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Detection of Methylated Tumor DNA in Serum as a Marker for the Colorectal Carcinoma

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1 INTRODUCTION AND AIMS

Colorectal Carcinoma (CRC) is one of the most common malignancies in Western countries, and several modifiable risk factors concerning lifestyle, nutrition and exercise are known. The estimated lifetime risk of CRC is 5-6% with almost 50% of CRC patients eventually dying of their disease. Despite advances in therapy, there has been only modest improvement in survival for patients with CRC. Hence, effective primary and secondary preventive approaches must be developed to reduce mortality (1).

Because of the frequency of the disease and better survival of patients with early-state lesions, CRC is suitable for screening. Several studies have shown the benefits of CRC screening (2). Unfortunately the established screening tests such as endoscoping examinations show a weak patient acceptance and other tests such as Fecal Occult Blood Test lack specifity and tend to lead to false positive or negative results. Because of the low prevalence of CRC screening, which is only approximately 60%, too many adults were not screened (3).

A new approach for the development of a tumormarker is the use of epigenetically altered DNA. In contrast to genetics, epigenetics is not explaining different phenotypes by a change in the DNA sequence, but by different other mechanism that can influence gene expression. One of these mechanism is the methylation of cytosine residues that precede guanines and which are called CpGs . The hypermethylation of CpG islands, which are CpG rich areas at the promoter region of a gene, leads to a suppressed transcription of the gene (4). The hypermethylation of different tumor suppressor genes has been found to be associated with CRC and other tumor entities. For example the genes *P16* and *RASSF1A* show a high methylation rate and can therefore be used for the development of a tumormarker (5).

Nutrition is one of the factors of the environment that possibly influences the methylation pattern of genes. The investigations are especially focused on folate, which acts as a donor and acceptor of methyl groups in DNA biosyntheses and provides methyl groups for the methylation of DNA (6).

For the detection of the methylation status of a certain gene different methods are known. One is based on the combination of sodium bisulfite treatment and PCR. For the

detection of methylated DNA in serum a qualitative approach has been designed. Here the sodium bisulfite treatment is followed by a Realtime-PCR. There are also different methods of calculating the level of methylation in a given sample. Another bisulfite conversion independent approach for the quantitative detection of mehtylated DNA is a method based on methylation sensitive enzymes. In the present work we will have to investigate, which of the different methods are the most efficient.

The overall aim of our work is to establish a non invasive blood test that uses epigenetically altered DNA as a biomarker for CRC. This testing method could be used not only for tumor prevention, but also to check for the efficiency of therapeutic interventions and for the follow-up.

To achieve our goal we had to accomplish following steps:

- Establish a suitable method to quantify DNA, which shows epigenetic changes.
    - Establishing of a positive and a negative control
    - Testing the linearity of our detection system for a wide dynamic range, the detection of a low amount of total DNA and the detection of methylated DNA diluted by a larger amount of unmethylated DNA
    - Establishing of a standard curve for quantification
    - Comparison of different calculation methods
- Optimize the extraction of the limited amount of DNA from serum
- Collect first clinical data

2 LITERATURE REVIEW

2.1 *Primary Prevention of Colorectal Cancer*

In Austria CRC is the second leading cause for death by malignant diseases in both sexes, only exceeded by breast cancer in women and lung cancer in men (7). The incidence of CRC varies up to 25-fold between countries. The highest rates are found in Westernized countries whereas the lowest rates are found in Africa and India. In addition it has been shown, that people who migrate from low- to high-risk areas of the world reach the incidence of cancer of a high-risk country even over one or two generations (8). These facts provided important evidence that lifestyle factors influence the development of this malignancy. Moreover, there is convincing evidence from epidemiological and experimental studies that dietary intake is an important etiological factor in colorectal neoplasia. Although the precise mechanisms have not been clarified, several lifestyle factors are likely to have a major impact on CRC development (9).

Different studies pointed out that physical inactivity, excess body weight (to a lesser extent), and abdominal fatness are consistent risk factors. Overall the overconsumption of energy is likely to be the major contributor to high rates of CRC in Westernized countries (10).

An analysis of different studies showed that also diabetes is associated with CRC. Thus, the association of abdominal obesity with colorectal cancer likely depends on hyperinsulinemia (11). Whereas many studies showed a relation between CRC and insulin, they found no evidence that a diet characterized by high glycemic index or glycemic load, or by a high intake of carbohydrate or sugars, increases the risk of CRC (12).

The Nurses' Health study provided evidence that red meat intake is related to risk of CRC. Willet et al found an association with beef, pork or lamb consumption. Other studies such as the Iowa Women's Study show an almost significant trend with processed meat (13). These results were confirmed by another study among middle-aged Americans, which suggests that dietary patterns characterized by a low frequency of meat consumption reduce the risk of CRC (14).

Consumption of tobacco products is another lifestyle factor that has been associated with small and large colorectal adenomas, which are generally accepted as being precursor lesions for CRC (15). Also substantial consumption of alcoholic drinks are found to be a cause for CRC (8).

On the other hand there are some nutritional factors that show a protective effect on colorectal cancer, e.g. calcium, vitamin D and folic acid (9).

A recent study showed that mice fed with a defined Westernized diet, which recapitulates intake levels of nutrients that are major dietary risk factors for human colon cancer, induced colonic tumors. But tumors were prevented by increasing the intake of calcium and vitamin D_3 comparable to upper levels of human intake. No alteration of tumorigenesis was found by similar elevation of other micronutrients, which are also at a low level in the Westernized diet such as folate, choline, methionine, or fibers (16).

Other studies about folate and its impact on CRC risk are inconclusive. Some reports show that groups of high intake of folate have a reduced risk of CRC compared with groups with low folate intake, whereas recent studies have not confirmed that inverse relationship. Hubner et al also assume that folic acid supplements must be taken for a prolonged period to impact on CRC risk. Such assertion is supported by findings from the Nurses' Health Study, which showed no inverse associated between CRC risk and supplementation when taken for less than 5 years, a non-significant inverse association when taken for 5-10 years, but a substantial and significant 75% reduction when taken for 15 years and more (17).

In addition, other dietary factors apart from folate and folic acid may also impact on folate metabolism, notably alcohol, choline, and methionine. Alcohol is a folate antagonist interfering with folate absorption and other aspects of folate metabolism, whereas choline and methionine are important sources of methyl groups. Studies that have analyzed combinations of these dietary components have generally found increased risk of CRC for 'methyl-poor' diets (diets high in alcohol and low in folate and methionine) compared with 'methyl-rich' diets (diets low in alcohol and high in folate and methionine) (18). The interactions of methyl groups supplied by nutrition with genetic systems are shown in chapter 2.5.

Whereas studies have provided inconsistent results about the protective effect of fibers, it could be shown that frequent consumption of fruits and vegetables decrease the risk of CRC (14). Ryan-Harshman et al have claimed that the intake of fibers is generally at such a low level that it is difficult to determine its influence on CRC. For example in one study with no association between dietary fiber intake and CRC the 10th percentile of dietary fiber intake was 5.4 g, and the 90th percentile was only 18.2 g (19). Another problem is that the definition of 'fibers' is not always clear. Observational studies may be unable to determine the relationships between fiber-rich foods and non-fiber dietary components, nutrients and micronutrients in fruits and vegetables. Fibers may also be a marker for unmeasured dietary substances that have anticarcinogenic effects. On the other hand a potential limitation of prospective randomized trials as often conducted is the length of the trials. It is not always clear how long an intervention needs to be present before an effect is evident (20).

Taking all these different risk factors under consideration it has been demonstrated that diet may cause or prevent approximately 80% of CRC incidence (13). The World Cancer Research Fund and the American Institute for Cancer Research systematically reviewed and assessed the body of evidence on diet, physical activity and cancer and published recently their findings. The conclusion about CRC and its risk factors are shown in Figure 2.1.

	Decreases Risk	Increases Risk
Convincing	Physical activity	Red meat Processed meat Alcoholic drinks (men) Body fatness Abdominal fatness Adult attained height
Probable	Foods containing dietary fiber Garlic Milk Calcium	Alcoholic drinks (women)
Limited – suggestive	Non-starchy vegetables Fruits Foods containing folate Foods containing selenium Fish Foods containing vitamin D Selenium	Foods containing iron Cheese Foods containing animal fats Foods containing sugar
Limited – no conclusion	Cereals (grains) and their products;potatoes; poultry; shellfish and other seafood; other dairy products; total fat; fatty acids composition; cholesterol; sugar (sucrose); coffee; tea; caffeine; total carbohydrate; starch; vitamin A; retinol; vitamin C; vitamin E; multivitamins; non-diary sources of calcium; methionine; beta carotene; alpha-carotene; lycopene; meal frequency; energy intakte	

Figure 2.1 Food, nutrition, physical activity, and cancers of the colon and the rectum. Summary of the conclusions of the "Expert Report, Food, Nutrition, Physical Activity and the Prevention of Cancer: a Global Perspective" by the World Cancer Research Fund and the American Institute of Cancer Research (available at www.dietandcancerreport.org).

2.2 *Secondary Prevention of Colorectal Cancer*

In 2008 the American Cancer Society updated the guidelines for screening and surveillance for the early detection of CRC. Recommended CRC tests are grouped in two categories: 1) tests that primarily detect cancer, which include both fecal occult blood testing (FOBT) and immunochemical-based FOBT and testing stool for exfoliated DNA and 2) tests that can detect cancer and advanced lesions, which include endoscopic examination and radiologic examination (3).

FOBTs are designed to detect the present of occult blood in stool. Blood in stool is an unspecific finding, but may originate from CRC. The proper use of stool testing requires annual testing that consists of collecting specimens from consecutive bowl movements. There are recommendations for a specific diet for three days previous to the testing:

Avoidance of exceed intake of vitamin C (either from supplements or from citrus fruits or juices), because it can result in false-negative results and avoidance of red meats, which is associated with false-positive results.

The limitation of FOBT is that many of the individual tests have limited sensitivity under best circumstances, and this sensitivity may be further compromised by poor and incomplete specimen and inadequate or improper processing and interpretation.

Endoscopic procedures allow the direct mucosal inspection of the entire colon (colonspectrometry) or the lower half of the colon lumen (sigmoidspectrometry). These methods require one or more days of bowl cleansing and usually one day dedicated to the examination. In addition a chaperon is needed for the transportation, because of sedation. There is also a risk of perforation, haemorrhage and subsequent hospitalization (2).

It must be taken under consideration that the power of a screening test is not only dependent on its specifity and sensitivity, but also on the people's willing to participate in a given screening program. This willingness is strongly influenced by whether the screening is easy to perform, safe and practicable in clinical routine. The American Society of Cancer reported that in 2006 only 56.3% of American women and men aged ≥ 50 years participated in screening of CRC with endoscopic procedures and the prevalence of having done a FOBT was 16.4%. The incidence of adults aged ≥ 50 years having had recent screening with either FOBT or endoscopy was 60.4% (3). The reason for such a low number of participations may be uncomfortable and unpleasant preparation procedures for endoscopy, sometimes painful examination procedures, complications during endoscopy and low sensitivity or specificity of FOBT.

Considering that the stage at diagnosis is one of the major determination of survival (21), it is crucial to find a non-invasive method to identify a marker for CRC that will achieve early diagnosis as well as predicting prognosis and response to treatment. To accomplish this goal, there are efforts to use epigenetic changes in the DNA of tumors as a tumormarker.

2.3 *Epigenetics*

Whereas classical genetics explains different phenotypes by a change in the DNA sequence, epigenetics concentrates on the changes in gene functions that cannot be explained by change in the primary DNA sequence. The main mechanisms in epigenetics comprise regulations of cytosine methylation and histon acetylation (22). Cytosine methylation occurs by enzymatic transfer of a methyl group from the methyl donor S-adenosylmethionin to the carbon-5 position of cytosine (Figure 2.2).

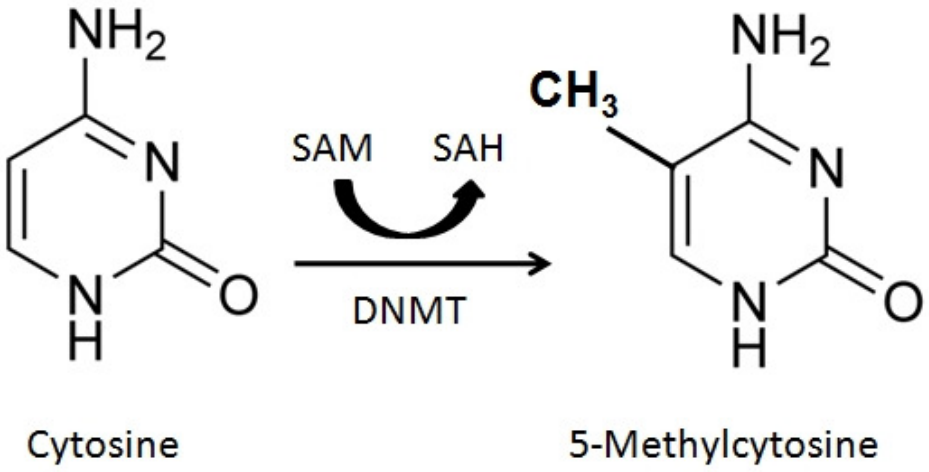

Figure 2.2 Methylation of cytosine residues. De novo methyltransferase (DNTM) catalyses the methylation at position 5 of cytosine, using S-adenosylmethionine (SAM) as methyl donor, which is released as S-adenosylhomocysteine (SAH).

DNA methylation has a critical role in the control of gene activity of the cells. It occurs in cytosines that precede guanines, which are called CpG sites. CpGs are underrepresented in the genome, because of CG suppression, but there are CpG-rich regions known as CpG islands which are located at the promoter regions of many genes (this is the case in about 60% of all human genes). Hypermethylation of these CpGs lead to inhibition of gene expression (4). For further details see Figure 2.3.

Several mechanisms have been proposed to explain the inactivation of gene expression by CpG island methylation. One is inhibition of direct interaction between methylated promoters and transcription factors. Another mechanism of silencing involves

methylated DNA binding proteins with transcriptional repression properties (e.g. MeCP2, MBD1) (23).

Hypermethylation is a physiological process active in all healthy cells and is involved in genomic imprinting and X-chromosome inactivation. But many studies have shown that an aberrant methylation pattern is associated with different diseases, e.g. cancer.

The low level of DNA methylation in tumors compared with the level of DNA methylation in normal-tissue counterparts was one of the first epigenetic alterations to be found in human cancers. The loss of methylation is mainly due to hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns. On the other hand the hypermethylation of CpG islands in the promoter regions of tumor-suppressor genes is a major event in the origin of many cancers (24). Thus, it can be said that tumor derived DNA is globally hypomethylated and focally hypermethylated (4).

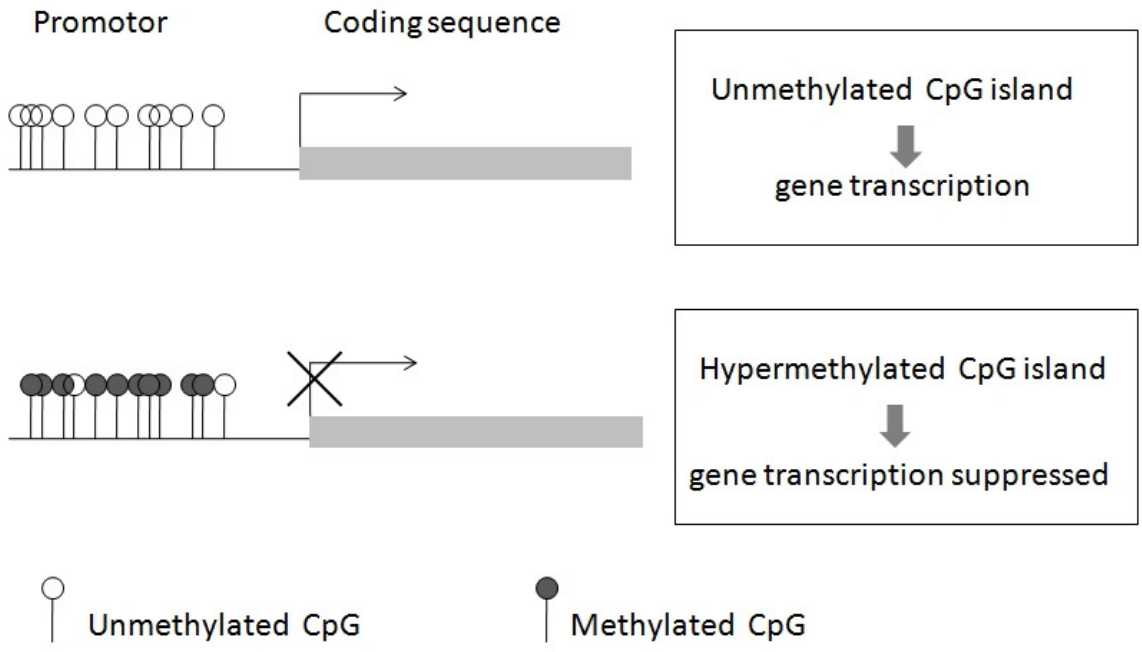

Figure 2.3 Consequence of CpG methylation. The upper panel shows a normal cell, where the CpG island remains unmethylated (pale pins). In absence of methylation of this CpG island, DNA in promoter region remains accessible to transcription factors, and the gene is expressed. In the lower panel a cancer cell shows a characteristic CpG island methylation, causing silencing of gene expression.

2.3.1 Methods of DNA methylation analysis

There are different methods to investigate epigenetic alterations. Herman et al developed a method, called methylation-specific PCR (MSP), which can rapidly asses the methylation status of the CpG sites within a CpG island. The isolated DNA is modified by sodium bisulfite treatment, converting unmethylated (but not methylated) cytosines to uracil. Subsequently the processed DNA is amplified by PCR using specific primers either for the methylated or the unmethylated form of the gene of interest (25, 26). For further details see chapter 3.6 and 3.7.

This method was later improved into a quantitative approach using Real time-PCR (RT-PCR). Here again sodium bisulfite conversion is needed, but then the PCR is performed using primers and fluorescence labeled probes. The increase of DNA can then be detected in real time and the treshhold cycle (Ct) is used for the quantification of input DNA, because it is related to the amount of input DNA.

To be specific, a standard curve is needed for the quantification of DNA and different methods to obtain such standard curves are published. For example the DNA used could be derived from cell lines (such as HeLa and LoVo (27) or NCI-H522 (28)), lymphocytes (29, 30), leucocytes ((31, 32) or salmon sperm (33). The standard DNA is mostly artificially methylated (32), but can also be derived from a cell line, which shows methylation itself (28, 34).

To assess the total amount of input DNA different methods are known. One determines the input DNA using a reference gene. Recently published studies used β-actin (*ACTB*) (29, 31, 32, 35), collagen, (*COL2A1*) (35) or *MYOD1* (28, 30) as such a reference gene. To ensure that the reference gene is quantified independent from its methylation status, the primers used must not harbour any CpGs (34).

Another approach is to calculate the total amount of input DNA by summing the amount of methylated DNA and the amount of unmethylated DNA of a certain gene. Two primersets and two different independent standard curves are needed (one containing fully methylated DNA, one unmethylated DNA) (27, 32).

The two different approaches lead to different methods of calculating the level of methylation in a sample, which are shown in detail in chapter 3.9. Fackler et al found no differences in the accuracy of the two methods at all (36).

An alternative technique has been described to rapidly profile the DNA methylation status without the use of sodium bisulfite. Here several methylation-sensitive restriction enzymes are used and again RT-PCR is performed (37-40). Further details are given in chapter 3.10. Oaks et al could show that this assay is a rapid and accurate way of determining levels of DNA methylation (41).

2.3.2 Quantitative detection of methylated DNA in serum

The new quantitative approach has been used to assess the methylation status not only in solid tumors, but also in body fluids such as serum or plasma.

It is known that the median DNA concentration in the serum of tumor patients is higher than in normal subjects. However, an increased nucleic acid level is not specific for a defined disease (26). Thus, the measurement of total DNA is not a suitable approach and a new method should be based on the detection of DNA that is released from the tumor. The different methylation patterns of DNA are used to distinguish between tumor-specific DNA and DNA from healthy cells. Based on this theory many studies have been done to investigate the potential of the detection of methylated DNA in serum/plasma and its value as a tumormarker.

Wallner et al found that the methylation of certain genes detected in serum was associated with tumor size, stage and extend of metastatic. They also found that the detection of methylation of specific genes in serum of patients with CRC is associated with higher mortality. Thus, they draw the conclusion that the determination of DNA methylation in serum has potential to become an independent pretherapeutic predictor of outcome (42).

Other studies have shown that there are differences in methylation of DNA according to different subgroups of CRC. For example the number of genes methylated was higher in proximal CRC or adenoma than in distal CRC (43). Others found that the serrated neoplasia pathway is associated with the methylation of different genes, one of them is *P16* (44).

Lofton-Day et al have also been working on the identification of new high-performing markers using CRC-specific methylated DNA in plasma. They suggest that an practicable blood-based test for the early detection of CRC followed by colonoscopy for individuals with positive results has the potential to be an effective tool for reducing mortality from this disease (45).

The quantitative approach of determining the hypermethylation even increases the possibilities of these methods, because also the ratio of methylated to unmethylated DNA can be evaluated. For example Nakayama et al found that the methylation score of *P16* significantly increased with the tumor stage. In addition the *P16* methylation score was significantly higher in patients with lymph node metastasis or tumor invasion to the veins and the group with a high *P16* methylation score showed significantly worse survival rates than the group with a low *P16* methylation score (46).

2.4 *Epigenetic and Colorectal Cancer*

For selected genes, epigenetic changes are tightly related to neoplastic transformation in CRCs. In the colon, aberrant DNA methylation arises very early, initially in normal appearing mucosa, and thus Kondo et al suggest that it may be part of age-related field defect observed in sporadic CRCs. They also define a certain CpG Island Methylator Phenotype (CIMP), because they observed that one group of CRCs showed rare methylation and another group showed methylation of several genes simultaneously (23). On the other hand the concept that patients with CIMP-positive tumors have a poorer prognosis has not been accepted by all researchers. There has been much debate whether the CIMP tumors represent a biologically distinct group of CRC or are an artificially selected group of tumors arising from a continuum of tumors with different degrees of methylation (4). Recent studies provided evidences for the CIMP theory. Nosho et al found that CIMP was significantly associated with female sex, older age, proximal location, poor differentiation, mucin, signet ring cells and inversely with stage I (47). The main unsolved issue with CIMP is which panel is the best for CIMP determination, or whether one panel will suffice. Samowitz et al misses a gold standard for CIMP which will make it easier to determine the correct CIMP status (48).

There is not only the influence of promoter hypermethylation itself, but there are also further genetic changes that are associated with it. There are studies that identified a number of relationships between CIMP and various other genetic changes, including microsatellite instability, mutant *Ki-ras*, wild-type *p53*, and the *BARF V600E* mutation (48).

One example is microsatellite instability, which is a status of defect mismatch repair systems often caused by hypermethylation of *MLH1* promoter. The consequences are a reduced repair of replication errors which are due to slippage of short nucleotide repeats during DNA replication and subsequently different mutations occur (49).

2.5 *Epigenetic, Diet and Lifestyle*

Diet is a major aspect of the environment that may influences DNA methylation, and studies on the role of specific foods, diet derived compounds and different types of dietary patterns on cellular mechanism and epigenetics in CRC are increasing. Of special interest are nutrients, which are needed for the nucleic acid synthesis and for the enzymes regulating their syntheses, e.g. essential amino acids, zinc, folate, and vitamins B6 and B12. Folate is the most studied nutrient in this area and many studies suggest that the effect of folate deficiency and supplementation on DNA are gene-specific (8). Cellular folate acts as a donor and acceptor for methyl groups in biosynthesis of nucleotide precursors used for DNA synthesis and provision of methyl groups for methylation of DNA, RNA and proteins. A derivative of folate (5-10 methylene THF) plays a role in the methylation of deosyuridine monophosphate (dUMP) to desoxythimidine monophosphate (dTMP), which is the sole de novo source of thymindine and the rate limiting step in DNA synthesis in mammalian cells.

Another effect is that, when dUMP accumulates because of a low level of folate, uracil misincorporations into DNA in place of thymine is induced. DNA repair enzymes act to remove misincorparated uracil from the DNA strand, causing temporary breakage in the DNA molecule that is sealed by DNA ligase. If folate is continually limited, uracil misincorporation and repair may occur repeatedly causing frequent breakage of the DNA molecule, chromosomal damage and malignant transformation (6).

It could be shown that long-term dietary deficiency of folate in humans result in global hypomethylation (which leads to chromosomal instability and increased mutational events, as mentioned before) in lymphocytes, which is reversible on repletion of folate status (17).

The mechanism leading to hypomethylation of DNA is contributed through methionine/homocysteine. To be specific, 5-methyltetrahydrofolate (5-methyl THF), the major circulating form of folate, acts as a cofactor in the conversion of homocysteine to methionine. Methionine is subsequently metabolised to S-adenosylmethionine, the principal methyl donor in the majority of biochemical reactions, including cytosine methylation in DNA.

A deficiency in vitamin B_{12} would be expected to induce DNA instability in the same way as folate deficiency. It is also required for the methylation of homocysteine to methionine. Although the geno-protective effect of vitamin B_{12} has been reported to act independently of folate, there is little evidence of a relationship between B_{12} and cancer (6).

Details for the folate metabolism and the flow of methyl groups towards either DNA synthesis or DNA methylation are shown in Figure 2.4.

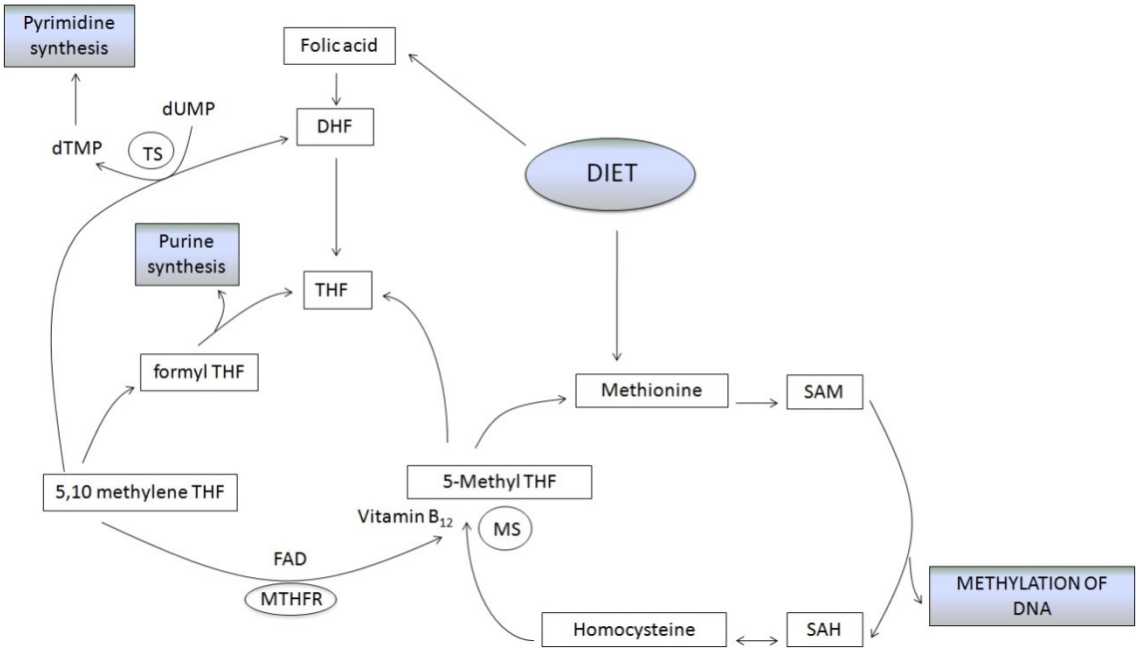

Figure 2.4 Schematic representation of folate metabolism illustrating how folate (and vitamin B12) are strategic cofactors in DNA methylation and DNA synthesis. TS, thymidylate synthase; THF, tetrahydrofolate; DHF, dihydrofolate; MTHFR, 5-10-methylenetetrahydrofolate reductase; MS, methionine synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; FAD, flavin adenine dinucleotide; dUMP, desoxyuridine monophosphate; dTMP, deoxythymidine monophosphate

There are also studies that show a decrease of the expression of certain genes in mice, when they are fed a defined Westernized Diet and in contrary a decrease of gene expression when calcium or vitamin D_3 were supplemented (16).

There is also a relationship between hypermethylation and cigarette smoking. Smoking has been associated with CpG methylation in lung cancer. Also in CRC a significant dose response relationship between the number of cigarettes smoked and the hypermethylation of promoters of certain genes could be shown (49).

2.6 *The role of RASSF1A/P16 hypermethylation in cancer*

2.6.1 *RASSF1A*

Ras-associated domain family 1, isoform A gene (*RASSF1A*) falls into the category of genes frequently inactivated by methylation rather than mutational effects.

The *RASSF1* gene locus spans about 11.151 bp of the human genome (located at locus 3p21.3) and is comprised of eight exons. Differential promoter usage and alternative

splicing generates seven transcripts (*RASSF1A-G*), the major ones are *RASSF1A*, *RASSF1C* and *RASSF1F*. Two CpG islands are associated with the promoter region of *RASSF1*, which are separated by approximately 3.5 kbp. The smaller of the two spans the promoter region of *RASSF1A* (50, 51). For details see Figure 2.5.

The loss of expression in tumors is due to selective CpG methylation of this promoter regions, whereas the bigger promoter region, which is *RASSF1C* specific, remains often unmethylated (52).

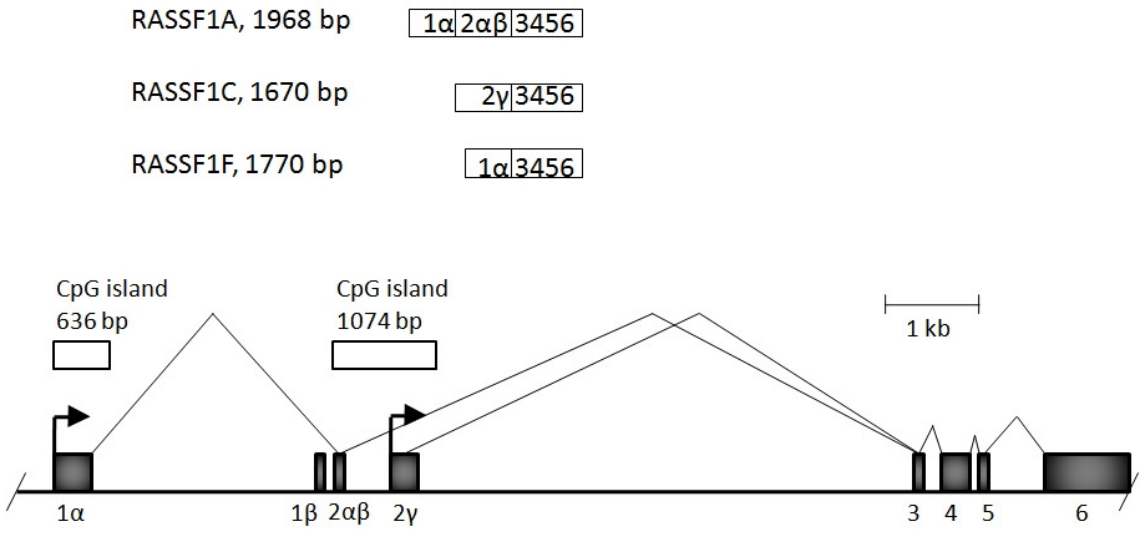

Figure 2.5 Genomic arrangement of *RASSF1A* at 3p21.3. The two major promoters of *RASSF1* (arrows) are located in two separated CpG islands (open squares). The three major isoforms (*RASSF1A*, *RASSF1C*, *RASSFFC*), which are made by alternative promoter usage and alternative slicing of the exons (black boxes) are shown.

Many studies could show that *RASSF1A* is a tumor suppressor gene and that it is epigenetically inactivated in a wide spectrum of tumors. The first time it was found to be silenced was in lung tumor cell lines, but also in at least 37 tumor types a high frequency of methylation is reported (50).

Frequent inactivation of *RASSF1A* in human cancers suggests that it must have a pivotal role in tumor prevention. This notion is supported by the phenotype of tumor cell lines with consecutive overexpression of *RASSF1A*. The observation indicate that *RASSF1A* expressing cells are less viable, growth suppressed, less invasive, and have reduced anchorage/substrate independence. In addition it was shown that *RASSF1A*-knocked out-mice are prone to develop cancer in an advanced age. Hypermethylation of

RASSF1A promoter correlated in certain tumor types with poor prognosis for the patients and an advanced tumor stage. It was also implicated in metastatic process. In small bowl carcinoids *RASSF1A* promoter methylation were more frequently found in metastatic than in primary tumors (50, 53).

RASSF1A polypeptides have a central role in regulating mammalian cell growth. Members of the RASSF family interact with active GTP-bound Ras by the Ras-association (RA) domain. The resulting protein complex mediates the pro-apoptotic effects of K-RasG12V (54).

In addition *RASSF1A* was reported to induce apoptosis via interaction with different other cell proteins and is linked to apoptosis through signalling pathways. But Richter et al have raised the concern that the data linking *RASSF1A* to cell death pathways were obtained under conditions where *RASSF1A* was overexpressed, and this may not reflect a situation that is biologically relevant (53).

Another important role of RASSF polypeptides is the interaction with the microtubule network. The microtubule network functions as a cell scaffold spanning cytoplasm and consisting of rigid but adjustable tubulin polymers. The network determines cell shape, takes part in cell motility, provides “tracks” for transport process, functions as a scaffold for protein-protein interaction and compartmentalizes the cell. Furthermore it is indispensable for the correct chromatin separation during mitosis. It was demonstrated that *RASSF1A* polypeptides co-localize with microtubule network during mitosis and thereby stabilizes microtubules and regulates the mitotic progression. Thus, the growth inhibitory function of *RASSF1A* could be at least partly depending on its modified interaction with the microtubule network (53).

The frequencies of *RASSF1A* promoter hypermethylation in CRC patients detected in different studies are shown in Table 2-1.

Table 2-1 *RASSF1A* promoter hypermethylation in CRC patients

Positive in tumor	***Positive in serum/plasma***	***Detection method***	***Reference/remark***
20% (n=202)		MSP	(5)
45% (n=29)		MSP	(55)
20% (n=222)		MSP	(56)
16% (n=149)		MSP	(43) CpG methylation plays a more important role in proximal than in distal CRC development
81% (n=48)		MSP	(57) *RASSF1A* methylation was also detected in 49 % (n=39) normal colonic mucosal tissue
30% (n=40)		MSP	(44)
31% (n=59)		MSP	(58)
	24% (n=17)	MSP	(59)
	29% (n=45)	MSP	(60) *RASSF1A* promoter hypermethylation in CRC patients were significantly higher than those in benign colorectal disease patients (6.7%) and healthy donors (0%)
	0% (n=16)	MSP	(61)

2.6.2 *P16*

P16^{INK4a} (referred to as *P16*) is part of the INK4a/ARF locus and is one of the genes most frequency lost in human cancer.

The INK4a/ARF locus spreads over 35 kbp of the genome and is located on chromosome 9p21. *P16* and ARF have different first exons which are spliced to a common second and third exon. Although the exons 2 and 3 are shared by *P16* and ARF, the proteins are encoded in alternative reading frames. As a consequence *P16* and ARF are no isoforms and do not share any amino acid homology (62). Details are shown in Figure 2.6 Genomic arrangement of *P16* at 9p21.

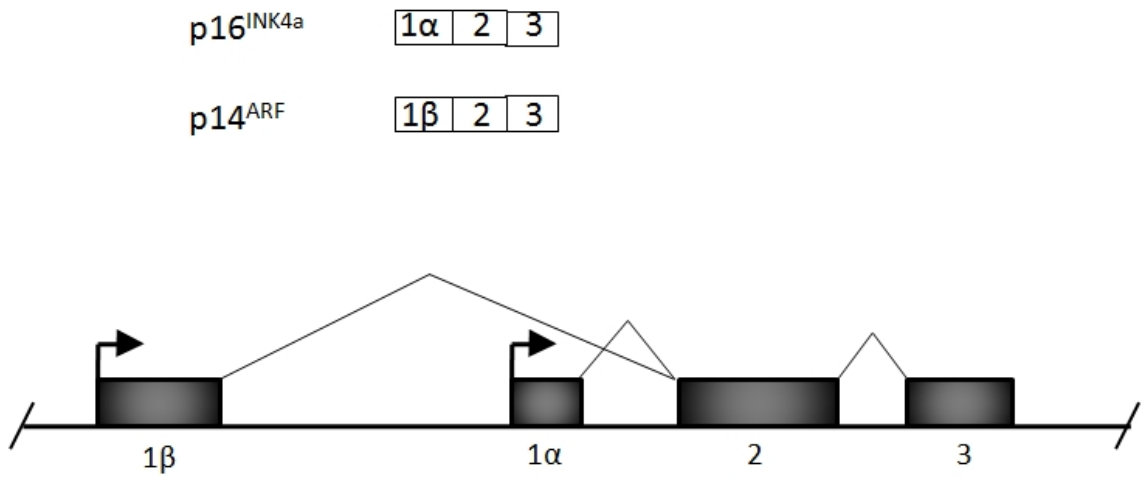

Figure 2.6 Genomic arrangement of *P16* at 9p21. *P16* and p14 have each their unique first exon than then splices to a common second and third exon, but in alternate reading frames.

Studies on *P16* and ARF knockout mice have revealed that they are more prone to spontaneous cancer than wild-type littermates, whereby single knockouts appear significantly less tumor prone than double knockouts (62). In addition also analyses of human tumors provide evidences that *P16* is an important tumor suppressor gene. Inactivation of *P16* by promoter methylation (or point mutation or deletion) is seen in approximately a third of human cancers, making its loss one of the most frequent

lesions of human malignancy (63), including melanoma, pancreatic adenocarcinoma, glioblastoma, certain leukaemia, non-small lung cancer, and bladder carcinoma (62).

One important function of *P16* is growth arrest which protects the cell from hyperproliferative signals (64). The influence of *P16* on proliferation is mediated by the regulation of RB, which is part of a potent anti-proliferative pathway. The expression of *P16* or other INK4 members produces decreased cdk4/6 kinase activity and Rb hypophosphorylation which in turn leads to E2F repression and growth arrest (63).

The frequencies of *P16* promoter hypermethylation in CRC patients detected in different studies are shown in Table 2-2.

Table 2-2 *P16* promoter hypermethylation in CRC patients

Positive in tumor	***Positive in serum/plasma***	***Detection method***	***Reference/remark***
33% (n=202)		MSP	(5) *P16* statistically altered in metastatic tumors
23% (n=40)		MSP	(44)
13% (n=149)		MSP	(43)
53% (n=58)	36% (n=58)	MSP	(65)
47% (n=94)	14% (n=94)	MSP	(66)
	71% (n=17)	MSP	(59)
	0% (n=16)	MSP	(61)
	40% (n=50)	qMSP	(67) *P16* methylation was significantly associated with tumor stage and lymphatic invasion

3 MATERIAL AND METHODS

3.1 *Sample collection*

Patients' blood samples were collected in Vacuette Serum Gel Tubes (Greiner Bio-One) and centrifugated at 1800 x g for 10 minutes at room temperature. Aliquots of 1ml were stored at -80°C.

3.2 *Cell culture*

To obtain DNA as standards for our experiments we cultivated two cell lines, Hep3B and HCC1.2. Hep3B is a human cell line, derived from a hepatocellular carcinoma and obtained from the American Type Culture Collection (ATCC). HCC1.2 has been established from a human hepatocellular carcinoma (68). Both cell lines were kept in RPMI –1640 Medium supplemented with 10% fetal calf serum (FCS) which is a rich source of nutrients. The cells were incubated in a 95% humid atmosphere with 5% CO_2 at 37°C. In intervals of approximately 7 days they were passaged by trypsinization. To be specific, the medium was discarded and the cells were detached from the plate by incubation for 5-10 minutes with 3 ml trypsin. The cell-trypsin mix was diluted with 7 ml medium (supplemented with FCS) and centrifuged for 10 minutes at 1540 rpm. The supernatant was discarded and the pellet resuspended in 10 ml medium (+FCS). The cells were split at different ratios (Hep3B 1:20; HCC1.2 1:10).

3.2.1 Determination of Cell number

Cells were counted in a counting chamber (Bürker Türk). The cover slip was placed on the chamber and a drop of cell suspension was transferred into the chamber which is divided into four quadrants. The number of cells was determined in two quadrants. The results obtained were used for the following calculation: number of cells in 1 quadrant x 10 000 = number of cells/ml cell suspension.

3.3 *Extraction systems of DNA*

Serum contains a limited amount of tumor DNA. To gain a sufficient amount of DNA for further analysis, it is crucial to apply efficient methods of DNA extraction. First, we

compared different kits, that are commercially available, i.e., QIAamp DNA Blood Mini Kit (Qiagen), Charge Switch gDNA 1 ml Serum Kit (Invitrogen), Methylamp Coupled DNA Isolation & Modification Kit (Epigentekt), and the High Pure Viral Nucleic Acid Kit (Roche Applied Science). In addition, we tested a kit that is designed for the use with an instrument for automated purification of DNA, i.e., Maxwell 16 DNA Purification Kit (Promega).

3.3.1 Approach 1: ChargeSwitch® gDNA 1 ml Serum Kit

The ChargeSwitch® gDNA 1 ml Serum Kit uses a magnetic bead-based, which principles are outlined in Figure 3.1.

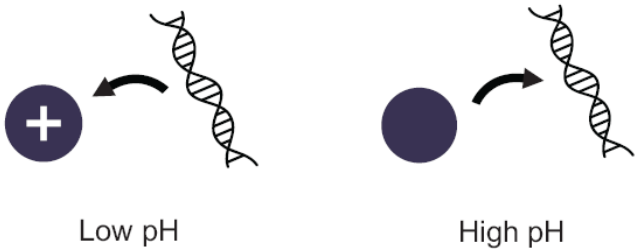

Figure 3.1 Magnetic Beads Technology. The surface of magnetic beads is charged depending on the pH of the surrounding. In low pH the beads bind the negatively charged nucleic acid backbone. To elute the DNA, the charge on the surface of the beads is neutralized by raising the pH to 8.5.

Lyses step: 1 ml of serum sample was placed in a microcentrifuge tube. 700 µl of lyses buffer and 30 µl of protease K were added and mixed by pipetting up and down 5 times. The mixture was incubated at room temperature for 20 minutes.

Binding to the beads: 250 µl of purification buffer and 30 µl of magnetic beads were added to the digested sample and mixed by pipetting up and down 5 times. To allow the DNA to bind to the magnetic beads the mixture was incubated 2 minutes at room temperature. Then the tube was placed in the MagnaRack® for 3 minutes. Without removing the tube from the MagnaRack® the supernatant was carefully removed without disturbing the pellet (for details see Figure 3.2).

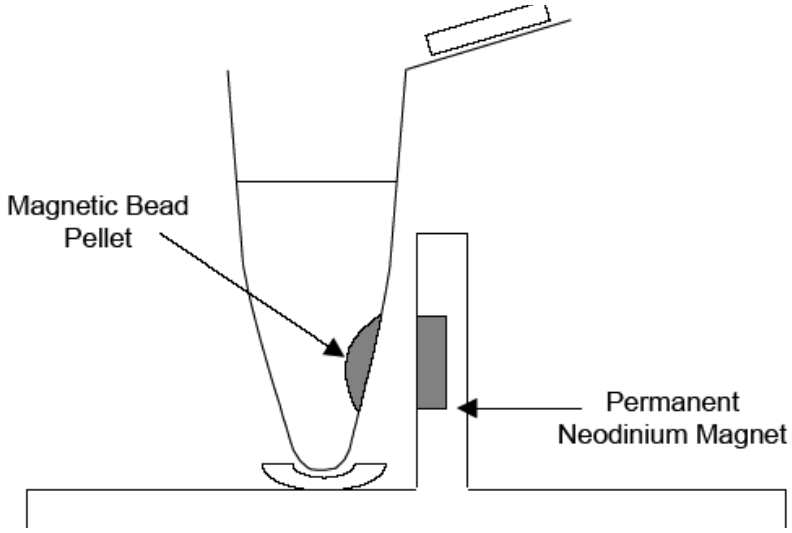

Figure 3.2 Principle of separation by magnetic bead technology. The MagnaRack® (Invitrogen) contains 6 neodinium magnets adjacent to the tube wall. The magnetic beads are attracted by the magnets and form a pellet on the tube wall. Thereafter the supernatant can be removed.

Washing steps: The tube was removed from the MagnaRack® and 1 ml Wash Buffer was added. For resuspension the magnetic beads were pipetted up and down 5 times. The tube was placed in the MagnaRack® again for 2 minutes and the supernatant was removed and discarded. The washing step was repeated once.

Elution step: 40 µl of elution buffer were added to the tube and pipetted up and down 10 times to resuspend the magnetic beads. After incubating for 2 minutes at room temperature the tube was placed in the MagnaRack® for 1 minute. The supernatant containing the DNA was removed and stored at -20°C.

3.3.2 Approach 2: QIAamp® DNA Blood Mini Kit

The QIAamp® DNA Blood Mini Kit uses spin columns to extract DNA from different body fluids. The manufacturers' protocol is designed for 200 µl and was modified to clean up 1 ml of serum in order to gain a sufficient amount of tumor DNA. The procedure consisted of the following steps (all centrifugation steps were carried out at room temperature):

Lyses step: 100 µl of Qiagen protease, 1000 µl of serum sample and 1000 µl of buffer "AL" were transferred to a 15 ml tube. The mixture was vortexed briefly and incubated

at 56°C for 10 minutes. Then 1000 µl of ethanol (96-100% v/v) were added and the tube was vortexed again.

Loading step: 500 µl of this mixture were applied to a QIAamp Mini spin column. The spin column was centrifuged at 6000 x g for 1 minute and the filtrate was discarded. These steps were repeated until all of the mixture was applied to the column.

Washing step: 500 µl of buffer "AW1" were added and the column was centrifuged at 6000 x g for 1 minute. The collection tube containing the filtrate was discarded and the spin column was placed into a new collection tube. In a second wash step, 500 µl of buffer "AW2" were added and the column was centrifuged at 20 000 x g for 3 minutes. Then the spin column was placed in a clean collection tube and centrifuged at 20 000 x g for another minute. This step reduces the probability of carry-over of buffer "AW2". Finally, the spin column was placed in a 1.5 ml microcentrifuge tube and the collection tube was discarded.

Elution step: 20 µl of buffer "AL" were added and the spin column was incubated at 45°C. After 15 minutes the spin column was centrifuged at 6000 x g for 1 minute and another 20 µl buffer "AL" were applied to the center of the spin column. A final centrifugation step at 6000 x g for 1 minute was carried out. The extracted DNA was stored at -20°C.

3.3.3 Approach 3: High Pure Viral Nucleic Acid Kit

The High Pure Viral Nucleic Acid Kit is specially designed to clean up small amounts of DNA in serum. The nucleic acids bind selectively to a glass fiber fleece in a special centrifuge tube in the presence of a chaotropic salt (guanidine HCl). The nucleic acids remain bound while a series of wash steps remove contaminating components. Finally, low salt elution removes the nucleic acid from the glass fiber fleece.

The manufacturers' protocol was modified to clean up not 200 µl but up to 1 ml of serum. In a first step we prepared a working solution by dissolving the carrier RNA in 0.5 ml elution buffer and adding 50 µl of this solution to 2.5 ml binding buffer.

Lyses step: 1 ml of this working solution and 250 µl of protease K were added to 1 ml of the serum sample. After incubating for 10 minutes at 72°C 500 µl of isopropanol were added.

Loading step: An aliquot of 600 µl was added to a spin column and centrifuged at 8000 x g for 1 minute. The flow-through was discarded and the spin column was placed back in the collection tube. This was repeated until all of the mixture was applied to the column. The filter tube was removed from the collection tube and placed in a new one. 500 µl of inhibitor removal buffer were added and the column was centrifuged at 8000 x g for 1 minute. The flow-through was discarded and the column was placed in a new collection tube.

Wash step: 450 µl of wash buffer were added to the upper reservoir of the column and centrifuged at 8000 x g for 1 minute. The column was placed in a new collection tube. This wash step was repeated once. Then the column was centrifuged at 20 000 x g for 10 seconds to remove any residual wash buffer. The collection tube and the flow-through were discarded and the column was placed in a clean 1.5 ml microcentrifuge tube.

Elution step: 40 µl elution buffer were added. The column was incubated at 45°C for 15 minutes before centrifugation at 8000 x g for 1 minute. The DNA was stored at -20°C.

3.3.4 Approach 4: Maxwell® 16 DNA Purification Kit

The Maxwell® 16 DNA Purification Kit is used in combination with the Maxwell® 16 System (Promega), which enables automated purification of genomic DNA. It is a magnetic particle-handling system that efficiently processes liquid samples (for details see Figure 3.3)

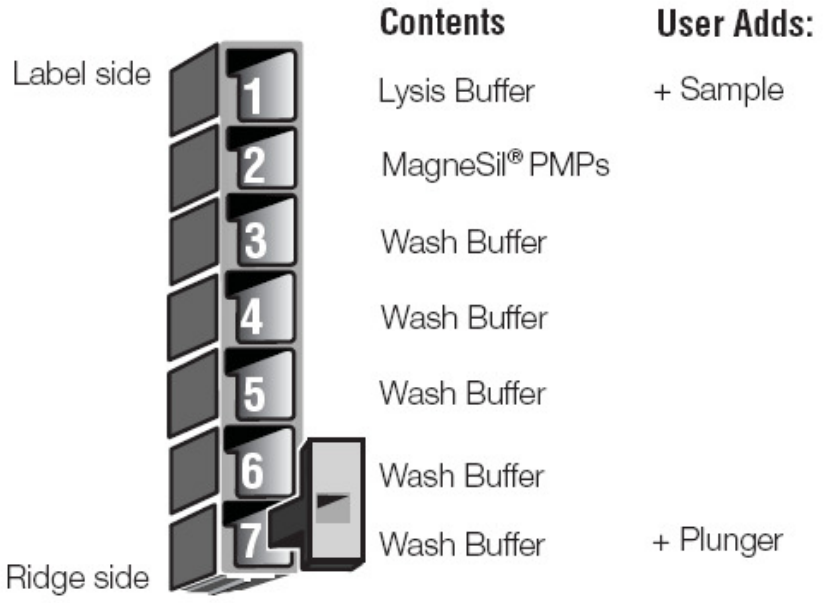

Figure 3.3 Maxwell® 16 DNA Purification Cartridge. This figure shows the content of the cartridge for the Maxwell® Blood Purification Kit. The Maxwell® system transports the magnetic particles through purification reagents in the prefilled cartridges, and mixes the samples during processing. The purified DNA can be used directly in downstream applications.

To perform the DNA extraction, at first a cartridge was placed in the Maxwell® 16 platform. 400µl of serum sample was added to the first well and a plunger was put in the last well. An elution tube was put in the rack and 40 µl of elution buffer were added. The platform was placed in the Maxwell® 16 instrument and a run was started. When the run was completed, the elution tube was removed and the cartridge was discarded. The DNA was stored at -20°C.

3.3.5 Approach 5: Methylamp™ Coupled DNA Isolation and Modification Kit

The Methylamp™ Coupled DNA Isolation and Modification Kit contains all reagents required for DNA isolation and bisulfite conversion.

Lyses step: 500 µl of solution "MR3" and 20 µl of a mixture of buffer "MR1" and "MR2" (1 ml of "MR1" was added to one vial of "MR1") were added to 500 µl of the serum sample. The solution was mixed and incubated at 65 °C for 10 minutes. 2 µl of buffer "MR4" were added to the mixture.

Loading step: 500 µl of the solution were transferred to the spin column. The column was centrifuged at 12 000 rpm for 30 seconds. After discarding the flow-through the remaining volume of the solution was added to the column. Again the column was centrifuged at 12 000 rpm for 30 seconds and the filtrate was discarded.

Washing steps: Thereafter 300 µl of ethanol (70% v/v) was added to the column and centrifuged at 12 000 rpm for 20 seconds. The flow-through was discarded and the column was replaced to the collection tube. The wash step was repeated with ethanol (90% v/v). The centrifugation was extended to 40 seconds.

Elution of the isolated DNA: For the elution of the DNA the column was put in a new 1.5 ml microcentrifuge tube and 24 µl of RNA-free water were added. The column was centrifuged at 12 000 rpm for 20 sec. 1 µl of solution "MR5" was added to the eluted DNA. The solution was mixed and incubated at 37°C for 10 minutes.

DNA Modification: Modification solution was prepared by adding 1.1 ml of solution "MR7" to one vial of "MR6". The solution was vortexed until it was clear. Another 35 µl of "MR5" were added to the solution. 125 µl of the MR5/MR6/MR7-solution were added to the sample, which was then incubated at 65°C for 90 minutes.

Purification of the modified DNA: 300 µl of solution "MR8" were added to the sample. After mixing, the solution was transferred to a new spin column and was centrifuged at 12 000 rpm for 15 seconds. The flow-through was discarded and the column was placed back to the collection tube. 200 µl of MR9 solution were added to the column, and centrifuged at 12 000 rpm for 15 seconds. 10 µl of MR5 were added to 1.1 ml of 90% ethanol and 50 µl of this mixture were applied to the column. After incubating for 8 minutes at room temperature the column was centrifuged at 12 000 rpm for 15 seconds. To perform the final wash steps 200 µl Ethanol (90% v/v) was added to the column and centrifuged at 12 000 rpm for 15 seconds. This step was repeated once and the centrifugation was performed for 35 seconds this time.

Elution of the modified DNA: The column was placed in a 1.5 ml microcentrifugation tube. 25 µl MR10 were added and incubated for 5 minutes at room temperature. Finally, the column was centrifuged at 12 000 rpm for 30 seconds. The modified DNA was stored at -20°C.

3.4 *Photometric Quantification of DNA*

For the quantification of DNA we used two systems: Eppendorf Biophotometer and NanoDrop ND-1000.

3.4.1 Eppendorf Biophotometer

The Eppendorf Biophotometer measures the absorption of the DNA at 270 nm. 2 µl of the sample were diluted with 200 µl of TE-buffer and transferred to a cuvette. After inserting the dilution factor the sample is measured and the concentration of the DNA is calculated automatically according to this factor.

3.4.2 NanoDrop ND-1000

The NanoDrop ND-1000 (Thermo Fisher Scientific) is a full-spectrum (220-750nm) photometer that requires not more than 1 µl of a sample. It utilizes a technology that employs a surface tension alone to hold the sample in place. This eliminates the need for cuvettes. In addition, the NanoDrop ND-1000 has the capability to measure low amounts of samples without diluting it.

1 µl sample was pipetted onto the end of a fiber cable. A second fiber cable was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. After the measurement the sample is removed and discarded.

3.5 *Methylation of genomic DNA*

To obtain completely methylated DNA as a positive control and for standard curves, we used *SssI* Methyltransferase (NewEngland BioLabs). The enzyme artificial methylates all cytosine residues recognising the sequence 5'...CG...3'.

Table 3-1 SssI Methyltransferase assay

Nuclease Free Water	220 µl
10x NEBuffer 2	50 µl
SAM 32 MM	10 µl

DNA (500µg/ml)	200 µl
SssI methylase (20U/µl)	20 µl
Final volume	**500 µl**

All reagents were mixed and incubated for 2 hours at 37°C.

3.6 *Sodium bisulfite conversion*

Standard molecular biology techniques to analyze individual gene loci, such as PCR, erase DNA methylation information. The solution to this problem is to modify the DNA in a methylation dependent way before amplification. This can be achieved by treating the genomic DNA with sodium bisulfite, which converts cytosine to uracil. Is the cytosine methylated the reaction is performed much slower and, as a result, the methylated cytosine remains unaffected. Therefore, bisulfite treatment gives rise to different DNA sequences for methylated and unmethylated DNA.

In subsequent PCR amplification, the uracil residues are replaced by thymine, and the methylcytosine residues are replaced by cytosine.

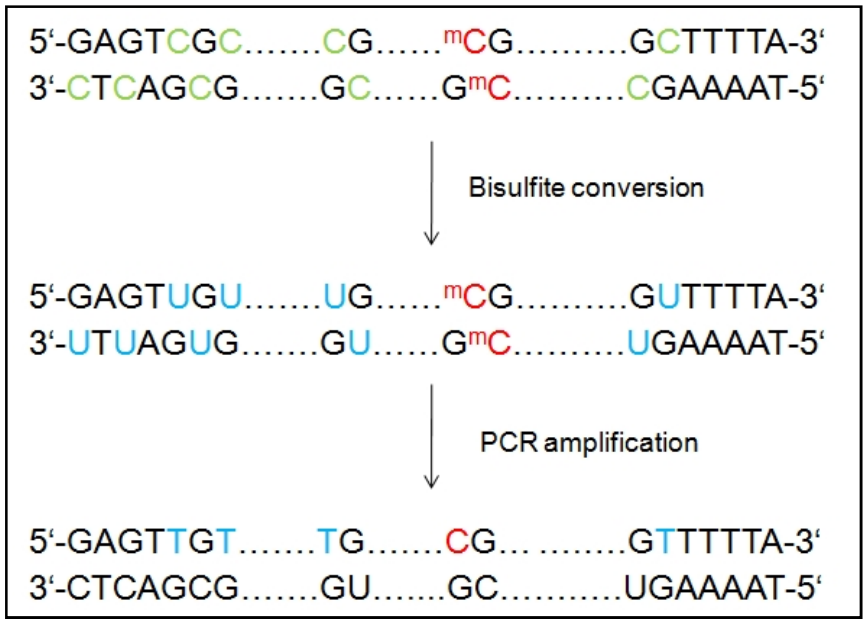

Figure 3.4 Bisulfite Conversion. During Bisulfite conversion the cytosine residues were altered to uracil, while methylated cytosine remain unchanged.

3.6.1 Protocol

The sodium bisulfite reaction was performed using the Epitect Bisulfite Kit (Qiagen). We used the protocol optimized for low concentrations of DNA, which enables use of larger input volumes. In a first step Bisulfite Mix aliquots were dissolved by adding 800 µl RNase-free water. The aliquots were vortexed until the Bisulfite Mix is completely dissolved, which can take up to 5 minutes. If necessary, the solution was heated to 60°C. The bisulfite reactions were prepared in 200 µl PCR tubes according to Table 3-2.

Table 3-2 Bisulfite Reaction Components

Extracted DNA	40 µl
Bisulfite Mix	85 µl
DNA Protect Buffer	15 µl
Final volume	**140 µl**

The PCR tubes were closed and the bisulfite reaction was mixed thoroughly. The bisulfite DNA conversion was performed using a thermal cycler programmed according Table 3-3.

Table 3-3 Thermal Cycler conditions for bisulfite conversion

Step	Time	Temperature
Denaturation	5 min	99°C
Incubation	25 min	60°C
Denaturation	5 min	99°C
Incubation	85 min	60°C

Denaturation	5 min	99°C
Incubation	175 min	60°C
Hold	Indefinite	20°C

Once the bisulfite conversion was completed, the PCR tubes containing the bisulfite reaction were centrifuged briefly. Then the complete mixture was transferred to a clean 1.5 ml microcentrifuge tube. 560 µl of freshly prepared Buffer BL containing 10 µl/ ml carrier RNA were added. The solution was mixed and centrifuged briefly. The whole solution was transferred to a EpiTect spin column and centrifuged at 20 000 x g for 1 min. The flow-through was discarded and the spin column was placed back in the collection tube.

Washing step: To wash the column 500 µl Buffer BW was added and the column was centrifuged at 20 000 x g for 1 minute. The filtrate was discarded and the spin column was placed back in the collection tube.

Desulfonation step: Then 500 µl of Buffer BD were added to the column, and after incubating for 15 minutes at room temperature the column was centrifugated at 20 000 x g for 1 minute. The flow through was discarded and the column was placed back in the collection tube.

Washing step: 500 µl Buffer BW were added to the column and centrifugated at 20 000 x g to wash away chemicals that would inhibit sequencing procedures. The filtrate was discarded and the column was placed back in the collection tube. The wash step was repeated once. Then the spin column was placed in a new collection tube and was centrifuged at 20 000 x g for 5 minutes to remove any residual liquid.

Elution step: To elute the DNA the spin column was placed in a clean 1.5 ml microcentrifuge tube and 20 µl Buffer EB were added to the center of the membrane. After incubating at 45°C for 15 minutes the column was centrifuged at 15 000 x g for 1 minute. Another 20 µl Buffer EB were added to the column an it was centrifuged again at 15 000 x g for 1 minute. The purified DNA was stored at -20°C.

3.7 *Polymerase Chain Reaction*

The MSP is a qualitative approach to determine the methylation status of CpG sites. The basis is the differences between methylated and unmethylated alleles arisen from sodium bisulfite treatment.

Two sets of PCR primers are needed: One of them is specific for bisulfite converted, methylated DNA (methylated cytosines are not altered), the other one is specific for bisulfite converted, unmethylated DNA (all cytosines are converted to uracil). The differences of the sequences of the former methylated or unmethalyted DNA after bisulfite conversion are shown in Figure 3.4. The primers for a methylationspecific PCR should contain at least two CpG sites and are located in the CpG islands of the promoter region of the gene (69).

The primerset specific for the methylated form of *RASSF1A* are termed as *RASSF1AM*, the one that detects the unmethylated form is called *RASSF1AU*. Respectively the primer sets for the gene *P16* are called *P16M* and *P16U*. The primerset for the gene *ACTB* is independent of the methylation status, because there are no CpG islands covered.

For the restriction enzyme assay no bisulfite conversion is needed and thus the primers are design for the original, not altered sequence of the *RASSF1A* gene.

Table 3-4 PCR reaction mix

Template	2 µl
Nuclease Free water	13,1 µl
DNTP	3,2 µl
PCR Buffer	2,5 µl
Primer Mix (containing reverse and forward primer, 10 pM)	1 µl
Taq Polymerase	0,2 µl
Final volume	**22 µl**

The PCR was performed on the GeneAmp PCR System 9700 (Applied Biosystem).

PCR conditions were set at 94°C for 12 minutes, followed by 40 cycles of 94°C for 30 seconds, 64°C for 50 seconds and 72°C for 30 seconds.

PCR products were separated on a 3% agarose gel at 170V applying a 1x TAE-solution as running buffer. Bands were stained with GelRed and visualized with UV-light.

3.8 *Realtime quantitative PCR*

3.8.1 The principle

Realtime quantitative PCR is based on the principles of polymerase chain reaction. Its key feature is that the amplified DNA becomes immediately detectable and thus can be quantified in real time. We used two different systems of DNA detection: TaqMan and SYBR Green.

TaqMan reaction requires a hybridization probe with two different fluorescent dyes. One dye is a reporter dye (FAM), the other is a quenching dye (TAMRA). When the probe is intact, fluorescent energy transfer occurs and the reporter dye fluorescent emission is absorbed by the quenching dye (TAMRA). During the extension phase of the PCR cycle, the fluorescent hybridization probe is cleaved by the 5'-3' nucleolytic activity of the DNA polymerase. On cleavage of the probe, the reporter dye emission is no longer transferred efficiently to the quenching dye, resulting in an increase of the reporter dye fluorescent emission spectra (70).

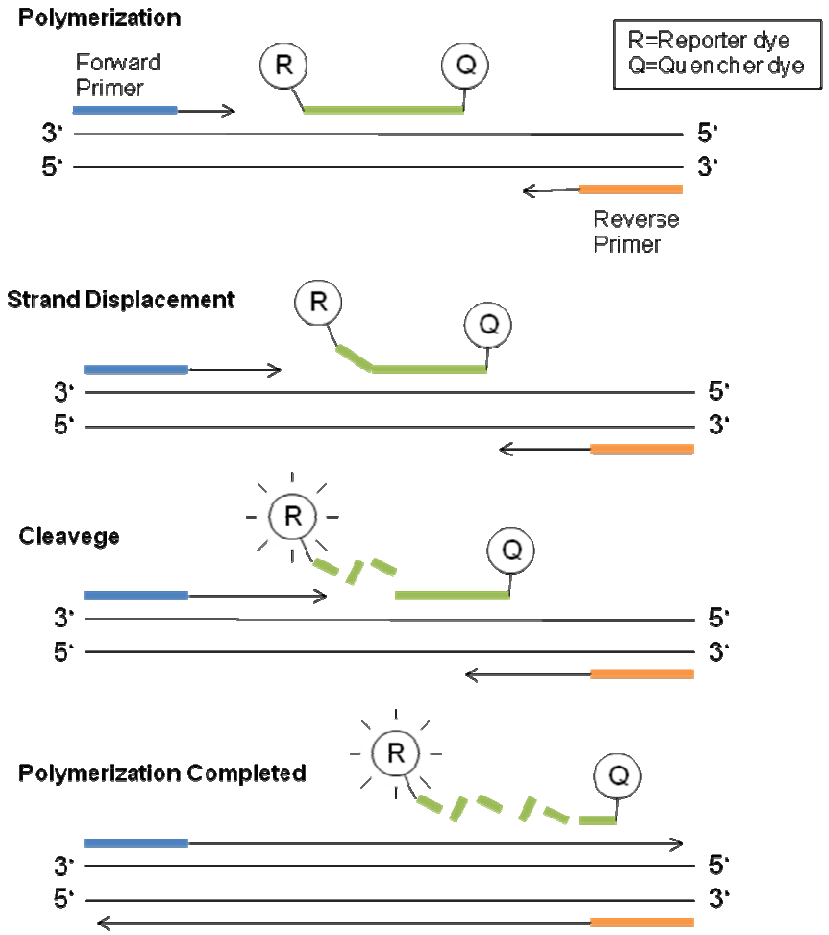

Figure 3.5 RT-PCR detection with TaqMan probe. During the annealing phase of the PCR cycle the primer and the labled TaqMan probe bind to the DNA. When the polymerase elongate the new DNA strand, the TaqMan probe is cleaved by the exonuclease activity of the polymerase. Because of the release of the reporter dye, fluorescence can now be detected.

For the second method of detection SYBR Green is used. Its fluorescence is enormously increased upon binding to double-stranded DNA. During the extension phase, more and more SYBR Green will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected (71).

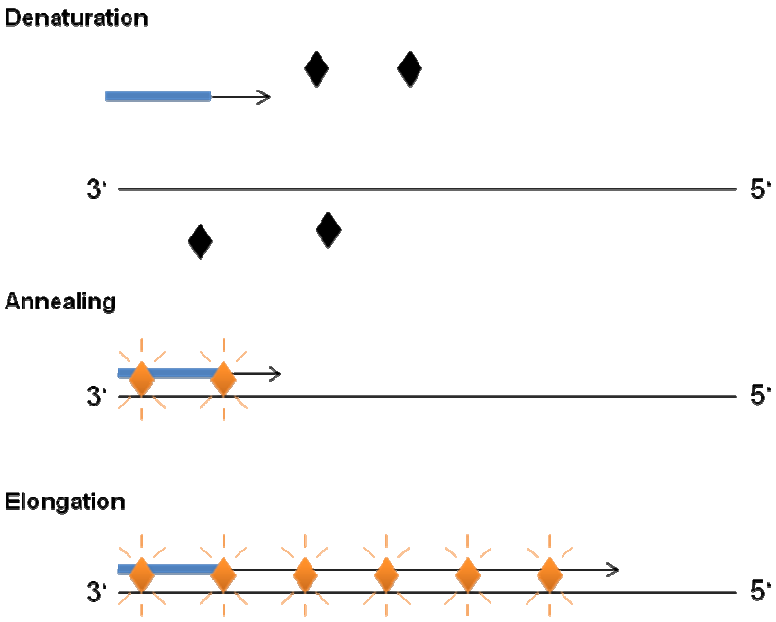

Figure 3.6 RT-PCR detection with SYBR Green. SYBR Green fluorescence is increased, when it binds to double-stranded DNA. Thus, the fluorescence signal is proportional to the amount of DNA.

For both systems the software of the realtime PCR instrument plots the intensity of fluorescent against the time represented by the cycle number. During the early cycles of the PCR the fluorescent remains at baseline. When sufficient hybridization probe has been cleaved by the Taq polymerase nuclease activity or enough SYBR Green has been bound to the DNA, the intensity fluorescent emission increases. After a certain number of cycles the PCR amplifications reach a plateau phase of fluorescence. The amplification plot is examined in the log phase of product accumulation and in this phase an arbitrary threshold is assigned. The cycle at which the sample reaches this level is called Cycle Treshold (Ct). The observed Ct-value is related to the initial amount of DNA. The higher the initial amount of DNA, the sooner accumulated product is detected in the PCR process, and the lower the Ct-value. Thus, an *increase* of the Ct-value of 3.33 represents a tenfold *decrease* of input DNA. To quantify a sample a standard curve was generated.

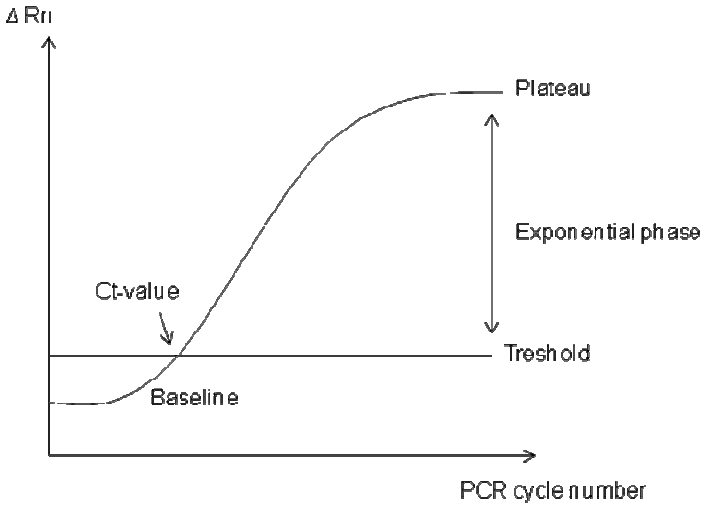

Figure 3.7 RT-PCR Amplification Plot. At early cycles the observed fluorescence remains at baseline, then amplification reaches the exponential phase and finally the fluorescence remains at a plateau. For the quantification the Ct-value is determined by an arbitrary defined threshold.

3.8.2 Absolute Quantification

To quantify the DNA on an absolute base we had to generate a standard curve (details in chapter 4.1.3). The correlation coefficient of the standard curve should be over 0.99 and the slope near -3.33.

3.8.3 Protocol

For the TaqMan assay 20 µl mastermix were prepared according to Table 3-5 and 4 µl bisulfite converted DNA were added.

Table 3-5 TaqMan assay Mastermix

		Final concentration *RASSF1A/ACTB*	Final concentration *P16*
TaqMan Master Mix	12.5 µl		
Primer forward	2.5 µl	600 nM	300 nM
Primer reverse	2.5 µl	600 nM	300 nM
Probe	2.5 µl	200 nM	25 nM
Final volume	**20 µl**		

The RT-PCR was performed in an ABI7000 Cycler (Applied Biosystems). The PCR conditions were set as 95°C for 15 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each plate contained a positive and a negative control.

For the SYBR-Green assay 20 µl mastermix were prepared by mixing 12.5 µl SYBRE Green mastermix and 7.5 µl primermix, containing primer forward and primer reverse. 4 µl restriction endonuclease treated DNA were added. The final concentration of the primer was 100 nM.

PCR conditions were set at 95°C for 15 minutes, followed by 45 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds. Each plate contained a positive and a negative control.

3.9 *PMR and %methylation procedure*

Two principle approaches for calculating the level of DNA methylation have been published. One of these is based on the calculation of the Percentage of Methylated Reference (PMR). Thereby two sets of primers and probes, designed specifically for bisulfite converted DNA, are used: one set for the methylated form of the gene of interest and one set for the reference gene. The primer set for the reference gene contains no CpG islands and therefor the amplification is not affected by the methylation status of the DNA. The amount of the reference gene was used to normalize

for the input DNA. In addition to the sample DNA a *Sss*I treated and hence fully methylated DNA is analysed. Finally the PMR is calculated by comparison of both results. (28, 29, 35, 72-74)

Another approach is to use a primer set for the methylated form and the unmethylated form of a gene. The percentage of methylation (%methylation) is calculated by dividing the amount of methylated DNA by the sum of methylated and the unmethylated DNA, which represents the input DNA (27, 36, 75).

Details of the principle of both approaches are shown in Figure 3.8.

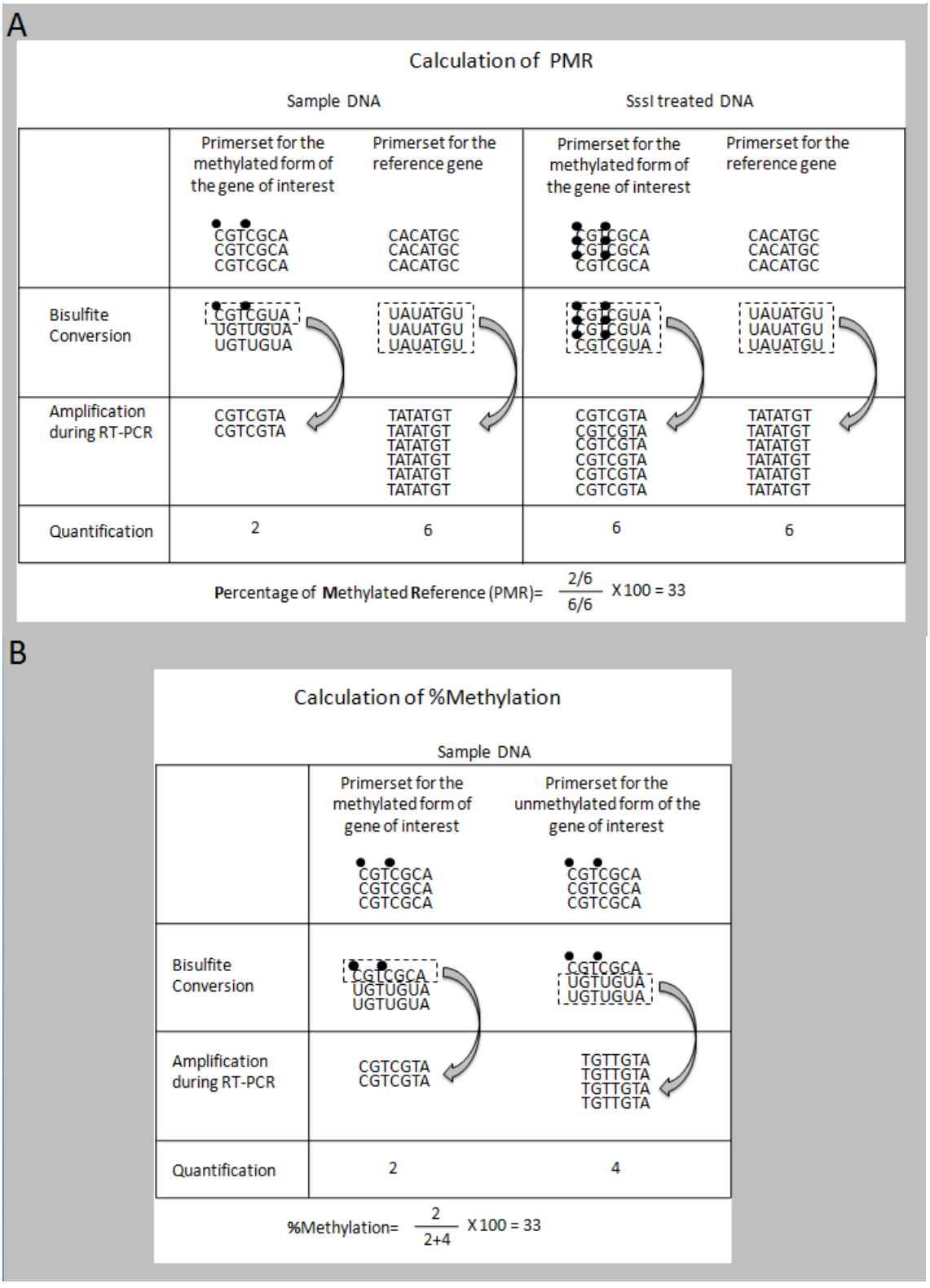

Figure 3.8 Calculation of PMR and %methylation (A) For the calculation of the PMR one primer set for the methylated form and one for a reference gen is used. The result for the sample is compared with the result of a fully methylated reference DNA. (B) To calculate the %methylation one primerset for the methylated form of a gene and a primerset for the unmethylated form of a gene is used. The sum of the amount of methylated and unmethylated DNA is used to determine the input DNA.

3.10 *Restriction endonuclease assay*

A sodium bisulfite conversion independent method to detect methylated DNA is the restriction endonuclease assay. We used a previously described method (38, 40, 41).

To perform the assay the extracted DNA was routinely split into three aliquots. One aliquot was digested with *Hpa*II, the second with *Msp*I and the third one was left undigested and served to quantify the total amount of DNA extracted from the samples.

*Hpa*II cuts the sequence CCGG only in its unmethylated form and no PCR product is to be expeted, if there is no methylated DNA in the sample. But if there is any methylated DNA, this assay serves to quantify the amount of this DNA.

In contrast, the restriction endonuclease *Msp*I cuts the unmethylated and the methylated form of CCGG and, thus, no PCR product should be detected at all. If there are RT-PCR curves observed although, they are considered as non-specifically amplified DNA.

The methylation index was calculated as: (amount of *Hpa*II digested DNA / amount of input DNA) – (amount of *Msp*I digested DNA / amount of input DNA) multiplied by 100 [i.e. (methylated DNA – non-specifically amplified DNA) x 100] (37)

The quantification of the DNA was performed using RT-PCR (for details see chapter 3.8). The principle of this assay and the calculation of the methylation index are also shown in Figure 3.9.

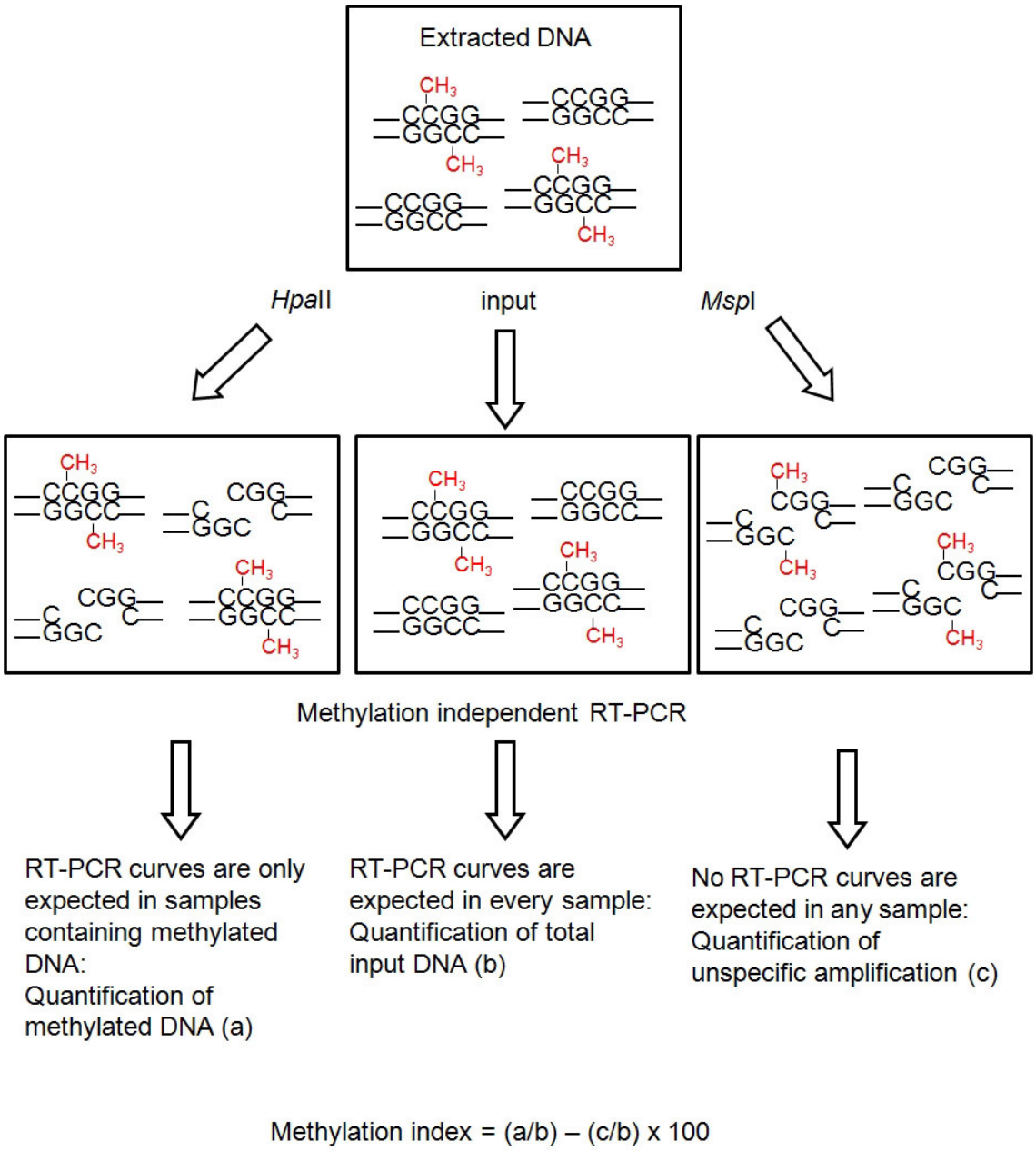

Figure 3.9 Restriction endonuclease assay. Extracted DNA is digested with two endonuclease enzymes: *Hpa*II and *Msp*I. *Hpa*II cuts only the unmethylated CCGG residue, while *Msp*I cuts CCGG independent if it is methylated or not. To assess the amount of total DNA input one determination is done without adding a restriction endonuclease.

3.10.1 Protocol

For the endonuclease assay the extracted DNA was split and the reaction mix was made according to Table 3-6. For the determination of input DNA the amount of restriction endonuclease was replaced by water.

Table 3-6 Restriction endonuclease assay

	*Hpa*II-assay	*Msp*I-assay	input
Nuclease Free Water	8 µl	8 µl	14 µl
10 x RE Buffer	2 µl	2 µl	2 µl
Extracted DNA	4 µl	4 µl	4 µl
*Hpa*II (10U/µl)	6 µl	-	-
*Msp*I (10U/µl)	-	6 µl	-
Final Volume	**20 µl**	**20 µl**	**20 µl**

Incubated at 37°C for 5 hours

To ensure complete digestion the reaction was repeated by adding another aliquot of the following reagent.

Nuclease Free Water	6 µl	6 µl	9 µl
10 x RE Buffer	1 µl	1 µl	1 µl
*Hpa*II (10U/µl)	3 µl	-	-
*Msp*I (10U/µl)	-	3 µl	-
Final Volume	**30 µl**	**30 µl**	**30 µl**

Incubated at 37°C over night

3.11 *Materials*

3.11.1 Reagents

Table 3-7 General Reagents

Reagent	Catalog number	Company
Ethanol	1.00983.1000	Merck
Isopropanol	1. 09634. 1011	Merck
Aqua bidestillata	15.533	Mayrhofer Pharmazeutika

Table 3-8 Cellculture

Reagent	Catalog number	Company
RPMI 1640	145238	Gibco
FCS	179822	Gibco
Trypsin EDTA	197812	Gibco

Table 3-9 Nucleic acid isolation and bisulfite conversion

Reagent	Catalog number	Company
QIAamp DNA Mini Kit	51104	Qiagen
ChargeSwitch gDNA 1 ml Serum Kit	CS11040	Invitrogen
Methylamp Coupled DNA Isolation &Modification Kit	P-1002	Epigentek Group
High Pure Viral Nucleic Acid	11858874001	Roche Applied Sience
Maxwell 16 DNA Purification Kit	AS1010	Promega

EpiTect Bisulfite Kit	59104	Qiagen

Table 3-10 DNA Methylation with *Sss*I Methylase

Reagent	Catalog number	Company
*Sss*I Methylase	M0226	Biolabs
10xNEB Buffer	B7002 S	Biolabs
SAM	B9003	Biolabs

Table 3-11 Reagents for PCR and gelelectrophoresis

Reagent	Catalog number	Company
dNTP set 100 mM solution	39025	Bioline
PCR Buffer	203205	Qiagen
HOT START Taq Polymerase	203205	Qiagen
Agarose	V-3125	Promega
Gelred 10 000x	41003	Biotium
50 x TAE-Buffer	1610743	Bio-rad
100 bp Dna ladder	15628-019	Invitrogen
Bromphenolblue	B-5525	Sigma Aldrich

Table 3-12 Reagents for Restriction endonuclease assay

Reagent	Catalog number	Company
*Hpa*II	ER0512	Fermentas
*Msp*I	ER0541	Fermentas
1xBuffer Tango	BY5	Fermentas

Table 3-13 Reagents for RT-PCR

Reagent	Catalog number	Company
SYBR Green PCR Master Mix	40309155	Applied Biosystems, USA
TaqMan Universal PCR Master Mix, No AmpErase UNG	4324018	Applied Biosystems, USA

3.11.2 Used oligonucleotids

Table 3-14 Oligonucleotides used for PCR

Primer name	Sequence
RASSF1AM forward	5'-GGGTTTTGCGAGAGCGCG-3'
RASSF1AM reverse	5'-GCTAACAAACGCGAAGGC-3'
RASSF1AU forward	5'-GGTTTTGTGAGAGTGTGTTTAG-3'
RASSF1AU reverse	5'-CACTAACAAACACAAACCAAAC-3'
P16M forward	5'-TTATTAGAGGGTGGGGCGGATCGC-3'
P16M reverse	5'-GACCCCGAACCGCGACCGTAA-3'
P16U forward	5'-TTATTAGAGGGTGGGGTGGATTGT-3'
P16U reverse	5'-CAACCCCAAACCACAACCATAA-3'

Table 3-15 Oligonucleotides used for the TaqMan RT-PCR assay

Primer name	Sequence
RASSF1AM forward	5'-GCGTTGAAGTCGGGGTTC-3'
RASSF1AM reverse	5'-CCCGTACTTCGCTAACTTTAAACG-3'
RASSF1AM probe	5'-ACAAACGCGAACCGAACGAAACCA-3'
RASSF1AU forward	5'-GGTGTTGAAGTTGGGGTTTG-3'
RASSF1AU reverse	5'-CCCATACTTCACTAACTTTAAAC-3'
RASSF1AU probe	5'-CTAACAAACACAAACCAAACAAAACCA-3'

P16M forward	5’-TTATTAGAGGGTGGGGCGGATCGC-3’
P16M reverse	5’-GACCCCGAACCGCGACCGTAA-3’
P16M probe	5’-AGTAGTATGGAGTCGGCGGCGGG-3’
P16U forward	5’-TTATTAGAGGGTGGGGTGGATTGT-3’
P16U reverse	5’-CAACCCCAAACCACAACCATAA-3’
P16U probe	5’-CTACTCCCCACCACCCACTACCT-3’
ACTB forward	5’-TGGTGATGGAGGAGGTTTAGTAAGT-3
ACTB reverse	5’-AACCAATAAAACCTACTCCTCCCTTAA-3’
ACTB probe	5’-ACCACCACCCAACACACAATAACAAACACA-3’

Table 3-16 Oligonucleotides used for the SYBR Green RT-PCR assay

Primer name	Sequence
RASSF1A forward	5’-CCTTCCTTCCCTCCTTCGT-3’
RASSF1A reverse	5’-ACCTCAAGATCACGGTCCAG-3’

4 RESULTS

4.1 *Quantification of DNA*

4.1.1 Establishment of positive and negative control

To analyze the methylation status of the cell lines Hep3B and HCC1.2, qualitative MSP was performed. Positive control for the *RASSF1AU* and *P16* primer was lymphozyte DNA from a healthy donor, which is unmethylated. As a negative control artificially methylated DNA was used. For the *RASSF1AM* and the *P16M* primer the artificially methylated DNA was used as a positive control and lymphocyte DNA as negative control.

The gelelectrophoresis picture (Figure 4.1) shows that *RASSF1A* is methylated in HCC1.2, but not in Hep3B cells. For *P16* the same result was observed, HCC1.2 shows methylation in this gene locus, whereas Hep3B is unmethylated. So we could use the DNA extracted from HCC1.2 as a standard for methylated DNA and HCC1.2 derived as unmethylated DNA standard.

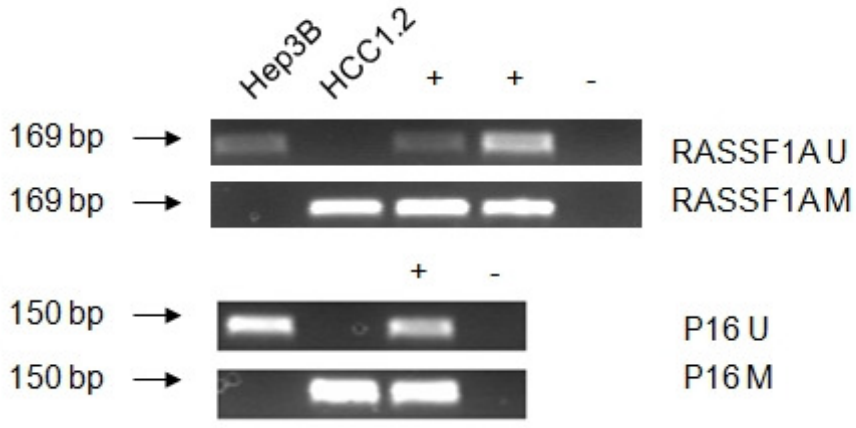

Figure 4.1 MSP analyses of the cell lines Hep3B and HCC1.2. DNA was extracted and sodium bisulfite converted. Then MSP was performed according to the protocol described in methods. As a positive control (+): lymphozyte DNA (*RASSF1AU*, *P16U*) and artificially methylated DNA (*RASSF1AM*, *P16M*) and for the negative control (-): artificially methylated DNA (*RASSF1AU*, *P16U*) and lymphocyte DNA (*RASSF1AM*, *P16M*) was used.

4.1.2 Determination of range and linearity

To establish an assay to detect methylated DNA in human serum, we had to investigate if our RT-PCR protocol is suitable to analyze a limited amount of DNA. To achieve this aim, we firstly determined the minimum amount of DNA that shows a positive RT-PCR signal. We found that we could detect DNA, when we added a minimum of 25 pg of DNA to the reaction mix (Figure 4.2). In addition, we evaluated, if our RT-PCR shows a linearity between the amount of input DNA and RT-PCR signal obtained. As shown in Figure 4.2, there is indeed linearity in a range of 5 logs. Thus, it is possible to create a standard curve to quantify DNA within this range (further details about the generation of the standard curve in chapter 4.1.3).

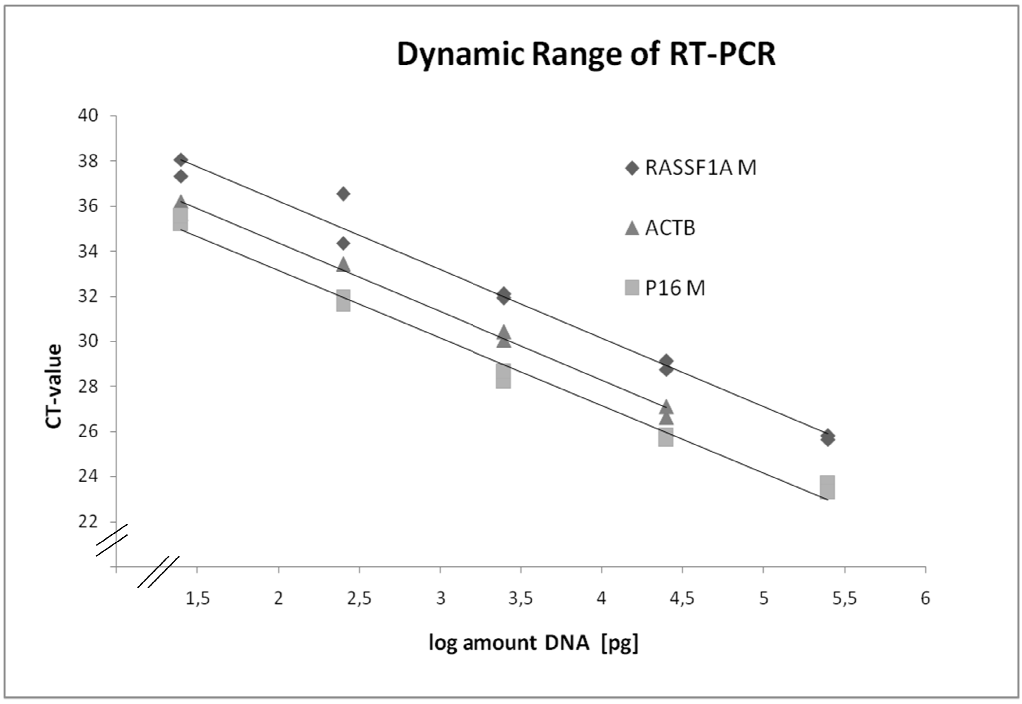

Figure 4.2 Dynamic range of the RT-PCR of the genes *ACTB* and *RASSF1A* and *P16* in their methylated form. Hep3B DNA was *Sss*I treated, bisulfite modified and serially diluted. The logarithm of the absolute amount of input DNA (250000 pg, 25000 pg, 2500 pg, 250 pg and 25 pg) was plotted against the measured CT-values. The data derive from one preparation, the RT-PCR was done in duplicate and the data represent the means +/-SD.

In serum of cancer patients most of the DNA fragments detectable, derive from healthy cells and are thus unmethylated. They dilute the small amounts of methylated DNA released from tumor cells. Therefore our assay had to detect methylated DNA even in

the presence of large amount of unmethylated DNA. Thus, we simulated this situation. We prepared different mixtures of 1 ng of methylated DNA/µl with 0 ng/µl, 1 ng/µl, 9 ng/µl, or 99 ng/µl of unmethylated DNA. So the percentage of methylated DNA was 100%, 50%, 10% or 1% of total DNA. Figure 4.3 shows that the Ct-values obtained were more or less identical over the concentration range, indicating that the unmethylated DNA does not interfere with the quantification of methylated DNA.

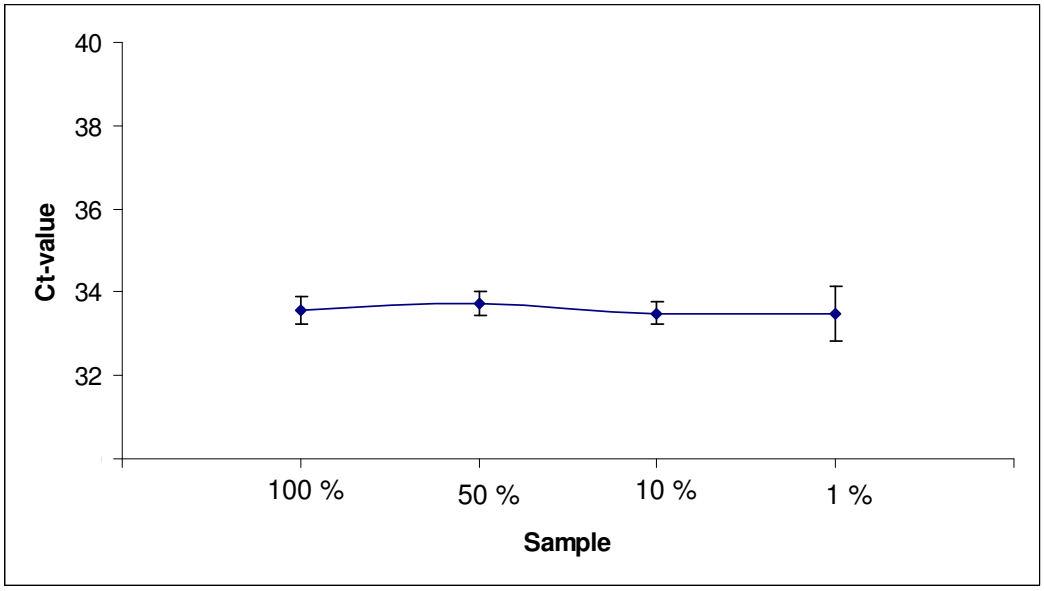

Figure 4.3 RT-PCR of methylated DNA (1 ng/µl) diluted with different amounts of unmethylated DNA, yielding a relative concentration of 100%, 50%, 10% and 1% methylated DNA. The total amount of methylated and unmethylated DNA in the reaction mixture was 1 ng/µl, 2 ng/µl, 10 ng/µl and 100 ng/µl. The data derive from one preparation, the RT-PCR was done in duplicate and the data represent the means +/-SD.

4.1.3 Establishment of a standard curve

The extracted DNA from serum was quantified using RT-PCR. To accomplish this, we used a standard curve to extrapolate the DNA concentration and then used two different methods of calculation of the methylation level, PMR and %methylation (see chapter 3.9).

To generate a standard curve, DNA from Hep3B cells was extracted, *Sss*I treated and then bisulfite modified. The extracted and modified DNA was quantified using NanoDrop ND-1000. Five 10-fold dilution steps (1:1, 1:10, 1:100, 1:1000 and 1:10000)

were made. These serial dilutions were included in each assay and served to calculate the DNA concentration in samples (see Figure 4.4).

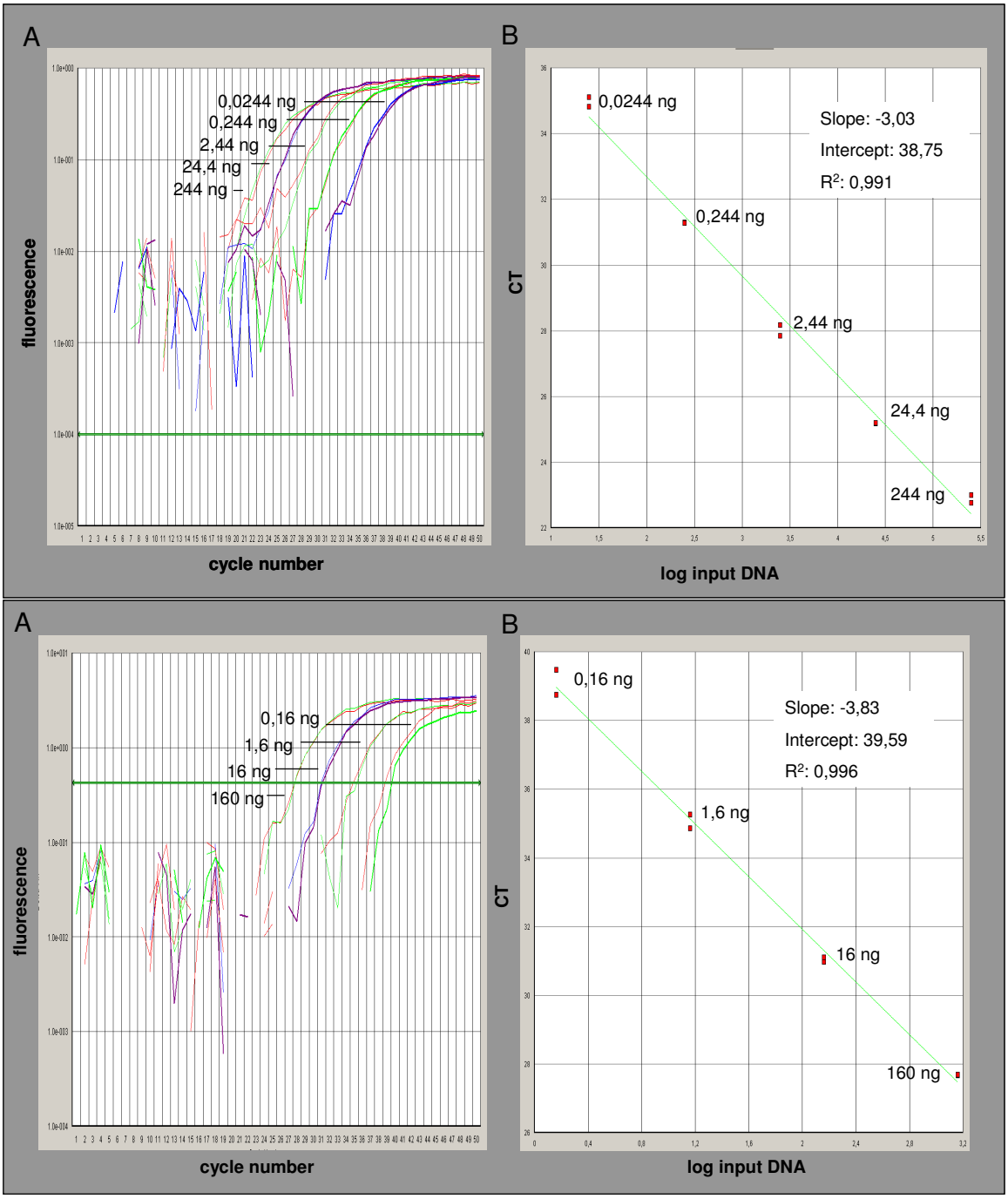

Figure 4.4 Creation of a standard curve using TaqMan probes for the methylated form of the genes *P16* (upper panel) and *RASSF1A* (lower panel). Dilutions of sodium bisulfite converted fully methylated DNA (1:1, 1:10, 1:100, 1:1000, 1:10000) were analyzed in duplex. (A) shows the RT-PCR curves obtained. For the generation of the standard curves (B) the observed Ct-values were plotted against the logarithm of the input DNA. The data derive from one preparation, the RT-PCR was done in duplicate and the data represent the means +/-SD.

In parallel we created an alternative internal DNA standard to be applied for the restriction endonuclease procedure (details see chapter 4.3). For this purpose, DNA was extracted from Hep3B cells, but was not *Sss*I treated or bisulfite converted, because no such alteration is needed for this assay.

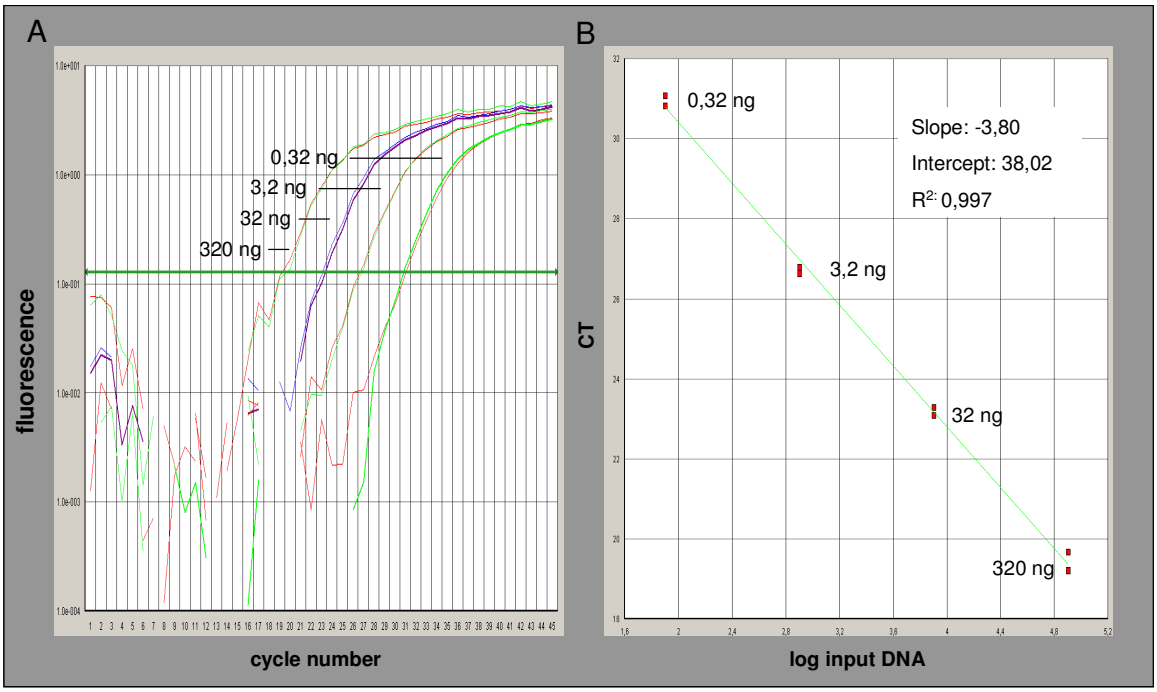

Figure 4.5 Creation of a standard curve using the SYBRE-Green assay for the gene *RASSF1A*. Dilutions of DNA (1:1, 1:10, 1:100 and 1:1000) were analyzed in duplex. (A) shows the RT-PCR curves measured. For the generation of the standard curve (B) the observed Ct-values were plotted against the logarithm of the input DNA. The data derive from one preparation, the RT-PCR was done in duplicate and the data represent the means +/-SD.

4.1.4 Comparison of PMR and %methylation procedures

We chose two methods to calculate the percentage of methylated DNA (for details see chapter 3.9). For this purpose we diluted fully methylated DNA with unmethylated DNA.

We prepared 8 different solutions with a total amount of DNA of either 3 ng/µl or 30 ng/µl and variable proportions of 99%, 50%, 10% or 1% of methylated DNA.

The DNA in the samples was quantified using RT-PCR and the concentrations were calculated by use of the standard curve.

The PMR of each sample was calculated using the following formula:

$$((\text{GENE:}ACTB)_{\text{sample}} / (\text{GENE:}ACTB)_{\text{fully methylated reference DNA}}) \times 100$$

The %methylation of the same samples was also calculated using the following formula:

GENE M/ (GENE M + GENE U) x 100

The principles of the two formulas are shown in Figure 3.8

Figure 4.6 compares the expected results (99%, 50%, 10% and 1%) with the results obtained by the two mathematical procedures. When applying the PMR approach the calculated percentages of DNA were mostly only 50% of the actual value. When using the %methylation procedure, the discrepancies were much less pronounced.

These results indicate that the calculation of the %methylation method yields more reliable data.

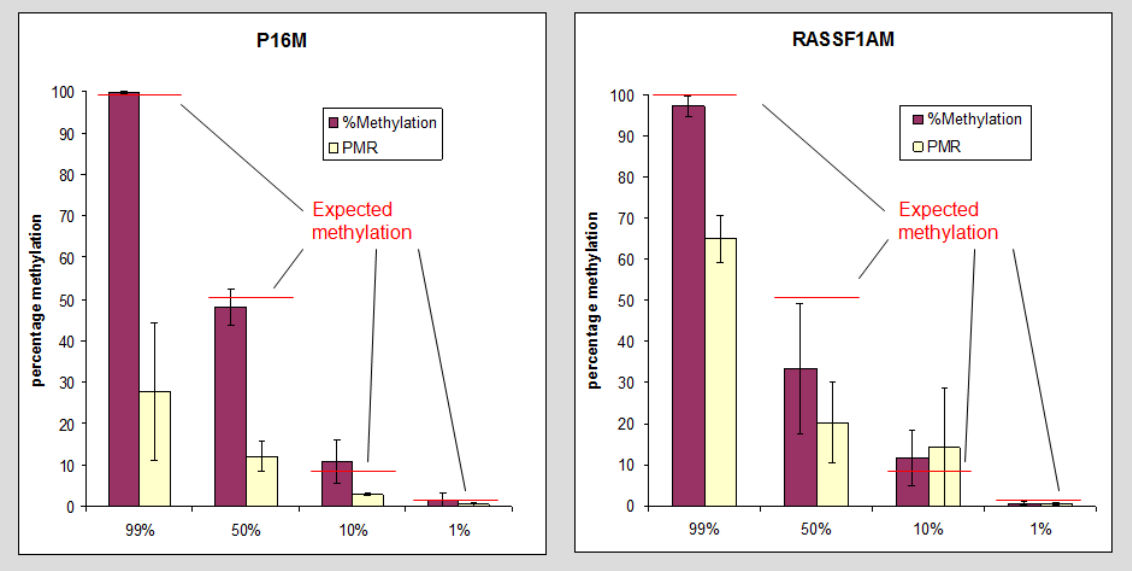

Figure 4.6 Comparison of PMR and %methylation procedures. The red lines indicate the expected results, i.e., 99%, 50%, 10%, or 1% of methylated DNA in 12 ng of total DNA. Preparations were analyzed in duplicates. The calculated results represent the mean +/SD of two independent preparations analyses.

4.2 *Optimization of extraction systems of DNA in serum*

Different methods for extraction of DNA from serum are known. Because of the limited amount of DNA in serum the application of the optimal extraction procedure appeared critical for the success of the present work. We therefore decided to compare different methods of isolation of DNA from serum.

For this purpose we used serum drawn from a healthy person and added a defined number of HCC1.2 cells. Then we applied the different extraction kits, which are commercially available (see chapter 3.3) and quantified the extracted DNA.

We found that the Approach 1 (Charge Switch gDNA 1 ml Serum Kit from Invitrogen) showed by far the best results. It enabled to extract in mean 12.21 ng of *RASSF1AM*-DNA or 12.04 ng of *ACTB*-DNA per ml serum supplemented with 1000 cells and 3.19 ng of *RASSF1AM*-DNA or 5.41 ng of *ACTB*-DNA per ml serum admixed with 100 cells.

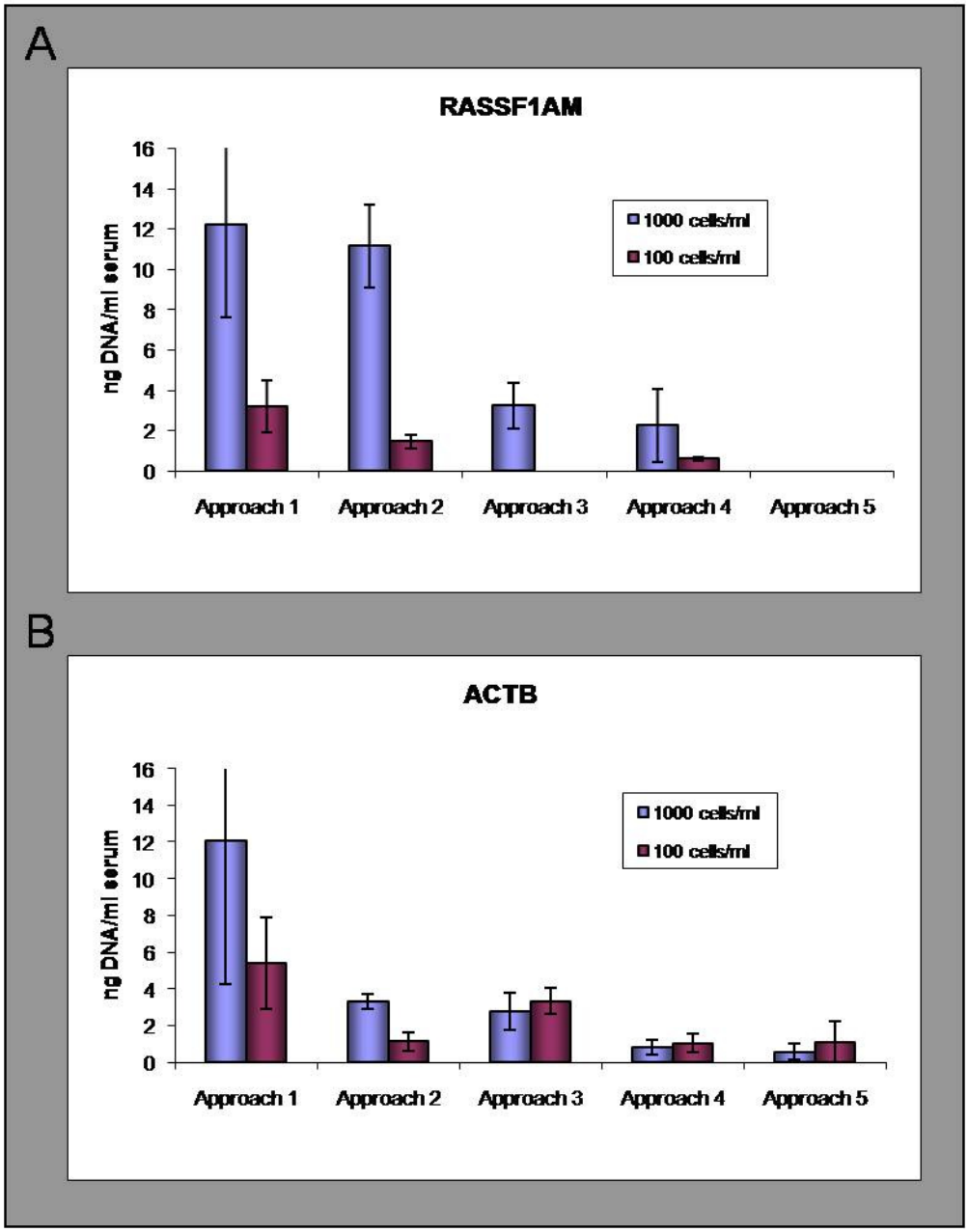

Figure 4.7 Comparison of the DNA yield after DNA isolation from serum supplemented with 100 and 1000 HCC1.2 cells/ml **using five different commercially available kits. With each kit isolation of 1** ml **serum was performed twice. The extracted DNA was quantified with RT-PCR in duplex. The mean values +/- SD are shown.**

In addition we evaluated if approach 1 shows linearity between the amount of input DNA and the output of the assay. Therefore, we mixed serum drawn from a healthy person with a defined number of HCC1.2 cells, i.e., 5000 cells/ml, 1000 cells/ml, 500 cells/ml and 100 cells/ml. Then the extracted DNA was quantified using RT-PCR and the primerset for *P16M*. The logarithm of amount of input cells was plotted on the x-

axis and the observed RT-PCR signal was plotted on the y-axis. Figure 4.8 shows that the RT-PCR signal is linear to the input of cells. This indicates that the DNA is extracted quantitatively.

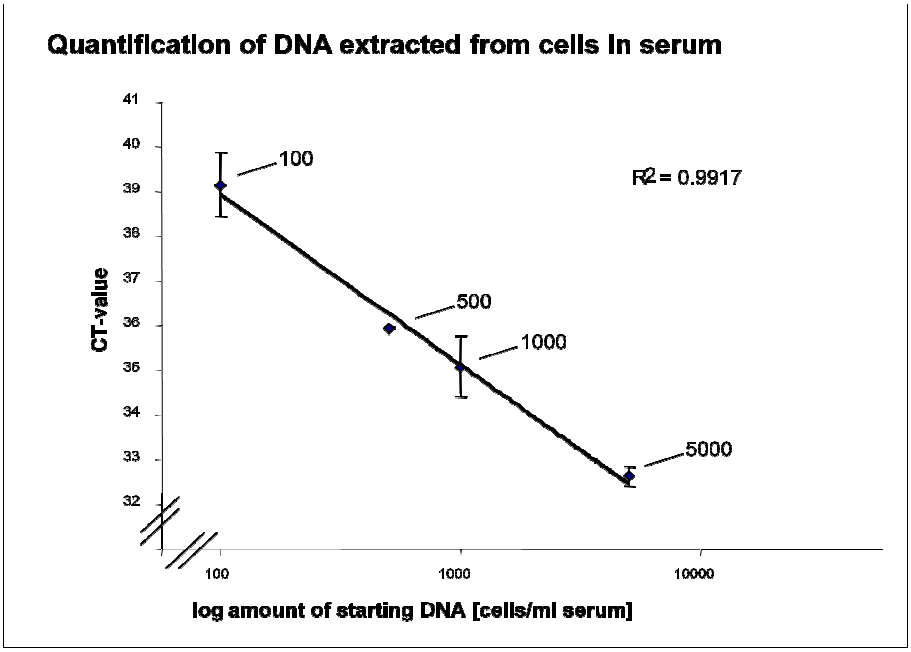

Figure 4.8 Linearity of amount of cells in serum and the RT-PCR signal. 100, 500, 1000, or 5000 HCC1.2. cells were added to 1 ml serum of a healthy donor. DNA was extracted, sodium bisulfite converted and RT-PCR was performed in duplex using the primer set for *P16M*. The mean values +/-SD are shown.

4.3 *Restriction Enzyme assay*

To assess, if the restriction endonuclease assay is a suitable alternative to the bisulfite-dependent methods, we firstly tested the linearity of this method. We used DNA extracted from HCC1.2 cells, in which the promoter region of *RASSF1A* is methylated as we could show before (details in chapter 4.1.1). Thus, we expected a positive RT-PCR signal, which we could detect as a matter of fact. We prepared a 1:5 dilution of the DNA and performed the restriction enzyme assay as described previously (for details see chapter 3.10).

The observed Ct-values were plotted against the logarithm of the amount of input DNA and the regression line is shown in Figure 4.9. Only the first 5 steps of these dilutions showed a positive RT-PCR signal. When we used the same dilutions for

the qMSP as described before also the sixth step (0.512 ng of input DNA) showed a positive signal, indicating that the bisulfite dependent method is more sensitive.

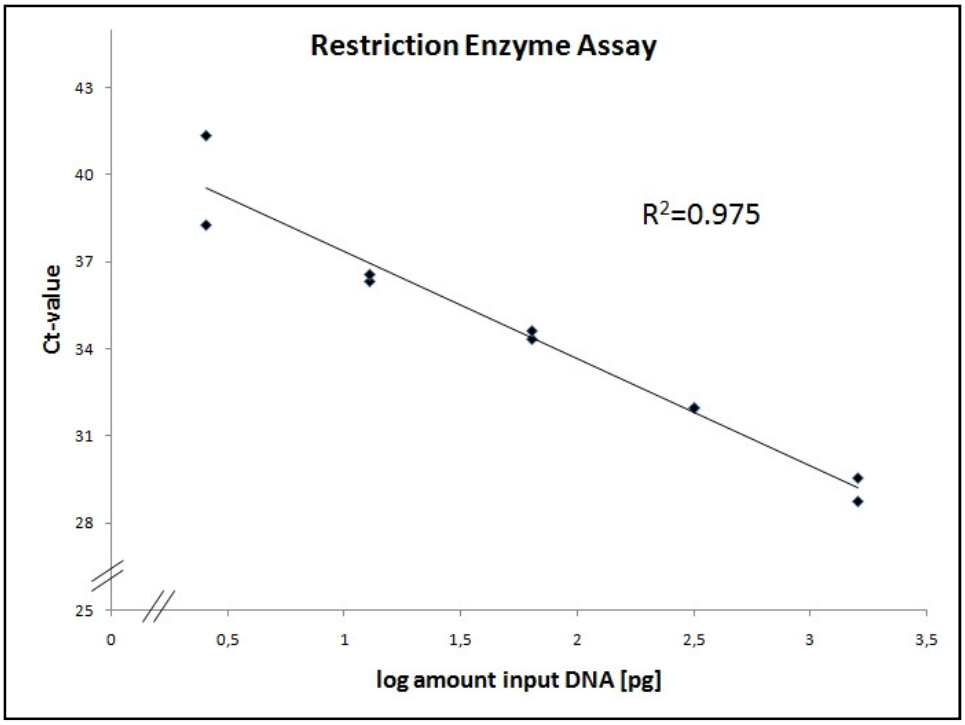

Figure 4.9 Linearity of amount of input DNA and observed Ct-value using the restriction enzyme assay. 1600, 320, 64, 12.8, 2.56 ng total DNA extracted from HCC1.2 cells were added to a restriction endonuclease assay. The RT-PCR was done in duplicate and the observed CT-values are plotted against the logarithm of input DNA.

4.4 *Patients*

In a final step we used the system tested previous to investigate the methylation status in serum samples drawn from 9 CRC patients. We also used samples from 8 lung cancer patients (to be precisely small cell lung cancer (SCLC)), because in lung cancer tumors more than 70 % were tested positive for methylated *RASSF1A* in recent studies and in CRC patient samples we could only expect 20 to 40 % positive samples (50).

The samples were cleaned up and then routinely split. Next the primers and probe for methylated *RASSF1A*, for methylated *P16* and for the according unmethylated form of

the genes were used. In addition also the primers and probe for the reference gene *ACTB* were used. Thus, the %methylation and also the PMR value could be calculated.

In Figure 4.10 the results of the methylation positive patients are shown. When a patient showed a positive signal a second blood sample was drawn to confirm the first result. There was no positive signal in the second sample of one patient. But the total amount of DNA was also extremely low, so it is possible that the amount of methylated DNA was too low to be detected.

In patient 2 the amount of *RASSF1AM*-DNA was 1.41+/-2.09 ng/ml in the first blood sample and 4.06+/-2.09 ng/ml in the second sample. The amount of *RASSF1AU*-DNA was 52.04+/-17.52 and 5.62+/- 3.41. Thus, the calculated %methylation was 3% in the first sample and 42% in the second sample. In addition to this the PMR of the second sample was also calculated as 14%. The PMR of the first sample could not be assessed, because of the low amount of serum that was available.

The amount of *RASSF1AM*-DNA in the sample of patient 5 was 5.73+/-1.38 ng/ml. The amount of *RASSF1AU*-DNA was 5.91+/-2.65 ng/ml in the first sample and 0.59+/-0.09 ng/ml in the second sample. The calculated %methylation was 51% for the first sample and 0% for the second sample and the PMR was 45% and 0%

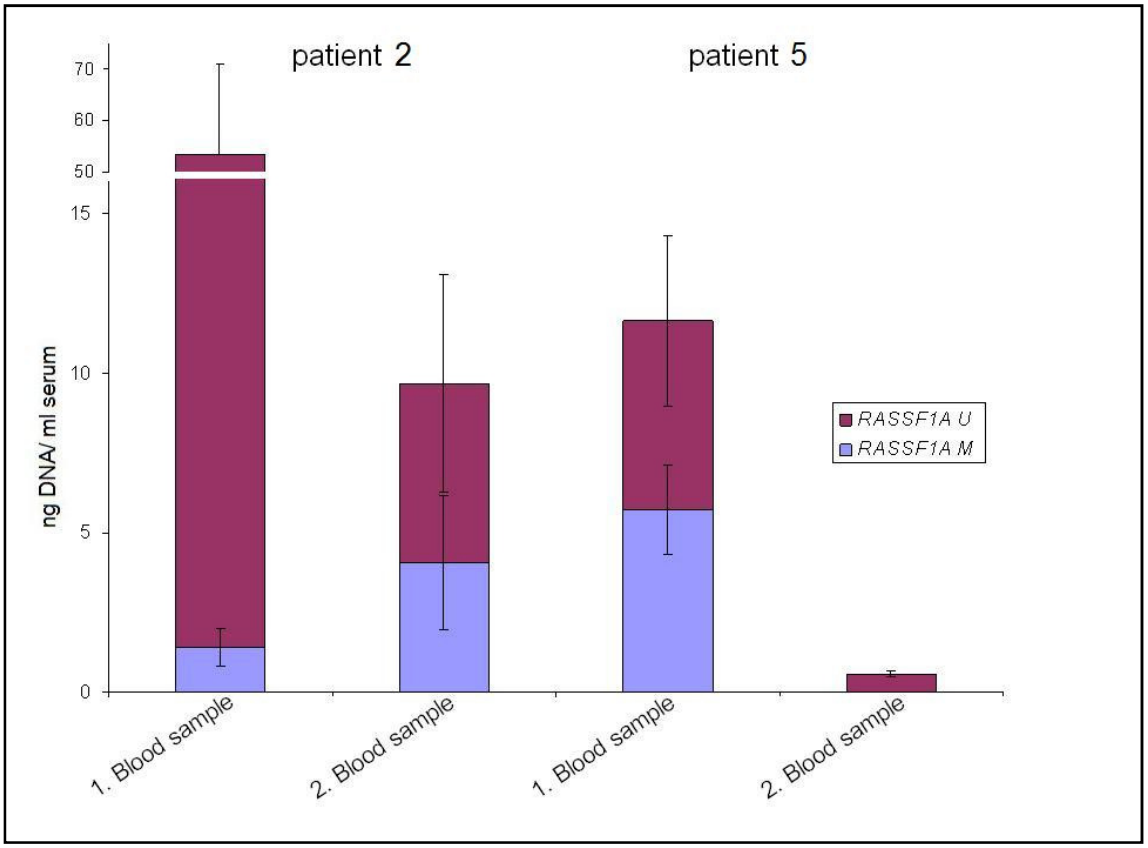

Figure 4.10 Amount of methylated and unmethylated DNA in methylation positive patients. Blood was drawn and analysed according to the protocol. The amount of methylated DNA is given in the lower part of the column, the amount of the unmethylated DNA is shown in the upper part of the column and the absolute height of the column gives the total amount of DNA. The data represent the mean +/- SD of two independent clean ups of DNA and RT-PCR analyses in duplex.

In Table 4-1 the clinical data and the results of the qMSP of the patients included in the present work were given.

Table 4-1 Clinical Data of patients

Sample number	**Sex**	**Age**	**Carcinoma**	**Metastasis**	**Methylation *RASSF1A***	**Methylation *P16***
1	f	58	SCLC	-	n	n
2	m	67	SCLC	-	pp	n
3	f	67	SCLC	-	n	n
4	m	50	SCLC	-	pn	n
5	f	65	SCLC	-	n	n

6	m	58	SCLC	-	n	n
7	m	73	CRC	liver, lung	n	n
8	m	64	CRC	liver, lung	n	n
9	m	77	CRC	liver	n	n
10	f	59	CRC	-	n	n
11	f	66	CRC	liver	n	n
12	m	70	CRC	liver	n	n
13	m	62	CRC	liver, lung	n	n
14	m	64	CRC	-	n	n
15	m	54	SCLC	-	n	n
16	m	78	CRC	liver	n	n
17	m	49	SCLC	-	n	n

m (male), f (female), n (negative result), p (positive result)

Each sample has also been analysed with the primerset for *ACTB* and the results are shown in Figure 4.11. These results represent the total amount of DNA in the serum samples, because the amplification is independent from the methylation status of the gene. We also used it as a positive control to confirm, if DNA is correctly cleaned up and modified. Each sample showed a positive signal when analysed with primers for *ACTB*. The amount of DNA varies from 17.8 +/- 10 ng *ACTB*-DNA/ml serum to 0.18 +/- 0.15 ng *ACTB*-DNA/ ml serum.

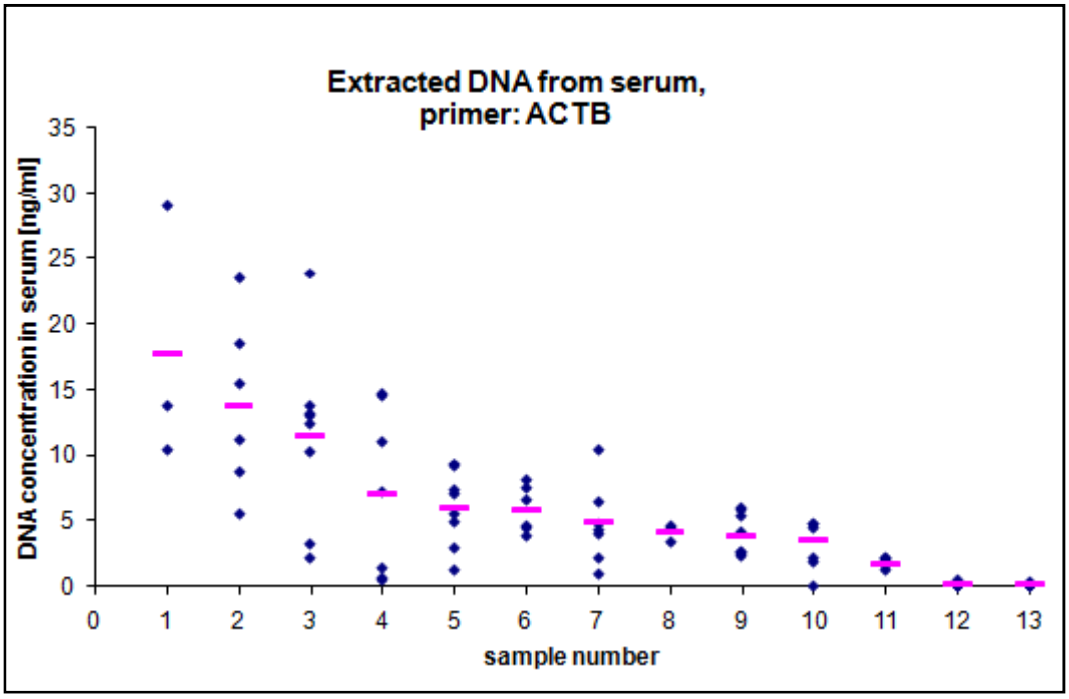

Figure 4.11 Total amount of DNA in serum samples. The DNA of the serum of 13 patients was extracted and quantified using the primers and probe for the reference gene *ACTB*. The measured values and their means are given.

5 DISCUSSION

CRCs are the third most common malignant tumors worldwide with an incidence of 570,000 per year. Therefore it is important to find sensitive and reliable methods for the diagnosis and the clinical monitoring. So far, numerous genes have been identified which are frequently methylated in colorectal carcinomas. The aim of our project was to establish quantitative methods for the detection of the amount of methylation of the genes *P16* and *RASSF1A* in serum, which is more easily available than tumor samples.

In a first step we determined that DNA extracted from the cell line HCC1.2 shows methylation of *RASSF1A* and *P16*. On the other hand the DNA extracted from Hep3B showed the unmethylated form of *RASSF1A* and *P16*. Therefore, we could use the DNA extracted from the two cell lines as a standard DNA in further investigations. Similar results concerning the *RASSF1A* patterns of the cell lines used were recently published by Macheiner et al (76).

In a next step we tested the sensivity and linearity of the RT-PCR approach. We found that we could detect as less as 25 pg of input DNA. Furthermore unmethylated DNA did not interfere with the detection of methylated DNA. It is shown in Figure 4.2 that there is also a sufficient linearity of input DNA and observed RT-PCR signal. These findings allowed us to create a standard curve, which could then be used to quantify DNA extracted from serum.

Standard curves published by Heid et al (70) or Shivapurkar et al (30) showed similar dynamic ranges and sensitivities. Also the requirements demanded by Fackler et al that the correlation coefficient of a standard curve should be 0.99 or higher and that the slope should be approximately -3.33 (indicating the 2-fold increases in PCR product per cycle in the linear phase of the quantitative PCR reaction) were reached by our method.

After we had confirmed that our standard curves are suitable for the quantification of methylated and unmehtylated DNA, we compared two different methods of calculating the level of methylated DNA in a certain sample. One is the PMR which compares the ratio of a gene of interest and a reference gene of the sample with the ratio of the gene of interest and a reference gene of a fully methylated reference. The other approach calculates the %methylation by dividing the amount of methylated DNA of a certain

gene by the sum of methylated and unmethylated DNA. The details of both formulas are given in chapter 3.9.

Our results indicate that the calculation of the %methylation method yields more reliable data, whereas Fackler et al found no differences in the accurency of the two methods (36).

The main variation of both approaches is the calculation of the total amount of input DNA. Using the PMR method the input DNA is determined by an independent reference gene. The %methylation uses two different primersets for the gene of interest (one for the unmethylated form and one for the methylated form) for calculating the input DNA.

The advantage of the latter approach is that the amount of a certain gene is determined directly. A disadvantage is that only two extremes of the whole range of methylation patterns are covered with this assay. Therefore, Eads et al speak of a semi-quantitative approach (33). For example, if there are 6 CpGs covered by the primers and the probe, there are $2^6 = 64$ different theoretical permutations of methylation status. If the primers and probe recognize only the fully methylated or the fully unmethylated version of a sequence, then only two out of 64 possible combinations are being investigated. If the other 62 permutations are prevalent in the genomic DNA sample, then obviously the signal will be low. This large number of possible permutation outlined above is the reason, why Binh et al suggest that methylation-independent control reaction should be used rather than relying on an unmethylated version of the reaction (77).

In our approach we used only mixtures of fully methyled and fully unmethylated DNA, which does not represent the circumstances in vivo. This may be the reason for the better outcome of the assay using methylated and unmethylated primers and probes under our experimental conditions.

Furthermore an advantage of the PMR method it that the use of a reference gene is reducing the analyses steps if more than one gene is determined simultaneously. In this case the reference gene can be assessed once and then be compared to all different genes and not each unmethylated form according to the different genes has to be assessed.

The next crucial step in the present work was to optimize the extraction of DNA, because of the low amount of DNA present in the serum. The best result showed a commercially available kit (Charge Switch gDNA 1 ml Serum Kit) that is based on a magnetic bead clean-up system, whereas the kits using a column system did not show such a good extraction rate. These findings are similar to the one published by Stemmer et al. They found that the column based Qiagen DNA isolation kit (see chapter 3.3.2) only extracted 13 ng DNA/ ml plasma in mean. When using a magnetic beads based system they could extract 46 ng/ml plasma from the same samples (78).

The Maxwell DNA-Cleanup, which is a magnetic beads based automated method, showed weaker results. On the other hand the advantage of such a method is the processing of more samples in a shorter time. It is also possible to clean up a higher amount of serum and to subsequently pool the DNA to obtain a higher yield of DNA. The automation of an assay is also an important milestone on the way to make an assay suitable for the routine analyses.

Finally, we chose the Charge Switch gDNA 1 ml Serum Kit for the processing of serum samples of patients. This kit showed not only the best results, when assessing the amount of cleaned-up DNA, also the linearity of amount of input of cells and output of DNA was sufficient (see Figure 4.8).

In a next step we also compared the bisulfite method with the the use of methylation restriction enzymes to assesses the methylation status of a sample.

The bisulfite dependent methods are somewhat prone to false-positive results, because incompletely converted sequences in the bisulfite treatment can be amplificated by primers designed to detect methylated DNA. Kristensen et al suggest that bisulfite treatment remains the main source of variability in the analyses of DNA methylation. Recent results show that incomplete conversion may be in order of 2%, even when a commercial kit is used (79).

In our experiments the bisulfite dependend methods showed a better applicability when samples contain low amounts of DNA, e.g. a dilution containing 128 pg DNA /µl showed a positive result, when using the bisulfite method, whereas no positive signal was obtained when the same dilution was processed using the methylation specific restriction enzymes. A possible reason for this weaker outcome is that no clean-up of

the digested DNA was performed. Thus, the RT-PCR could be inhibited by reagents present in the sample. A proper method of cleaning up the DNA may improve the result.

As a final step, we also wanted to test our system with serum samples drawn from cancer patients. We determined the methylation status of 9 samples from CRC patients and 8 samples from patients suffering from SCLC. We investigated also lung cancer patients, because we expected a higher rate of positive results. Agathanggelou et al reviewed the percentage of tissue samples of different tumor entities showing *RASSF1A* methylation. He reported that about 70% of SCLC and 20 - 40% of CRC patients investigated were positive with respect to *RASSF1A* methylation (50). One study even showed 81% positive CRC patients, but normal colonic mucosal tissue was also methylated in 49% of cases studied. Thus, the latter result has to be evaluated critically. Data of the methylation status of *P16* in CRC patients show ratios of 13 - 47% in tumor samples (for further details see Table 2-2).

In serum samples we expected a lower number of patients with positive results than in tumor tissue samples, as there are reports that the percentage of methylated *RASSF1A* in serum of CRC patients ranges from 0 to 29% only (for further details see Table 2-1). The percentage of methylation of *P16* has been reported to range from 0 to 40% (Table 2-2).

Actually, we found methylated *RASSF1A* in 25% (n=8) of the serum samples drawn from SCLC patients and in 0% (n=9) of the samples derived from CRC patients. *P16M* was negative for all samples of both groups of cancer.

This relatively low number of cases, could possibly be explained by a too low amount of DNA present in the serum.

For CRC patients the reason may be the unique physiological circumstances. In contrast to tumors of other organ systems, CRCs drain predominately via the mesentric/portal veins to the liver. Taback et al compared the frequency of hypermethylation of certain tumor-suppressor genes (e.g. *P16* and *RASSF1A* and three other genes) in serum collected from the mesenteric/portal system or the peripheral vein. They found that DNA methylation was more frequently detected in serum derived from the mesenteric/portal system. For *RASSF1A* the frequency was 6% in the mesenteric/portal system compared to 0% in the peripheral vein. In total 27% of the patients showed

methylation of any of the examined genes in the mesenteric/portal system compared to 6% in peripheral vein. The authors of this study considered this as a reason for the lower frequency of tumor DNA in CRC patients' serum/plasma compared with solid tumors of other organ systems (61).

Recently we found no methylated DNA in serum samples of hepatocellulare carcinoma patient before start of treatment. However chemoembolisation, which causes necrosis of a relatively large amount of tissue, lead to a detectable amount of methylated DNA (data not shown). This is further evidence that there is not enough tumor DNA present in serum of patients prior to any cytotoxic treatment.

We also used the primerset for *ACBT* to evaluate the level of total DNA in the serum samples, which ranged from 17.8 +/- 10 to 0.18 +/- 0.15 ng DNA/ ml serum. These values are somewhat below those published by others, e.g. 12 ng/ml in mean in serum samples of 16 CRC patients (80); 105-709 ng/ml in plasma samples of 48 CRC patients (81); 46 ng/ml in mean in plasma from lung or CRC patients (78).

We conclude that we were faced with considerable difficulties to detect methylated DNA in serum of patients before treatment. This limitates the application of the assay established in the present work for screening purpose. However, the positive data of liver cancer patients after chemoembolisation are first evidence that our approach could reliably indicate response to therapy. This aspect should be investigated in further studies on a larger number of patients.

5.1 *Conclusions*

- RT-PCR is a valuable tool to asses the methylation status of genes; it shows linearity over a wide range of input DNA and produces results with high accuracy.

- The best method for cleaning up DNA from serum is based on the use of magnetic beads.

- The calculation method %methylation yields more accurate results under the present experimental conditions. However, the calculation of the PMR is more reasonable for samples derived from patients and is more practicable, because of the reduced amount of work.

- The application of methylation specific restriction enzymes did not show any enhance to the sensitivity of the assay. Maybe some further improvements of the protocol could lead to a better result.

- The relatively low rate of positive results in the samples derived from CRC and SCLC patients could be explained by the low amount of tumor DNA present in serum. This points toward major limitation of the assay established in the present work.

6 ABSTRACT

Colorectal carcinomas (CRCs) are the third most common malignant tumors worldwide with an incidence of 570,000 per year. Several risk factors concerning lifestyle, nutrition and exercise are known. Thus, effective primary and secondary prevention must be developed to reduce mortality.

It is important to find sensitive and reliable methods for the diagnosis and the clinical monitoring of this disease. An approach for this purpose is the use of epigentically altered DNA as a marker for the CRC. So far, numerous genes have been identified which are frequently methylated in CRCs. Diet is a major aspect of the enviroment that may influences DNA methylation and studies on the role of dietary patterns in epigenetic alterations in CRC are increasing.

The aim of our project was to establish methods to detect and quantify the methylated forms of the genes *P16* and *RASSF1A* in serum, which is more easily accessible than tumor samples.

First we had to investigate if the approach to quantitatively detect methylated *RASSF1A* and *P16* is suitable to analyze DNA in serum. We were able to detect even a low amount of DNA (25 pg). Moreover, a linearity of input DNA and real-time-PCR signal was observed over a wide range of input DNA (5 logs). These findings enabled us to establish a standard curve to quantify the DNA in a given sample.

For calculating the methylation level of a certain gene in serum we compared two published approaches. We could show that the method of calculating the %methylation obtained more accurate results under our experimental conditions. On the other hand the second approach of calculating the Percentage of Methylation (PMR) is more reasonable and is more practicable to perform, especially when a larger number of genes has to be investigated.

Second we compared the efficiency of five different, commercially available DNA isolation kits. We found that the kit applying magnetic beads provided the best extraction rates. An automated version of a clean-up kit has also been tested, and even though the amount of extracted DNA was lower, this method could be very useful in establishing a routine test system.

We tested also another, sodium-bisulfite independent, restriction enzyme based method to quantitatively detect methylated DNA sequences in serum. We observed better results with the bisufite-dependent method. But maybe some further improvements of the protocol could lead to better results of the restriction enzyme assay.

Using the most efficient protocol, we analyzed serum samples of small cell lung cancer (SCLC) and of CRC patients. We could detect methylation of *RASSF1A* in 2 of 8 (25%) lung cancer patients and in 0 of 9 (0%) of CRC patients. When using the primer and probes for *P16* all samples of both cancer groups were negative.

In conclusion, we were able to detect methylated DNA in serum of tumor patients, but only in a limited number of cases. As a possible reason for this poor outcome we suppose the low amount of DNA present in the samples. Because it is likely that the DNA concentration is higher in serum of patients, after a response to a therapy the method established in the present work could be of use for monitoring a clinical intervention.

7 ZUSAMMENFASSUNG

Kolorektale Karzinome sind die dritt häufigsten bösartigen Tumore weltweit mit einer Inzidenz von 570.000 Erkrankungen pro Jahr. Verschiedene Risikofaktoren sind bekannt, die die Lebensweise, die Ernährung und die körperliche Bewegung betreffen. Daher müssen wirkungsvolle primäre und sekundäre Interventionen entwickelt werden um die Mortalität zu reduzieren.

Es ist wichtig eine sensitive und verlässliche Methode für die Diagnose und die Verlaufskontrolle dieser Erkrankung zu finden. Ein Ansatz für diesen Zweck ist, epigenetisch veränderte DNA im Serum als Marker für das kolorektale Karzinom zu nützen. Es wurde schon eine Reihe von Genen identifiziert, die in kolorektalen Karzinomen methyliert sind. Die Ernährung ist ein wesentlicher Aspekt, der die DNA Methylierung beeinflussen könnte und die Zahl der Studien, die die Rolle des Ernährungsmusters in den epigenetischen Veränderungen in kolorektalen Karzinomen untersuchen, nimmt zu.

Das Ziel unseres Projektes war es, eine Methode für die Detektion und Quantifizierung von methyliertem *RASSF1A* und *P16* im Serum zu finden. Serum bietet den Vorteil, dass es leichter zugänglich ist als Tumorproben.

In einem ersten Schritt untersuchten wir, ob der Ansatz zur quantitativen Detektion von methylierten *RASSF1A* und *P16* geeignet ist DNA aus Serum zu analysieren. Wir konnten auch eine geringe Menge von DNA (25 pg) detektieren. Außerdem konnten wir zeigen, dass der Zusammenhang zwischen eingesetzter DNA-Menge und RT-PCR Signal über einen weiten Bereich linear ist (fünf 10er Potenzen). Diese Ergebnisse ermöglichten es uns eine Standardkurve zu etablieren um DNA Proben zu quantifizieren.

Für die Berechnung des Methylierungslevels einer Probe verglichen wir zwei verschiedene publizierte Methoden. Bei den von uns gewählten Versuchsansatz zeigte sich, dass die Berechnung des %Methylierung Werts akkuratere Ergebnisse lieferte. Hingegen ist der theoretische Hintergund der zweiten Methode, des PMR, vernünftiger. Diese Methode ist weiters praktikabler, da sie vor allem bei einer größeren Anzahl von untersuchten Genen einen zeitlichen Vorteil bringt.

In einem weiteren Schritt untersuchten wir die Effizienz fünf verschiedener kommerziell erhältlicher Kits für die Aufreinigung von DNA. Wir konnten zeigen, dass der Kit, der auf einer Methode basiert, die *magnetic beads* nützt, die besten Ergebnisse erzielte. Eine automatisierter Aufreinigungskit lieferte eine geringere Menge an isoliertet DNA. Diese Methode könnte jedoch nützlich für die Etablierung eines routinemäßigen, klinischen Tests sein.

Wir testeten auch einen Bisulfit-unabhängigen, auf Restriktionsenzymen basierende Methode für die quantitative Detektion von methylierten DNA Sequenzen im Serum.

Wir beobachteten bessere Ergebnisse mit der Bisulfite-abhängigen Methode. Aber eine Verbesserung des Arbeitsprotokolls könnte zu besseren Ergebnissen des Restriktionsenzyms Ansatz führen.

Anschließend benutzen wir das geeignetste Protokoll um Serumproben von Patienten mit kleinzelligen Lungenkarzinom und kolorektal Karzinom zu untersuchen. Wir konnten bei 2 von 8 (25%) Bronchialkarzinom Patienten und 0 von 9 (0%) kolorektal Karzinom Patienten methyliertes *RASSF1A* detektieren. Bei der Untersuchung auf *P16* waren alle Proben aus beiden Karzinom Gruppen negativ.

Zusammenfassend kann man sagen, dass es uns gelungen ist eine Methode zu etablieren mit der wir methylierte Tumor DNA im Serum nachweisen können, aber nur bei einer relativ geringen Anzahl von Patienten. Der Grund für dieses niedrige Ergebnis könnte der geringe Gehalt von DNA im Serum sein. Da die Konzentration der Tumor DNA nach einer Therapie vermutlich ansteigt, könnte die in dieser Arbeit etablierte Methode von Nutzen für die Verlaufskontrolle von klinischen Interventionen sein.

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Abbreviations

ACTB	β-Actin
ACTB-DNA	Amount of DNA detectet with the primerset for β-Actin
ATCC	American Type Culture Collection
CIMP	CpG Island Methylator Phenotype
CpG	5'-CG-Dinucleotide
CRC	Colorectal Carcinoma
Ct	Cycle treshhold
DHF	Dihydrofolate
DNA	Desoxyribonucleinacid
DNTM	De novo methyltransferase
DNTP	Deoxyribonucleotide triphosphate
dTMP	deoxythymidine monophosphate
dUMP	desoxyuridine monophosphate
FAD	flavin adenine dinucleotide
FAM	Fluorescein
FCS	Fetal calf serum
FOBT	Fecal occult blood testing
MBD1	Methyl-CpG-binding domain
MeCP2	Methyl-CpG-binding protein
MS	Methionine synthase
MSP	Methylation-specific PCR
*Msp*I	Methylation-spesific restriction enzyme
MTHFR	5-10-methylenetetrahydrofolate reductase
P16	$P16^{INK4a}$
P16M-DNA	DNA quantified with primer for the unmethylated form of *P16*
P16U-DNA	DNA quantified with primer for the unmethylated form of *P16*
PCR	Polymerase Chain Reaction
PMR	Percentage of Methylated Reference
qMSP	Quantitative methylation-specific PCR
RASSF1A	RAS-association domain family 1, isoform A gene
RASSF1AM	Methylated form of *RASSF1A*

RASSF1AM-DNA	DNA quantified with primer for the methylated form of *RASSF1A*
RASSF1AU	Unmethylated form of *RASSF1A*
RASSF1U-DNA	DNA quantified with primer for the unmethylated form of *RASSF1A*
RT-PCR	Realtime-Polymerase Chain Reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SCLC	Small cell lung cancer
*Sss*I	CpG Methyltransferase
TAE Buffer	Tris-acetate EDTA Buffer
TAMRA	Tetramethyl-6-Carboxyrhodamine
THF	Tetrahydrofolate
TS	Thymidylate synthase
%methylation	Percentage of methylation

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August 2006	Start of the thesis at the Institute of Cancer Research

Work experience

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