissue types (Wong, *et al.* 1999; An, *et al.* 2002; Laird, 2003; Tong and Lo, 2006). The method is based on the sodium bisulfite conversion of unmethylated cytosines to uracils, without affecting 5-methylcytosine

(Laird, 2003; Toyooka and Shimizu, 2004). PCR is then performed, using two sets of primers, a specific primer sequence for each of the two methylation-forms of the gene.

Since the aim was to determine the methylation levels of DNA in various tissues, it was important to isolate good quality DNA from these tissues. For this reason several methods were evaluated to isolate genomic DNA and the average yield and purity of the DNA isolated with each method was subsequently calculated and compared to each other to determine which method was most suitable for each application (see Table 3.7).

It was concluded that the Wizard[®] Genomic DNA purification kit was the best method to isolate genomic DNA from smaller volumes of whole blood, while larger amounts of DNA is better isolated with the Möhr method (detailed in sections 3.8.1 and 3.8.2). The Wizard[®] kit is very quick and easy, and avoids exposure of DNA to hazardous chemicals. It produces high concentrations good quality DNA from very small sample volumes. The DNA can immediately be used for various applications with no additional purification steps. Since phenol:chloroform extraction is not suitable for paraffin-embedded tumour tissue samples, the E.Z.N.A.[®] Tissue DNA mini kit was more suited for tumour tissue DNA isolation in this study (detailed in section 3.8.4). Although only one paraffin-embedded sample was available, the method was successful, even though the isolated DNA concentration of 31.5 ng/μl (purity of 1.75) was slightly lower than the optimal concentration possible with the kit (E.Z.N.A.[®] Tissue DNA mini kit – Instruction manual).

The successful isolation of pure DNA from the tumour tissue was subsequently confirmed with PCR amplification of the *p16* and *RASSF1A* promoter sequences (see Figures 3.3 and 3.4), although the *RASSF1A* amplicon was larger than the amplicon obtained with the DNA isolated from the blood. Sequencing of this amplicon did not resolve its origin and it was, therefore, proposed that the tumour tissue DNA contains a tumour and/or tissue specific isoform of the *RASSF1A* gene, or a mutated form, as it is a malignant growth (see Figure 3.6 and Appendix F). Another alternative explanation is that the amplified sequence is a non-specific DNA fragment. No

publications of similar cases could be found. The tumour derived DNA was, nonetheless, treated with bisulfite and the *p16* MSP amplification was successful (see Chapter 5, Table 2 and Figure 6.1). The Wizard[®] Genomic DNA purification kit was further used for the isolation of DNA from various cell cultures. The method, just as with the whole blood samples, produced high quantities DNA from the various cell cultures used (see Table 3.6), and it was, therefore, decided to continue the DNA isolations from the cell cultures with this kit.

In the initial experiments, the MSP assay was performed according to the original method described by Herman, *et al.* (1996), treating the DNA with sodium bisulfite, and after purification using the two primer sets for the *p16* promoter as published to perform PCR amplification. The results were, however, not satisfactory. During my study in Germany I applied the method (as optimized in their laboratory) with much success and I was able to produce very good results (see Figure 4.5 and 4.9) using their reagents and PCR machine. The method did unfortunately not produce the same quality of results when repeated in our laboratory with local reagents, consequently several alterations were necessary to obtain satisfactory results. This was done by altering the annealing temperatures, changing the MgCl₂ concentration in the PCR reactions, using different DNA polymerases, etc. (see Figures 4.6 - 4.8). The method was eventually established and optimized with satisfactory results for the *p16* promoter CpG island sequence and was used to study DNA methylation in various breast cancer patients (see Figure 6.1).

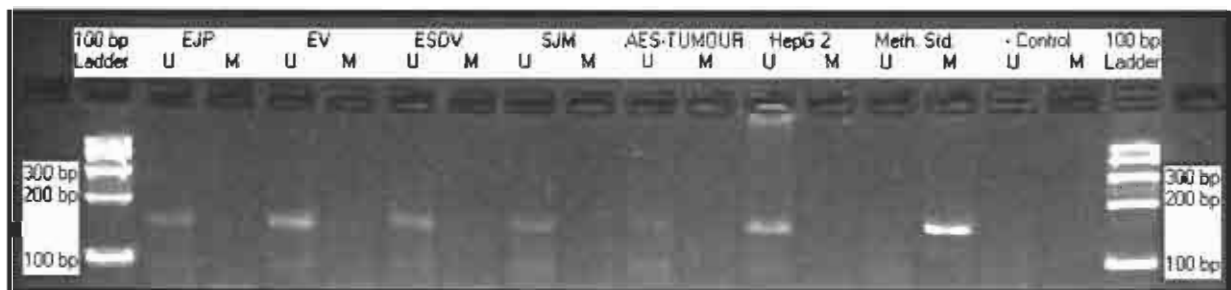


Figure 6.1. MSP analysis of various breast cancer patient samples. Lane 1 and 18: 100 bp Molecular weight marker. Lane 2 to 11: Samples (U/M); lane 12 and 13: Unmethylated HepG2 DNA (U/M); lane 14 and 15: Methylated Standard (M/U); and lane 16 and 17: negative controls with 4 mM MgCl₂ (U/M).

The RASSF1A MSP assay was also modified extensively, but satisfactory results for subsequent application could not be achieved (see Figures 4.10 and 4.11). The

unmethylated-specific primer set gave specific, accurate amplification of the expected amplicon, but in spite of numerous alterations and efforts, the methylation-specific primer set continue to lack specificity. The cause for this non-specific amplification is unknown, but may lie in the design of the primer pair. Since this application of the MSP assay is used frequently in various laboratories, it is possible that I have not yet identified the optimal conditions for our laboratory. It is, however, proposed that the two primer sets for this assay be reviewed, and if necessary, redesigned. It is further proposed that more reagents from other suppliers are obtained and tested.

The established *p16* promoter CpG island methylation analysis was applied to various types of samples from both diagnosed breast cancer patients and control individuals. The samples included genomic DNA isolated from whole blood, plasma and paraffin-embedded tumour tissue.

Several publications reported that tumour DNA is released into the circulation and that this free-circulating DNA can potentially be used as a diagnostic tool. It was further reported that alterations in methylation profiles could be detected in free-circulating DNA (An, *et al.* 2002; Johnson and Lo, 2002; Laird, 2003; Tong and Lo, 2006; Verma and Manne, 2006). This would implicate that the free-circulating DNA can be used as a non-invasive representative of the biological composition of the tumour. Laird (2003) hypothesised that the presence of DNA methylation markers in plasma or serum simply indicates "...the release of sufficient amounts of tumour DNA into the bloodstream, which is likely to be correlated with invasiveness. For this purpose, any tumour-specific marker would be efficient and its presence in the tumour itself would not necessarily be correlated with clinical outcome". Others, however, stated that an increase in free-circulating DNA is inversely linked with prognosis (Silva, *et al.* 1999).

The methylation status of the *p16* promoter sequence for the 13 control persons and 10 clinically diagnosed breast cancer patients was analysed using the MSP assay. DNA was isolated from various tissues, as mentioned above. Among the 16 patient and control whole blood DNA samples, 14 were unmethylated to some extent, and of the 16 patient and control free-circulating DNA samples, the *p16* promoter sequence of only two were methylated. As seen in Chapter 5, Table 1, all of the DNA samples

from the normal individuals were unmethylated for the *p16* promoter, which is to be expected, since it is evident from the literature that this sequence is hypermethylated in malignant tissue (Esteller, 2005; Miyamoto and Ushijima, 2005). In line with this, it would be expected that more of the patient DNA samples should be methylated to some degree, since aberrant *p16* methylation has been noted to be present with a frequency of 23 to 30 % in DNA isolated from breast cancer tissue (Silva, *et al.* 1999; Ferguson and Nass, 2000; Tong and Lo, 2006).

It was observed that free-circulating DNA had the same methylation status as genomic DNA for the normal individuals (Chapter 5, Table 1), but four of the six patient samples had an opposite *p16* DNA methylation status for both tissues. This may suggest that there is an alteration in the methylation status of either the genomic DNA or the free-circulating DNA of patients, which is not reflected in the other. This variation may also indicate that the free-circulating DNA represents the methylation status of the *p16* promoter in the tumour tissue, which is to be expected if the hypothesis that free-circulating DNA is derived from the tumour is accurate, although the tumour tissue sample contradicted this hypothesis (Chapter 5, Table 2). In line with the hypothesis put forward by Fleischhacker (Fleischhacker and Schmidt, 2006), it was concluded that the tumour was epigenetically heterogeneous, since PCR products were obtained with both sets of primers for the free-circulating DNA and tumour tissue samples.

The whole blood DNA sample of patient AES also have a methylation score of only -0.906, while the unmethylated standard has a score of -2.245, therefore, it is presumable that there may be a degree of hypermethylation present, although not visible as an amplicon. Since four of the six patient whole blood DNA samples also presented amplicons for both sets of primers, it would indeed support the findings of other publications that there is a degree of hypermethylation of the *p16* promoter sequence in breast cancer patients, since none of the control individuals presented this result. It was observed that for all the patients with a positive ER receptor status, only 20 % of the whole blood samples were methylated, while for the negative ER receptor status, 100 % were methylated. The patient whole blood samples with a positive PR receptor status presented no methylated samples, while 66 % of the samples with a negative PR receptor status were methylated. It is, therefore,

proposed that there may be a potential correlation between the expression of the ER and PR receptors and the methylation status of specific gene promoters.

The majority of calculated methylation scores, for both the genomic DNA samples, and the free-circulating DNA samples, were observed between 0 and -1 (Chapter 5, Figure 1). The methylation score is, however, more of a qualitative indication of the level of methylation of a DNA fragment than a quantitative measure (Chapter 5, section 2.4). There was some concern about the borderline between significant and insignificant methylation scores, and it was proposed that potential borderline values of <-0.05 and >0.05 may be useful to indicate significance of a specific score. This suggestion was, however, based on a small number of samples, and it would be advisable to use a greater number of samples to determine these parameters more reliably.

It is also proposed that a series of DNA samples with known increments of methylation increase may be useful to examine the accuracy of the methylation score determination, and to create a fixed quantity as a reference point for further methylation status evaluations. Although a limited number of patient samples were used in this study, the results obtained correlate well with those found in the literature and, therefore, validate to a large degree the results obtained with MSP in our laboratory, e.g. the average ages of the patients in correlation to their methylation status, as well as the percentage *p16* promoter methylation of the patient free-DNA samples (Chapter 5, section 4).

The aim of this study was to establish and optimize a method to measure gene-specific DNA methylation. Methylation-specific PCR was established and applied to analyse methylation of the *p16* and *RASSF1A* promoter CpG islands. We conclude that the method has tremendous potential to be further optimized and applied in numerous applications. These might include the investigation of other genes whose methylation status has not yet clearly been identified, analysis of various methylation profiles of other types of cancer (including cancers significant in South Africa) and the establishment of methylation profiles for various inherited metabolic diseases with associated carcinogenesis, as this has not yet been described.

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APPENDIX A.

p16 Promoter sequence (H.sapiens promoter region of CDKN2 gene): (X94154)

```
1   attcaagagc taacaggtat tagcttagga tgtgtggcac tgtttottaag gcttatatgt
61  attaatacat catTTAAact cacaacaacc cctataaagc aggggggact catattccct
121 tccccccctta taattacgaa aaatgcaagg tattttcagt aggaaagaga aatgtgagaa
181 gtgtgaagga gacaggacag tatttgaagc tggcttttgg atcactgtgc aactctgctt
241 ctagaacact gagcactttt tctgggtctag gaattatgac ttTgagaatg gagtccgtcc
301 ttccaatgac tccctcccca ttttccatc tgcctacagg cagaattctc ccccgTccgt
361 attaaataaaa cctcatcttt tcagagtctg ctcttatacc aggcaatgta cactctgag
421 aaacccttgc cccagacagc cgtttttacac gcaggagggg aaggggaggg gaaggagaga
481 gcagtccgac tctccaaaag gaatcctttg aactagggtt tctgacttag tgaacccgc
541 gctcctgaaa atcaagggtt gagggggtag ggggacactt tcttagtctg acaggtgatt
601 tcgatttctc ggtggggctc tcacaactag gaaagaatag ttttgctttt tcttatgatt
661 aaaagaagaa gccatacttt cctatgaca ccaaacaccc cgattcaatt tggcagttag
721 gaaggttgta tcgcggagga aggaaacggg gcgggggagg atttcttttt aacagagtga
781 acgcactcaa acacgccttt gctggcagga gggggagcgc ggctgggagc agggaggccg
841 gagggcggtg tggggggcag gtggggagga gccagtcct ccttcttgc caacgctggc
901 tctggcgagg gctgcttccg gctgggtccc ccgggggaga cccaacctgg ggcgacttca
961 ggggtgccac attcgctaag tgctcgaggt taatagcacc tcctccgagc actcgctcac
1021 agcgtccctt tgcttgaaa gataccggg tccctccaga ggatttgagg gacagggctg
1081 gagggggctc ttccgccagc accggaggaa gaaagaggag gggctggctg gtcaccagag
1141 ggtggggcgg accgagtgcg ctccggcggc gcggagaggg gtagagcagg cagcggcgg
1201 cggggagcag catggagccg gcggcgggga gcagcatgga gccttcggct gactggctgg
1261 ccacggccgc gg
```

APPENDIX B.

RASSF1A Promoter sequence (Human cosmid clone LUCA12 from 3p21.3): (AC002481.1)

```
1   ACCCCAAGTG AAGGCTCGAG ACTTCCTGCC CCACCCAGTG GGTAGGCCAA GTGTGTTGCT
61  TCAGCAAACC GGACCAGGAG GGCCAGGGCC GGATGTGGGG ACCCTCTTCC TCTAGCACAG
121 TAAAGCTGGC CTCCAGAAAC ACGGGTATCT CCGCGTGGTG CTTTGCGGTC GCCGTCGTTG
181 TGGCCGTCCG GGGTGGGGTG TGAGGAGGGG ACGAAGGAGG GAAGGAAGGG CAAGGCGGGG
241 GGGGCTCTGC GAGAGCGCGC CCAGCCCCGC CTTCGGGCCC CACAGTCCCT GCACCCAGGT
301 TTCCATTGCG CGGCTCTCCT CAGCTCCTTC CCGCCGCCCA GTCTGGATCC TGGGGGAGGC
361 GCTGAAGTCG GGGCCCGCCC TGTGGCCCCG CCCGGCCCCG GCTTGCTAGC GCCCAAAGCC
421 AGCGAAGCAC GGGCCCAACC GGGCCATGTC GGGGGAGCCT GAGCTCAATTG AGCTGCGGGA
481 GCTGGCACCC GCTGGGCGCG CTGGGAAGGG CCGCACCCGG CTGGAGCGTG CCAACGCGCT
541 GCGCATCGCG CGGGGCACCG CGTGCAACCC CACACGGCAG CTGGTCCCTG GCCGTGGCCA
601 CCGCTTCCAG CCCGCGGGGC CCGCCACGCA CACGTGGTGC GACCTCTGTG GCGACTTCAT
661 CTGGGGCGTC GTGCGCAAAG GCCTGCAGTG CGCGCGTGAG TAGTGGCCCC GCGCGCCTAC
721 GAGAGCGGAA
```

APPENDIX C.

Alignment of forward and reverse (reverse complement) sequences of the RASSF1A sequence obtained for patient AES – genomic DNA:

Raw forward sequence	-----
Raw reverse (reverse complement)	TCCCCACAGTCCCTGCACCCAGGTTTCC
EDIT Blood sequence	TCCCCACAGTCCCTGCACCCAGGTTTCC
Raw forward sequence	-TTGCGCGGCTCTCCTCAGCTCCTTCCC
Raw reverse (reverse complement)	ATTGCGCGGCTCTCCTCAGCTCCTTCCC
EDIT Blood sequence	ATTGCGCGGCTCTCCTCAGCTCCTTCCC
Raw forward sequence	GCCGCCCAGTCTGGATCCTGGGGGAGGC
Raw reverse (reverse complement)	GCCGCCCAGTCTGGATCCTGGGGGAGGC
EDIT Blood sequence	GCCGCCCAGTCTGGATCCTGGGGGAGGC
Raw forward sequence	GCTGAAGTCGGGGCCCGCCCTGTGGCCC
Raw reverse (reverse complement)	GCTGAAGTCGGGGCCCGCCCTGTGGCCC
EDIT Blood sequence	GCTGAAGTCGGGGCCCGCCCTGTGGCCC
Raw forward sequence	CGCCCGGCCCGCGCTTGCTAGCGCCCAA
Raw reverse (reverse complement)	CGCCCGGCCCGCGCTTGCTAGCGCCCAA
EDIT Blood sequence	CGCCCGGCCCGCGCTTGCTAGCGCCCAA
Raw forward sequence	AGCCAGCGAAGCACGGGCCCAACCGGGC
Raw reverse (reverse complement)	AGCCAGCGAAGCNCGGGCCCAACCGGG-
EDIT Blood sequence	AGCCAGCGAAGCNCGGGCCCAACCGGG-
Raw forward sequence	CATGTCGGGGGAGCCTGAGCTCATTGAG
Raw reverse (reverse complement)	CATGTCG-----
EDIT Blood sequence	CATGTCGGGGGAGCCTGAGCTCATTGAG
Raw forward sequence	CTGCGGGAGAA
Raw reverse (reverse complement)	-----
EDIT Blood sequence	CTGCGGGAGAA

APPENDIX D.

BLAST results for patient AES genomic DNA RASSF1A sequence:

BLASTN 2.2.15 [Oct-15-2006]

Reference:

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1161943228-16273-75843301034.BLASTQ4

Database: All GenBank+EMBL+DBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences)
4,451,611 sequences; 18,244,403,944 total letters

Query= Length=206

Sequences producing significant alignments:		Score (Bits)	E Value
gi 90761235 gb DQ444319.1	Homo sapiens RASSF1 (RASSF1) gene,...	383	2e-103
gi 2340092 gb AC002481.1 AC002481	Human cosmid clone LUCA12 from	383	2e-103
gi 25777689 ref NM_170717.1	Homo sapiens Ras association (Ra...	309	2e-81
gi 25777687 ref NM_170716.1	Homo sapiens Ras association (Ra...	309	2e-81
gi 25777685 ref NM_170715.1	Homo sapiens Ras association (Ra...	309	2e-81
gi 25777683 ref NM_170714.1	Homo sapiens Ras association (Ra...	309	2e-81
gi 25777678 ref NM_007182.4	Homo sapiens Ras association (Ra...	309	2e-81
gi 114587015 ref XM_001168270.1	PREDICTED: Pan troglodytes R...	307	8e-81
gi 114587025 ref XM_001168402.1	PREDICTED: Pan troglodytes R...	293	1e-76
gi 109658477 gb BC117153.1	Homo sapiens Ras association (Ra...	262	4e-67
gi 109039442 ref XM_001100583.1	PREDICTED: Macaca mulatta si...	214	8e-53
gi 109039454 ref XM_001100950.1	PREDICTED: Macaca mulatta si...	206	2e-50
gi 55242226 gb AF132675.1 AF132675	Homo sapiens Ras associatio...	125	6e-26
gi 9055146 gb AF102772.2	Homo sapiens pancreas-specific ras ...	121	9e-25
gi 9055144 gb AF102771.2	Homo sapiens cardiac-specific ras a...	121	9e-25
gi 9055142 gb AF102770.2	Homo sapiens ras association (RaGb...	121	9e-25
gi 9965374 gb AF291719.1 AF291719	Homo sapiens Ras associatio...	121	9e-25
gi 9965310 gb AF286217.1 AF286217	Homo sapiens Ras associatio...	121	9e-25
gi 42415631 gb AC025353.9	Mus musculus chromosome 9, clone RP23	77.8	1e-11
gi 71533421 gb AC162905.4	Mus musculus BAC clone RP24-281B12...	77.8	1e-11
gi 13383927 gb AF333027.1 AF333027	Mus musculus Blu protein (...)	77.8	1e-11
gi 19699690 emb AL672219.7	Mouse DNA sequence from clone RP2...	77.8	1e-11
gi 50500837 emb CR620030.1	full-length cDNA clone CS00K005YK...	73.8	2e-10
gi 50485991 emb CR605184.1	full-length cDNA clone CS00K012YI...	73.8	2e-10
gi 78483009 dbj AB212726.1	Rattus norvegicus Rassf1 gene for...	69.9	3e-09
gi 115550897 dbj AK238758.1	Sus scrofa mRNA, clone:THYD10026G07	44.1	0.18
gi 109496661 ref XM_001074022.1	PREDICTED: Rattus norvegicus...	40.1	2.8
gi 109495303 ref XM_001055286.1	PREDICTED: Rattus norvegicus...	40.1	2.8
gi 90652761 dbj AB236437.1	Mesocricetus auratus Rassf1 gene ...	40.1	2.8
gi 50731198 ref XM_417211.1	PREDICTED: Gallus gallus similar...	40.1	2.8
gi 14786798 gb AC091214.20	Homo sapiens 12q BAC RP11-13F9 (R...	40.1	2.8
gi 18958641 gb AC091958.3	Homo sapiens chromosome 5 clone RP11-	40.1	2.8
gi 62000944 gb AC157521.2	Mus musculus BAC clone RP23-103M13...	40.1	2.8
gi 55771382 dbj AF008226.1	Thermus thermophilus HB8 genomic DNA	40.1	2.8
gi 82524279 ref NM_204534.4	Gallus gallus retinoic acid rece...	40.1	2.8
gi 2121321 gb AC002123.1 AC002123	Human BAC clone GS1-312P20 fro	40.1	2.8

<u>gi 46197919 gb AE017221.1 </u>	Thermus thermophilus HB27, complete g	<u>40.1</u>	2.8
<u>gi 29725735 gb AY247792.1 </u>	Cricetulus griseus mitotic checkpo...	<u>40.1</u>	2.8

Alignments

gi|90761235|gb|DQ444319.1| Homo sapiens RASSF1 (RASSF1) gene, promoter region
and partial
cds
Length=2001

Score = 383 bits (193), Expect = 2e-103
Identities = 202/204 (99%), Gaps = 1/204 (0%)
Strand=Plus/Plus

Query	2	CCCCACAGTCCCTGCACCCAGGTTTCCATTGCGCGGCTCTCCTCAGCTCCTTCCCGCCGC	61
Sbjct	593	CCCCACAGTCCCTGCACCCAGGTTTCCATTGCGCGGCTCTCCTCAGCTCCTTCCCGCCGC	652
Query	62	CCAGTCTGGATCCTGGGGGAGGCGCTGAAGTCGGGGCCCGCCCTGTGGCCCCGCCCGGCC	121
Sbjct	653	CCAGTCTGGATCCTGGGGGAGGCGCTGAAGTCGGGGCCCGCCCTGTGGCCCCGCCCGGCC	712
Query	122	CGCGCTTGCTAGCGCCCAAAGCCAGCGAAGCNCGGGCCCAACCGGG-CATGTCGGGGGAG	180
Sbjct	713	CGCGCTTGCTAGCGCCCAAAGCCAGCGAAGCACGGGCCCAACCGGGCCATGTCGGGGGAG	772
Query	181	CCTGAGCTCATTGAGCTGCGGGAG	204
Sbjct	773	CCTGAGCTCATTGAGCTGCGGGAG	796

gi|2340092|gb|AC002481.1|AC002481 Human cosmid clone LUCA12 from 3p21.3,
complete sequence
Length=28244

Score = 383 bits (193), Expect = 2e-103
Identities = 202/204 (99%), Gaps = 1/204 (0%)
Strand=Plus/Plus

Query	2	CCCCACAGTCCCTGCACCCAGGTTTCCATTGCGCGGCTCTCCTCAGCTCCTTCCCGCCGC	61
Sbjct	17918	CCCCACAGTCCCTGCACCCAGGTTTCCATTGCGCGGCTCTCCTCAGCTCCTTCCCGCCGC	17977
Query	62	CCAGTCTGGATCCTGGGGGAGGCGCTGAAGTCGGGGCCCGCCCTGTGGCCCCGCCCGGCC	121
Sbjct	17978	CCAGTCTGGATCCTGGGGGAGGCGCTGAAGTCGGGGCCCGCCCTGTGGCCCCGCCCGGCC	18037
Query	122	CGCGCTTGCTAGCGCCCAAAGCCAGCGAAGCNCGGGCCCAACCGGG-CATGTCGGGGGAG	180
Sbjct	18038	CGCGCTTGCTAGCGCCCAAAGCCAGCGAAGCACGGGCCCAACCGGGCCATGTCGGGGGAG	18097
Query	181	CCTGAGCTCATTGAGCTGCGGGAG	204
Sbjct	18098	CCTGAGCTCATTGAGCTGCGGGAG	18121

APPENDIX E.

Alignment of forward and reverse (reverse complement) sequences of the RASSF1A sequence obtained for patient AES – Tumour tissue DNA:

```
raw forward sequence -----
raw reverse (reverse complement) TCCCCACAGTCCCTGCACCCAGGTAGGC
EDIT Tumour sequence -----

raw forward sequence -----A-GGAAGG-AG--TAATCCTGT
raw reverse (reverse complement) CAGAGAAGGGAAGGGAGGATA-TCCTGT
EDIT Tumour seq. -----GGAAGG-AG--TA-TCCTGT

raw forward sequence TTCCAGGAACTGAAACCTAACACCATGA
raw reverse (reverse complement) TTCCAGGAACTGAAACCTAACACCATGA
EDIT Tumour seq. TTCCAGGAACTGAAACCTAACACCATGA

raw forward sequence ACCTGTCCTCTATCACATGGAGAAAGTG
raw reverse (reverse complement) ACCTGTCCTCTATCACATGGAGAAAGTG
EDIT Tumour seq. ACCTGTCCTCTATCACATGGAGAAAGTG

raw forward sequence GGCCCCCTGACGAACGCCCAGGAATCTT
raw reverse (reverse complement) GGCCCCCTGACGAACGCCCAGGAATCTT
EDIT Tumour seq. GGCCCCCTGACGAACGCCCAGGAATCTT

raw forward sequence CCTACCATTGGCAGGTGCAGGGAACCTA
raw reverse (reverse complement) CCTACCATTGGCAGGTGCAGGGAACCTA
EDIT Tumour seq. CCTACCATTGGCAGGTGCAGGGAACCTA

raw forward sequence GGACCTAATATCCTCGTGATCCTTGGGA
raw reverse (reverse complement) GGACCTAATATCCTCGTGATCCTTGGGA
EDIT Tumour seq. GGACCTAATATCCTCGTGATCCTTGGGA

raw forward sequence GGCTGAGTCCCCAGGAGAGGGTGCCAGG
raw reverse (reverse complement) G-----
EDIT Tumour seq. G-----

raw forward sequence CCAAGGGGAGCCTGAGCTCATTGAGCTG
raw reverse (reverse complement) -----
EDIT Tumour seq. -----

raw forward sequence CGGA
raw reverse (reverse complement) ----
EDIT Tumour seq. ----
```

APPENDIX F.

BLAST results for patient AES tumour tissue DNA RASSF1A sequence:

BLASTN 2.2.14 [May-07-2006]

Reference:

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1160653566-28470-95895357041.BLASTQ2

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences)
4,437,839 sequences; 18,128,857,162 total letters

Query= Length=223

Sequences producing significant alignments:			Score (Bits)	E Value
gi 18307335 emb AL162595.17	Human DNA sequence from clone RP...	333	4e-89	
gi 25777689 ref NM_170717.1	Homo sapiens Ras association (Ra...	48.1	0.003	
gi 25777687 ref NM_170716.1	Homo sapiens Ras association (Ra...	48.1	0.003	
gi 25777685 ref NM_170715.1	Homo sapiens Ras association (Ra...	48.1	0.003	
gi 25777683 ref NM_170714.1	Homo sapiens Ras association (Ra...	48.1	0.003	
gi 25777678 ref NM_007182.4	Homo sapiens Ras association (Ra...	48.1	0.003	
gi 109658477 gb BC117153.1	Homo sapiens Ras association (Ra...	48.1	0.003	
gi 90761235 gb DQ444319.1	Homo sapiens RASSF1 (RASSF1) gene...	48.1	0.003	
gi 9055146 gb AF102772.2	Homo sapiens pancreas-specific ras ...	48.1	0.003	
gi 9055144 gb AF102771.2	Homo sapiens cardiac-specific ras a...	48.1	0.003	
gi 9055142 gb AF102770.2	Homo sapiens ras association (RalGD...	48.1	0.003	
gi 50500837 emb CR620030.1	full-length cDNA clone CSODK005YK...	48.1	0.003	
gi 50485991 emb CR605184.1	full-length cDNA clone CSODK012YI...	48.1	0.003	
gi 9965374 gb AF291719.1 AF291719	Homo sapiens Ras associatio...	48.1	0.003	
gi 9965310 gb AF286217.1 AF286217	Homo sapiens Ras associatio...	48.1	0.003	
gi 5524226 gb AF132675.1 AF132675	Homo sapiens Ras associatio...	48.1	0.003	
gi 2340092 gb AC002481.1 AC002481	Human cosmid clone LUCA12 from	48.1	0.003	
gi 17425227 dbj AP001258.4	Homo sapiens genomic DNA, chromos...	44.1	0.048	
gi 18307725 dbj AP002358.3	Homo sapiens genomic DNA, chromos...	44.1	0.048	
gi 13991969 emb AL355353.23	Human DNA sequence from clone RP...	42.1	0.19	
gi 50475351 emb CR594544.1	full-length cDNA clone CSODA004YD...	42.1	0.19	
gi 9685998 gb AC008555.5 AC008555	Homo sapiens chromosome 19 clo	42.1	0.19	
gi 17155029 gb AC008971.6	Homo sapiens chromosome 5 clone CTD-2	40.1	0.75	
gi 16944878 emb AL158826.23	Human DNA sequence from clone RP...	40.1	0.75	
gi 7161758 emb AL109838.11 HSDJ79618	Human DNA sequence from ...	40.1	0.75	
gi 29336214 gb AC139783.3	Homo sapiens chromosome 5 clone RP11-	40.1	0.75	
gi 29336213 gb AC139777.3	Homo sapiens chromosome 5 clone RP11-	40.1	0.75	
gi 32964866 gb AF235103.5	Homo sapiens chromosome 8 multiple...	39.2	2.9	
gi 4156134 gb AC005091.1	Homo sapiens BAC clone CTA-318C11 from	38.2	2.9	
gi 33440540 gb BC056219.2	Homo sapiens DEAH (Asp-Glu-Ala-His...	38.2	2.9	
gi 31044379 gb AC139103.3	Homo sapiens chromosome 8, clone, ccm	38.2	2.9	
gi 22726090 gb AC105449.8	Homo sapiens chromosome 8, clone RP11	38.2	2.9	
gi 21490285 gb AC104982.7	Homo sapiens chromosome 17, clone ...	38.2	2.9	
gi 21321982 gb AC010650.9	Homo sapiens chromosome 16 clone RP11	38.2	2.9	
gi 19849376 gb AC104996.4	Homo sapiens chromosome 17, clone ...	39.2	2.9	
gi 21261794 emb AL512637.18	Human DNA sequence from clone RP...	38.2	2.9	

APPENDIX G.

Relative quantification of MSP results:

1. Assign a calibrated quantity

Following analysis of the PCR product with agarose gel electrophoresis, relative quantification of the levels of DNA methylation is performed with the Syngene ChemiGenius Bio-Imaging System, Genetools (3.06.04) software. The various electrophoresis lanes are identified and marked, and specific values can be allocated to specific fragments (Figure G.1). For this study, the indicated 100 bp fragment of a 100 bp molecular weight ladder was assigned a calibrated quantity of 100 for each agarose gel.

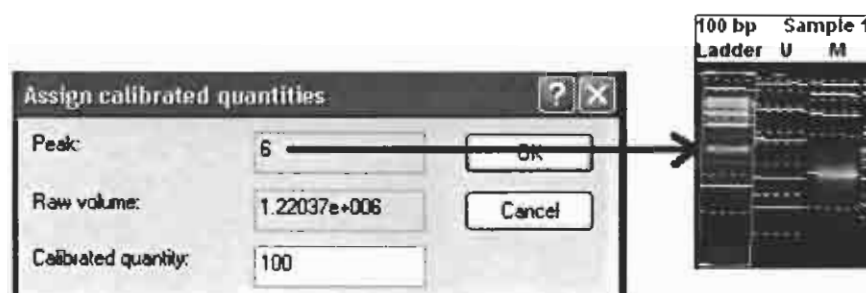


Figure G.1. Assignment of a relative quantity to a specific peak to calibrate further quantifications. The indicated fragment of the 100 bp ladder has been assigned with a calibrated quantity of 100 for each gel.

2. Selection of specific peaks to be quantified

Following the calibration, specific peaks are selected to quantify specific amplicons relative to the calibrated peak. As illustrated in Figure G.2, these peaks are measured to have a constant base-width of 5 mm. The peaks selected are the peaks corresponding to the area where the expected fragment appears, or should appear

(Figure G.3). By choosing the same area each time, independent of the presence of an amplicon, background variation is minimized.

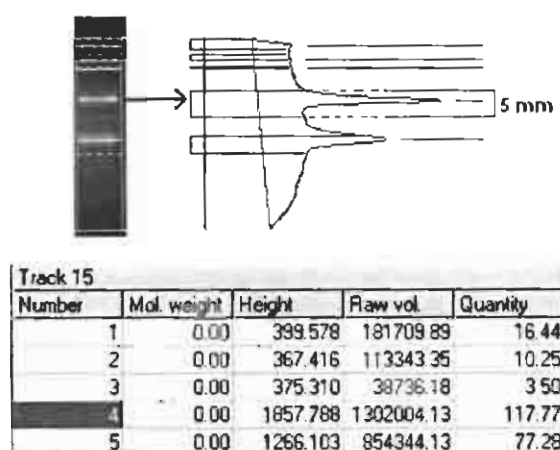


Figure G.2. Relative quantification of a selected fragment by selecting the corresponding peak. The base-width of the peak has been measured as 5 mm, and the relative quantification of the peak can be seen in the table where the selected peak is highlighted.

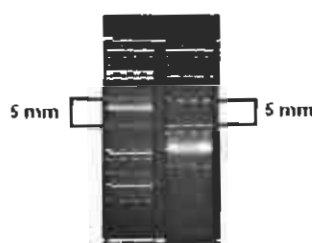


Figure G.3. Definition of corresponding areas, independent of the presence of an amplicon.

Once every peak has been defined, all the relative quantities of those peaks can be used to calculate the methylation score of the sample.

3. Calculate the methylation score

The methylation score of each DNA sample can be calculated with the following equation:

$$[1 - (U/M)] \quad (1)$$

This equation will produce a relative level of methylation. This result can be either positive (more methylated) or negative (more unmethylated).

APPENDIX H.

Buffers and solutions:

- **50 X TAE buffer (1l)**

242 g Tris-base (Mr = 121.14 g/mol)

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

Fill up to 1 litre with ddH₂O.

- **5 X TBE buffer (1l)**

54 g Tris-base (Mr = 121.14 g/mol)

27.5 g Boric acid (Mr = 61.83 g/mol)

20 ml 0.5 M EDTA (pH 8.0)

Adjust to pH 8.3.

Fill up to 1 litre with ddH₂O.

- **0.5 M EDTA (1l)**

186.12 g EDTA (Mr = 372.24 g/mol)

900 ml ddH₂O

Adjust to pH 8.0 with 5 N NaOH

Fill up to 1 litre with ddH₂O.

- **5 N NaOH (0.5 l)**

100 g NaOH (Mr = 40 g/mol)

Fill up to 0.5 litres with ddH₂O.

<!-- Keep on ice while dissolving.

- **1 % Agarose gel (Volume 0.04 l; TAE buffer)**

0.4 g	Agarose
40 ml	1 x TAE buffer
5 µl	Ethidium bromide

Running buffer: 1 X TAE buffer.

- **2.5 % Agarose gel (Volume 0.2 l; TBE buffer)**

6 g	Agarose
200 ml	0.1 x TBE buffer
20 µl	Ethidium bromide

Running buffer: 0.1 X TBE buffer.

- **Ethidium Bromide (10 mg/ml)**

0.1 g	Ethidium bromide
10 ml	H ₂ O

Wrap container in aluminium foil.

<!-- Carcinogenic.

- **6 X gel-loading dye**

0.25 % (w/v)	Bromophenol blue
30% (v/v)	Glycerol

Fill up to 10 ml with sterile ddH₂O.

- **PBS (1l)**

137 mM	NaCl
2.7 mM	KCl
10 mM	Na ₂ HPO ₄
2 mM	KH ₂ PO ₄

Adjust to pH 7.8

Store at 4°C.

- **TE buffer**

10 mM Tris-Cl (*pH* 8.3)

1 mM EDTA

- **TNE buffer**

10 mM Tris-Cl (*pH* 8.3)

0.15 M NaCl

5 mM EDTA

- **Lysis buffer**

1 mM MgCl₂

1 mM Na₂SO₄ (*pH* 6.5)

0.8 % (v/v) NP-40

0.4 g % (m/v)DOC

- **10 x PCR sulphate buffer**

16.6 mM (NH₄)₂SO₄

67 mM Tris-Cl (*pH* 8.8)

6.7 mM MgCl₂

10 mM 2-mercaptoethanol

APPENDIX I.

Table I.1. Supplier companies and catalogue numbers of reagents used

Reagent	Company	Catalogue number
Acetic acid glacial	Merck, BDH AnalAR [®]	BB100017P
Agarose	Whitehead Scientific, Hispanagar	D-1 LE
Ammonium acetate	Merck, BDH AnalAR [®]	10013
Ammonium sulphate	Saarchem, uniLAB [®]	112 400
Biotoools DNA polymerase	Biotoools	10.001
Boric acid	Merck, uniVAR [®]	140 520
Chloroform	Sigma	C2432-500ML
Dimethylformamide	Merck, BDH AnalAR [®]	BB103226L
Dinucleotide mix	New England Biolabs	N0447S
DMSO	Merck, pro Analysi	1.02952.1000
EDTA	Merck, Fluka	93304
Ethanol	Merck, pro Analysi	1.00983.2500
E.Z.N.A. [®] Tissue DNA mini kit	Optima Scientific, PEQLAB	12- 3396-01
Formamide	Merck, pro Analysi	9684
Glycerol	Merck, pro Analysi	1.04092.1000
Glycogen	Merck	4202
Herring sperm DNA	Boehringer Mannheim GmbH	223646
Hydroquinone	Separations, MP Biomedicals	150131
Isoamylalcohol	Merck, pro Analysi	979.1000
Magnesium chloride	Merck, pro Analysi	1.05833.0250
2-Mercaptoethanol	Merck, BDH Biochemicals	44143
NP 40	Merck, Fluka	74385
Phenol	Riedel-deltaen [®]	16017

Table I.1. (Continue)

Phusion™ High-fidelity DNA polymerase	Finnzymes	F-530S
Potassium chloride	Merck, pro Analyti	AB004936.500
Potassium hydroxide	Merck, pro Analyti	1.05033.0500
Potassium dihydrogen orthophosphate	Saarchem, uniVAR®	504 36 00
Propan-2-ol (Isopropanol)	Bio-zone chemicals	1184001
RNase A	Sigma	R875-100MG
SDS	Sigma	L4390-1kg
Sodium bisulfite	Separations, MP Biomedicals	152573
Sodium chloride	Merck, pro Analyti	1.06404.0500
Sodium deoxycholate (DOC)	Sigma	D6750
Sodium hydroxide	Merck, pro Analyti	1.06498.0500
Sodium perchlorate	Sigma	410241-500G
Sodium phosphate	Merck, pro Analyti	6649
di-Sodium hydrogen orthophosphate	Merck, uniVAR®	5822870
Taq I	Roche	10 567 671 001
Taq polymerase	Whitehead Scientific, Promega	M1865
Tris-base	Sigma	93304
Tris-chloride	Sigma	T5941-1KG
Universal methylated human DNA standard	Inqaba, Zymo research	D5011
Vac-Man® Laboratory Vacuum manifold	Whitehead Scientific, Promega	A7231
Water – HiPerSolv for HPLC™	Merck, BDH AnalaR®	BC152736D
Wizard® DNA Clean-Up system	Whitehead Scientific, Promega	A7280
Wizard® Genomic DNA purification kit	Whitehead Scientific, Promega	A1120
Wizard® SV gel and PCR Clean-Up system	Whitehead Scientific, Promega	A2981
Xylol	Merck, uniVAR®	722 1120