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**Epigenetic Regulation of the *pKi-67* Gene Promotor:
DNA Methylation Analysis of its CpG island**

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1. Introduction

The Ki-67 protein (pKi-67) is a widely applied marker in routine pathology for determination of cell proliferation activity, particularly in tumors. Over-expression of the Ki-67 protein has been observed in many types of malignancies and the expression level of this protein has often been demonstrated to correlate with the clinical course and prognosis of tumors (Brown and Gatter, 2002). In colorectal cancer, the expression of the Ki-67 protein has been intensively investigated: the protein expression increases progressively from hyperplasia, through different degrees of dysplasia in adenomas, to reach the highest level in adenocarcinoma (Kikuchi et al., 1997). Numerous studies have been performed to examine the correlation between the Ki-67 protein expression and the prognosis of colorectal cancer patients as well. However the results were inconsistent (Allegra et al., 2002; Jansson and Sun, 1997; Palmqvist et al., 1999). Recently, the *pKi-67* mRNA was reported to have significant prognostic value in colorectal cancer: lower level of the *pKi-67* mRNA was associated with favorable prognostic outcome (Duchrow et al., 2003). This result pointed us towards the importance of the *pKi-67* mRNA transcription regulation mechanisms, which have not been studied thoroughly yet. Interestingly, the promoter region of the *pKi-67* gene (GenBank accession number gi: 1944550) has been found to harbor a large CpG dinucleotide rich fragment, which is generally considered to be a potential target for DNA methylation modification. The methylation status of these CpG dinucleotides within the *pKi-67* promoter is however unrevealed. Thus, the aim of this study was to examine if DNA methylation modification of the *pKi-67* promoter CpG-rich region can be observed and if such would impact on the *pKi-67* gene transcription level as an epigenetic regulation.

1.1 The Ki-67 protein

The Ki-67 protein was discovered by the prototype monoclonal antibody Ki-67 (Gerdes et al., 1983). This antibody was generated by immunizing mice with nuclei of the Hodgkin lymphoma cell line L428.

The name “Ki-67” was derived from the city of origin (Kiel) and the clone number of the 96-well plate (No.67). As the antigen of Ki-67 was not initially characterized, it was just referred to as the “Ki-67 antigen”. The nature of this antigen remained unclear until it was identified as a protein by screening a cDNA expression library (Gerdes et al., 1991). The complete primary structure of the Ki-67 protein was published two years later after cloning and sequencing the entire cDNA (Schluter et al., 1993). The computer deduced amino acid sequence of the Ki-67 protein revealed two putative nuclear targeting signals (Chelsky et al., 1989; Silver, 1991) and eight potential “bipartite nuclear targeting signals” (Dingwall and Laskey, 1991), indicating that this antigen seems to be a nuclear protein.

The Ki-67 protein has been found to be present in all active phases (G_1 , S, G_2 and mitosis) of proliferating cells, but to be absent in quiescent cells (Gerdes et al., 1984). This characteristic expression pattern made the protein an interesting marker to assess proliferation activity in a given cell population, particularly in tumors. However, the application of the prototype Ki-67 antibody was limited because it could not be used in formalin-fixed paraffin sections in routine histopathology. Later on, a panel of new monoclonal antibodies against Ki-67 protein were raised and designated as MIBs (Molecular Immunology Borstel). One of the monoclonal antibody, MIB-1, can be used on paraffin sections and thus overcoming the major drawback of the original Ki-67 antibody (Cattoretti et al., 1992).

1.1.1 Expression of the Ki-67 protein

1.1.1.1 Expression pattern

The Ki-67 protein is exclusively expressed in proliferating cells (G_1 , S, G_2 and mitosis). Quiescent or resting cells in G_0 phase do not express Ki-67 protein. The Ki-67 protein staining increases during S phase (Bruno and Darzynkiewicz, 1992; du Manoir et al., 1991) and G_2 phase (du Manoir et al., 1991) and reaches its maximum intensity in the metaphase (du Manoir et al., 1991; Starborg et al., 1996; Verheijen et al., 1989). The staining signal decreases from anaphase and telophase to G_1 phase (Braun et al., 1988; Starborg et al., 1996).

The half-life of the Ki-67 protein is estimated to be 90 minutes (Heidebrecht et al., 1996). The topographical distribution of the Ki-67 protein is also cell cycle related. In the early part of G₁ phase the Ki-67 protein staining is restricted to numerous foci throughout the nucleoplasm. Such foci have been suggested to correspond to the sites of reforming nucleoli (du Manoir et al., 1991; van Dierendonck et al., 1989). Later in the mid-G₁ phase, the Ki-67 protein is mainly localized in the reassembled nucleoli. During S and G₂ phase, the protein is detected in the nucleoli and later throughout the nucleoplasm (Braun et al., 1988; du Manoir et al., 1991; van Dierendonck et al., 1989). A redistribution of the Ki-67 protein occurs during mitosis. In the prophase, the protein forms a fine meshwork beside the condensing chromatin (Verheijen et al., 1989). During metaphase, a bright Ki-67 protein staining is visible covering the surface of individual chromosomes (Braun et al., 1988; du Manoir et al., 1991; Starborg et al., 1996; van Dierendonck et al., 1989). Afterwards, the Ki-67 protein staining decreases rapidly during anaphase and telophase.

1.1.1.2 Different isoforms of the Ki-67 protein

The Ki-67 protein can be isolated from proliferating cells as two isoforms of 395 and 345 kDa respectively. This is due to alternative splicing of the *pKi-67* mRNA with or without exon 7 (Duchrow et al., 1994). Recently, three new splicing variants of human *pKi-67* mRNA were reported (Schmidt et al., 2004).

1.1.2 Biological significance of the Ki-67 protein

Although the Ki-67 protein is a widely used proliferation marker, its biological function is not completely understood. Functional research of the Ki-67 protein has been hampered by (1) its large size, (2) high susceptibility to protease cleavage, and (3) lack of apparent homolog proteins of known function that could act as a comparable reference during functional characterization (Duchrow et al., 1994). However, it has been demonstrated that the Ki-67 protein has an essential role in cell proliferation: Schluter et al. (1993) reported that incubation of cultured IM-9 cells with oligonucleotides complementary to *Ki-67* mRNA led to inhibition of DNA synthesis in a dose dependent manner.

Moreover, microinjection of antibodies against murine Ki-67 equivalent into the nuclei of Swiss-3T3 fibroblasts resulted in a decreased rate of cell division (Starborg et al., 1996). Similar results were obtained by microinjection of antibodies against human Ki-67 antigen (Heyden, 1997). Further investigations indicated that the Ki-67 protein might be responsible for transition of cells from the G₁ to the S phase and for completion of S phase (Duchrow et al., 2001). The observations by Schmidt et al. (2003) suggested that the Ki-67 protein was a Ran-associated-protein regulating disintegration and reformation of the nucleolus and thereby determining entry and exit of the M phase.

1.1.3 Application of the Ki-67 protein as a proliferation marker

Over-expression of the Ki-67 protein has been detected in many kinds of malignant tumors (Brown and Gatter, 2002). Numerous immunohistochemical studies have been performed to examine the correlation between the Ki-67 labeling indices and the prognosis of different types of malignancies using monoclonal Ki-67 antibodies, e.g., MIB-1. An investigation of multiple myeloma patients revealed that Ki-67 expression was correlated with staging and prognosis of this disease (Drach et al., 1992). Another study suggested that Ki-67 expression could help to distinguish multiple myeloma from monoclonal gammopathy of unknown significance (Miguel-Garcia et al., 1995). For soft tissue sarcomas, the Ki-67 index has also been demonstrated to be a good indicator for prognosis (Heslin et al., 1998; HUUHTANEN et al., 1999; Rudolph et al., 1997; Ueda et al., 1989). A similar correlation was observed in breast cancer (Molino et al., 1997). Among prostate cancer patients, Ki-67 expression has been determined to be an independent and significant prognostic factor for disease specific survival (Aaltomaa et al., 1997; Borre et al., 1998). In addition, it was suggested to be an independent predictor of disease recurrence and progression in those patients who underwent radical prostatectomy (Bai et al., 1999; Bettencourt et al., 1996; Bubendorf et al., 1996; Keshgegian et al., 1998; Moul et al., 1996) or transurethral resection (Stattin et al., 1997).

For colorectal cancer, the prognostic relevance of the Ki-67 protein expression remained uncertain. Analyses of several large series of colorectal cancer patients did not find prognostic differences depending on the high or low expression level of Ki-67 protein (Allegra et al., 2002; Jansson and Sun, 1997). Palmqvist et al. (1999) however observed an independent prognostic role of the Ki-67 protein in 56 Dukes' B stage colorectal cancer patients when determined at the invasive margin. When evaluating the *pKi-67* mRNA level in colorectal cancers, our own group could show that lower levels of the *pKi-67* mRNA were associated with a more favorable prognostic outcome (Duchrow et al., 2003). Thus, these data suggest that rather the *pKi-67* mRNA than the Ki-67 protein level could aid as prognostic markers in colorectal cancer. This finding pointed our interest towards the mRNA transcription regulation of this gene.

1.2 The *pKi-67* gene

1.2.1 Gene structure

The *pKi-67* gene has been localized on chromosome 10q25-ter. (The *pKi-67* gene is always printed in italic type in this work to distinguish it from the protein). Cloning of the *pKi-67* cDNA and the gene locus revealed a very large gene of about 12,500 bp comprising fifteen exons (Duchrow et al., 1996; Schluter et al., 1993). The central part of the *pKi-67* cDNA exists of one single exon (exon 13). This exon contains sixteen tandemly repeated elements, the "Ki-67 repeats". Each of these "Ki-67 repeats" harbors a highly conserved 66 bp motif (from 72% to 100% identical), the "Ki-67 motif". The "Ki-67 motif" encodes for the epitope of the Ki-67 antigen that is recognized by the prototype Ki-67 antibody and MIB1 (Kubbutat et al., 1994).

1.2.2 The promoter region of the *pKi-67* gene

The gene's 5' flanking region covering its putative promoter had been cloned by Gerdes et al (1996). They obtained a 14,041 bp DNA fragment by hybridizing the lambda EMBL-3 library of HeLa-S3 chromosomal DNA with a 300 bp probe from the 5' untranslated part of the *pKi-67* cDNA. The full sequence of this fragment was determined and published in the GenBank Database under the accession number of gi: 1944550.

A total of 11,797 bp of the entire sequence are located upstream of the first exon (Gerdes, 1996).

1.2.2.1 Functional analysis of the *pKi-67* promoter by luciferase experiment

Previously, luciferase experiments had been performed in our laboratory to identify the functional activity of the *pKi-67* gene's promoter region. Truncated segments of the promoter were inserted into luciferase reporter genes, transiently transfected, and analyzed for luciferase activities. The obtained luciferase value represented the transcriptional activity of the inserted promoter segment. As shown in **Figure 1**, relative luciferase activity of *fragment 1* (598 bp), which was arranged between a downstream *Alu*-repeat and the transcription start, was used as a reference. A 100 bp extension of exon 1 (*fragment 2*) generated a three fold higher promoter activity than fragment 1. In *fragment 3*, further addition of the complete exon 1 and intron 1 resulted in a six times increase in the luciferase activity as compared with *fragment 1*. However, further extension beyond exon 2 resulted in a three-fold inhibition of the promoter activity (*fragment 4*). This inhibition may be explained by binding of some putative inhibitors to the direct repeated elements in the untranslated region of exon 2. Extension into the 5' direction beyond the *Alu*-repeat only mildly enhanced luciferase activity (*fragment 5*) as compared to *fragment 3*. The highest promoter activity was obtained in *fragment 6*, which was extended to both sides while leaving out the region of exon 2. Taken together, these data suggested that the region of *fragment 3* (from base 11244 to 12276, GenBank gi:1944550) in the *pKi-67* promoter was of superior importance for the transcriptional regulation (unpublished data, personal communication).

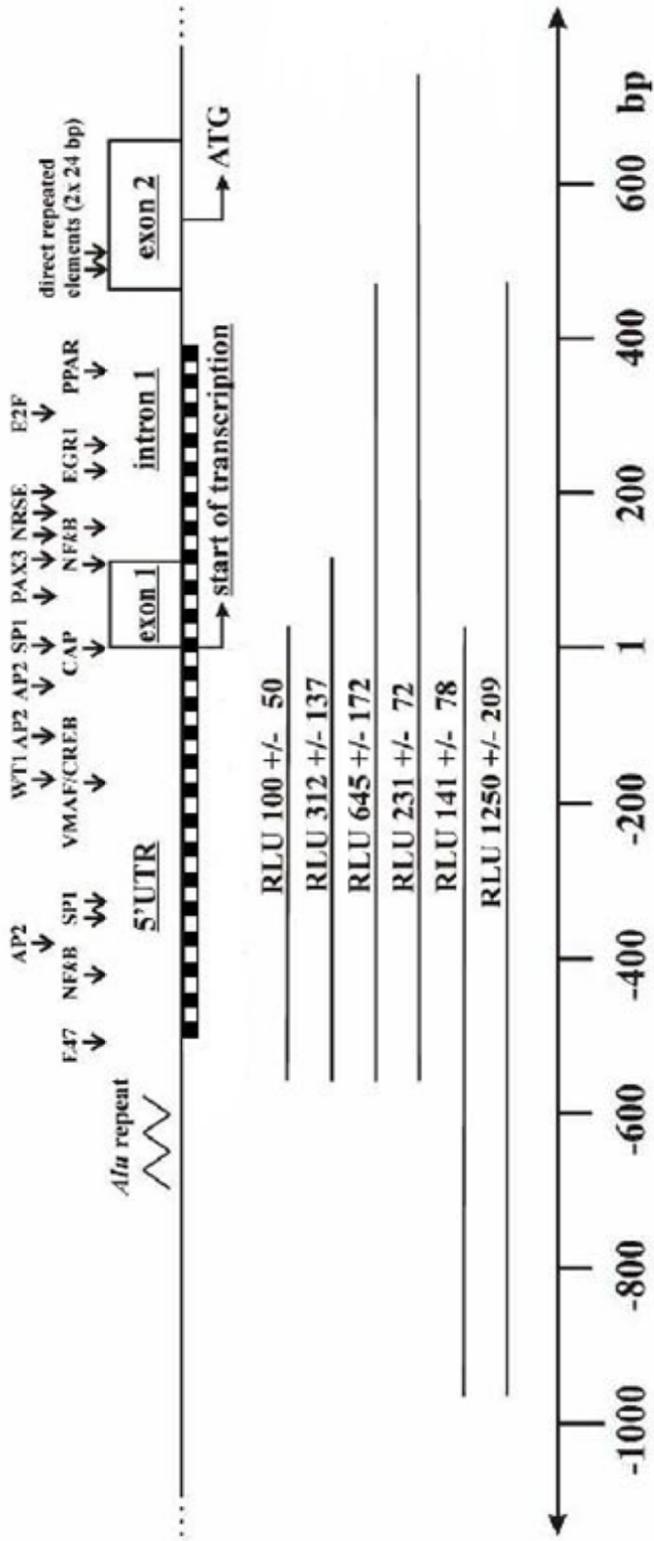


Figure 1 Functional analysis of the the *pKi-67* gene's promoter region. Truncated segments of the *pKi-67* gene promoter were generated by PCR within the frame from -956 bp to +754 bp and inserted in forward orientation upstream of luciferase reporter genes, transiently transfected into HeLa cells and analyzed for luciferase activities after 48 hours. Reporter gene activities of different fragments were expressed as relative luciferase activities (RLU) against the internal control. The luciferase activity of fragment 1 was defined as 100 RLU. Potential transcription factor binding sites in the promoter region of *pKi-67* are indicated by arrows. The CpG-rich fragment (CpG island) is indicated with dappled box. Sequence positions are numbered relative to the transcription start site. (personal communication).

1.2.2.2 CpG-rich fragment in the *pKi-67* promoter region

Interestingly, quite overlapping with this *fragment 3* -the active part of the *pKi-67* promoter- is a large CpG dinucleotide rich fragment of 900 bp. It spans the gene's 5' promoter, the first exon and the first intron (roughly from base 11280 to 12200, GenBank gi: 1944550). CpG dinucleotides are generally considered to be potential targets for DNA methylation modification which is a major form of epigenetic regulation in mammalian cells (Holliday and Grigg, 1993). However, the methylation status of this CpG-rich fragment within the *pKi-67* promoter region is unknown.

A high density of potential binding sites for transcription factors were found in this CpG-rich fragment: three potential binding sites for Ap-2, a cis acting factor, were identified at -388/-377, -140/-129 and -48/-37. Two binding sites for NF κ B were found at +93/+106 and +160/+169 (positions are numbered relative to the transcription start site). The CpG-rich fragment also contains potential binding sites for Sp1, CREB (cAMP response element binding protein), E2F (E2F transcription factor), EGR-1 (early growth response 1), NRSF (neuron-restrictive silencer element), PPAR (peroxisome proliferator-activated receptor), Maf (Maf oncoprotein), and WT1 (Wilms' tumour suppressor protein WT1) (Wehrstedt, 2004) (**Figure 1**).

1.2.3 Mechanisms about the *pKi-67* gene's transcription regulation

The *pKi-67* mRNA has been reported to be transcribed at a much higher level in malignant tumors as compared to their normal counterparts (Wu et al., 2001). In addition, the *pKi-67* mRNA level was suggested to have a prognostic value: a lower level of the *pKi-67* mRNA was associated with favorable prognostic outcome in colorectal cancer patients (Duchrow et al., 2003). These results indicated the potential importance of the regulation mechanisms of the *pKi-67* mRNA transcription.

DNA methylation of CpG motifs, especially of those located in promoter regions, have been shown to play an important role in the regulation of gene transcription by numerous publications (Scarano et al., 2005). Thus, it was the objective of this thesis to test if the CpG-rich fragment within the *pKi-67* promoter is involved in the epigenetic regulation of this gene's transcription.

1.3 DNA methylation

1.3.1 DNA methylation, CpG island, and DNA methyltransferase

DNA methylation is an important epigenetic modification that affects various cell processes including embryogenesis (Monk et al., 1987), genomic imprinting (Reik and Walter, 2001), X-chromosome inactivation (Avner and Heard, 2001), and tumorigenesis (Worm and Guldborg, 2002). DNA methylation in the human genome occurs nearly exclusively at cytosine residues (at the 5- position of the pyrimidine ring, m⁵C) within the symmetric dinucleotides CpG (Holliday and Grigg, 1993) (**Figure 2A**). The m⁵C accounts for 0.75-1% of the total DNA bases. Approximately 70% of all CpG dinucleotides are methylated and follow a rather diffused distribution throughout the genome (Worm and Guldborg, 2002). However, some DNA regions have particularly higher densities of CpG dinucleotides. These regions are named as CpG islands. A formal definition of a CpG island is a DNA region comprising more than 200 bp, with a guanine/cytosine content above 50% and an observed versus expected presence of CpG above 60% (Gardiner-Garden and Frommer, 1987). The human genome contains roughly 30,000 to 45,000 CpG islands that are believed to preferentially occur at the promoter and first exon regions of protein encoding genes. Most transcriptionally active genes, especially housekeeping genes, have their CpG islands unmethylated (housekeeping genes are genes involved in basic functions needed for the survival of the cell: thus, they are constitutively expressed). Methylation of certain CpG islands is correlated with gene inactivation and has been shown to be important during gene imprinting and tissue-specific gene expression (Venter et al., 2001). In general, it can be concluded that there is an inverse relationship between the density of promoter methylation and the transcriptional activity of a gene (Worm and Guldborg, 2002). Early experiments indicated that methylation of certain DNA sequences could suppress gene transcription by interfering directly with the binding of transcription factors (Comb and Goodman, 1990; Huntriss et al., 1997). Some transcription factors, such as Ap-2, CREB, E2F, and NFκB can not bind to DNA when their target sequences are methylated leading to inactivation of transcription (Kondo and Issa, 2004).

Recently, alternative models for methylation dependent gene inactivation suggested the conversion of chromatin structure from a transcriptional competent pattern into a closed pattern mediated by a cascade of histone modification events (Bird, 2002; Kondo et al., 2003). However, the exact mechanism by which DNA methylation inactivates gene expression still remains to be determined. It is however known, that the methylation pattern of a cell can be faithfully transmitted to the daughter cells during cell division (Urnov and Wolffe, 2001; Vercelli, 2004). Herefor, the enzyme DNA methyltransferase 1 (DNMT1) is responsible for copying the methylation pattern after DNA replication. It methylates DNA containing hemi-methylated CpGs (postreplicative maintained methylation) by transferring methyl groups from the universal methyl donor S-adenosylmethionine (SAM) to cytosines in the newly synthesized DNA strand (**Figure 2B**). In addition, two *de novo* methyltransferases (DNMT3A and DNMT3B) as well as DNA demethylases might also be involved in the inheritance of the DNA methylation pattern (Bestor, 2000).

1.3.2 DNA methylation in cancer

Neoplastic cells exhibit different DNA methylation patterns as compared to normal cells. Generally, this can be summarized as genomic global hypomethylation accompanied by region-specific hypermethylation events (Baylin and Herman, 2000; Jones and Laird, 1999). Global hypomethylation has been proposed to lead to genetic instability (Chen et al., 1998). The regional hypermethylation events in neoplasia usually happen in the CpG islands and are often associated with silencing of tumor suppressor genes. Transcriptional inactivation of tumor suppresser genes by promoter CpG island hypermethylation has been well documented in many human cancers (Momparler and Bovenzi, 2000; Robertson, 2001). *De novo* methylation of these genes may occur early in tumor progression and lead to abnormal function of important cellular pathways including those controlling cell cycle, DNA repair, apoptosis and cell to cell growth signaling (Robertson, 2001). In colorectal cancer, examples of this process include inactivation of the *p16* cell cycle regulator, the *THBS1* angiogenesis inhibitor, the *TIMP3* metastasis suppressor, and the *MLH1* DNA mismatch repair gene (Rashid and Issa, 2004).

1.4 Methodology for DNA methylation analysis

A variety of approaches has been established to detect the methylation status of specific genomic sequences. These approaches can be roughly divided into two types: methylation-sensitive restriction enzyme based approaches and bisulfite modification dependent approaches.

1.4.1 Methylation-sensitive restriction enzyme based approaches

The methylation-sensitive restriction enzymes possess CpG dinucleotides containing recognition sites and cut substrates with unmethylated recognition sequence only. In contrast, their methylation-**ins**ensitive isoschizomers cut both, methylated substrates as well as unmethylated ones. Therefore, cutting DNA with both, a methylation-sensitive endonuclease and a methylation-insensitive isoschizomer will obtain different products if the recognition site of the sample is methylated.

Digested products can be analyzed by southern-blot with specific probes. **Figure 3** is a sketch map depicting this method. Southern-blot assay allows rapidly analyzing a large number of specimens at sufficient sensitivity (10% of methylated sequences in 10 μ g of the initial DNA template). The sensitivity of this technique can be increased (starting at 0.1% of methylated sequences in 10ng of the initial DNA) using PCR (Azhikina and Sverdlov, 2005). A pair of primers is designed to flank the recognition site and only the methylated and thus non-cleaved fragment of DNA will be amplified exponentially (**Figure 3B**).

Unfortunately, the methylation-sensitive restriction enzyme based approach has some obvious shortcomings: it can detect only the methylation status of the recognition sites of the restriction enzymes used, thus limiting the amount of information gained. Even the PCR combined approach is associated with possible artifacts that are caused by incomplete digestion of the sample DNA (Oakeley, 1999).

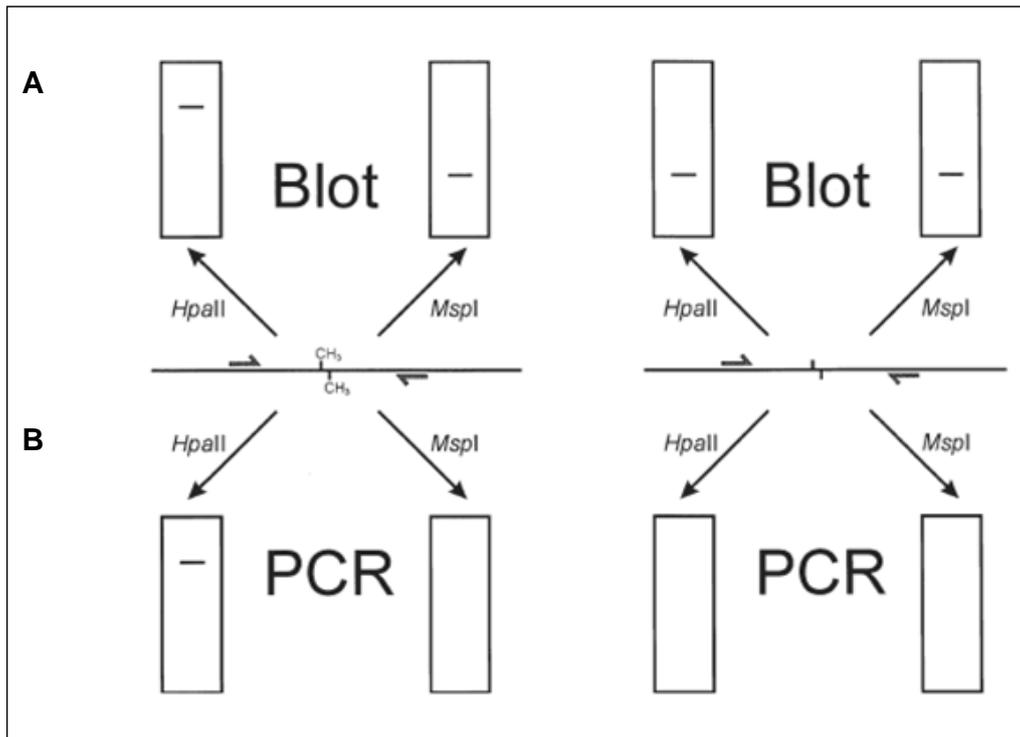


Figure 3 Use of methyl-sensitive restriction enzymes for analyzing methylation of specific CpG sites (modified from Oakeley, 1999). DNA is digested in parallel with *HpaII* and *MspI* (*MspI* will cut the sequence CCGG, whether or not the internal second cytosine is methylated, whereas *HpaII* will only cut if the internal cytosine is unmethylated). **(A)** The digested products are then analyzed by southern-blot with specific probes. If the recognition sequence is methylated, a larger hybridizing band in the *HpaII* track and a smaller band in the *MspI* track will be observed. If the recognition sequence is unmethylated, the two hybridizing bands will be of the same size. **(B)** The digested products are further analyzed by PCR with primers flanking the recognition sequence. A product will be observed in the *HpaII* track only if the DNA is methylated at the recognition site (see (B) to the left). No band should ever appear in the *MspI* track. The latter can be taken as a control for complete digestion.

1.4.2 Bisulfite modification dependent assays

These approaches depend on the ability of sodium bisulfite to efficiently convert cytosine residues to uracil in single-stranded DNA under conditions whereby m⁵C remains essentially non-reactive. The bisulfite reaction was first described in early 1970s (Hayatsu, 1976; Shapiro and Weisgras, 1970) and involves the following steps: (1) addition of bisulfite to the 5-6 double bond of cytosine, (2) hydrolytic deamination of the resulting cytosine-bisulfite derivative to gain an uracil-bisulphite derivative, and (3) removal of the sulphonate group by a subsequent alkali treatment, to obtain uracil (**Figure 4**).

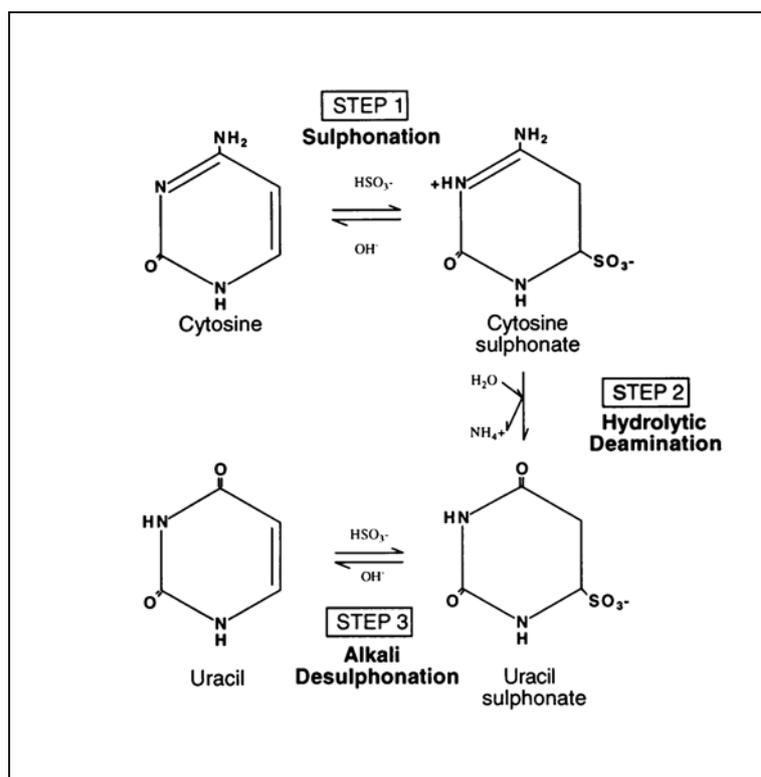


Figure 4 Schematic diagram of the bisulfite reaction (modified from Clark et al., 1994). Three steps are involved: (1) addition of bisulfite to the 5'-6' double bond of cytosine, (2) hydrolytic deamination of the resulting cytosine-bisulfite derivative to gain a uracil-bisulphite derivative, and (3) removal of the sulphonate group by a subsequent alkali treatment, to obtain uracil.

Based on the same principle of bisulfite modification, many methods for mapping of cytosine methylation have been developed including, e.g., bisulfite sequencing assay (Clark et al., 1994), methylation specific PCR (Herman et al., 1996), combined bisulfite analysis (Xiong and Laird, 1997) and methylation-sensitive single nucleotide primer extension (Gonzalzo and Jones, 1997).

1.4.2.1 Bisulfite sequencing

The bisulfite sequencing procedure can provide an accurate display of every methylated cytosine on both strands of any target sequence, using DNA isolated from less than 100 cells (Clark et al., 1994). In this procedure, the modified DNA sample is PCR amplified using specific primers that anneal to the converted sequence.

Primers should not contain any CpG sites within their sequences to avoid discrimination against methylated or unmethylated DNA. Each strand of the DNA sample should be amplified separately because the two strands lose their complementation after conversion of all unmethylated cytosines. The resulted PCR products can be used either in cloning and sequencing to study the methylation status of individual DNA molecules, or in direct sequencing to examine strand-specific methylation for the population of molecules (Clark et al., 1994; Frommer et al., 1992). The main disadvantage of the bisulfite sequencing procedure have been described to be technically demanding and labor intensive especially when a larger number of samples needs to be investigated (Worm and Guldborg, 2002).

1.4.2.2 Other bisulfite modification dependent assays

Other bisulfite modification dependent assays include: methylation-specific PCR (MSP), methylation-sensitive single nucleotide primer extension (Ms-SNuPE), and combined bisulfite restriction analysis (COBRA). These methods are high throughput approaches and simple to use compared to the bisulfite sequencing procedure but suffer from analyzing only a limited number of CpG sites per assay (Gonzalzo and Jones, 1997; Herman et al., 1996; Xiong and Laird, 1997).

In the current work, methylation status of the *pKi-67* promoter CpG-rich fragment was detected by a methylation-sensitive restriction enzyme based method (methylation-specific restriction-PCR-assay) before confirmed by an alternative method of bisulfite sequencing.

2. Aims of the current study

The Ki-67 protein is a well established and widely used marker to assess cell proliferation activity. Over-expression of the Ki-67 protein has been detected in many kinds of malignant tumors. A similar observation has been made on the mRNA level: the *pKi-67* mRNA is transcribed at a markedly higher level in malignant cells than in normal somatic cells. Recently, the *pKi-67* mRNA level was suggested to have prognostic value in colorectal cancer. These results indicate the potential importance of the regulation mechanisms of the *pKi-67* mRNA transcription especially in colorectal cancer. The promoter region of the *pKi-67* gene harbors a CpG-rich fragment (CpG motifs are generally considered to be potential targets for DNA methylation modification). However, the methylation status of these CpG dinucleotides is unknown. The overall aim of the current study therefore focused on the DNA methylation pattern of this CpG-rich fragment as a potential epigenetic regulation of the *pKi-67* gene transcription. In particular, the following questions were addressed:

- (1) What is the methylation status of the CpG-rich fragment within the *pKi-67* promoter region in normal human somatic cells?
- (2) Is there a different methylation pattern of this CpG-rich fragment in cancer cells, especially in colorectal cancer cells?
- (3) Is the promoter methylation involved in the regulation of the human *pKi-67* gene's transcription?

3. Materials and methods

3.1 Materials

3.1.1 Cell lines

The human colon cancer cell lines SW480, DLD1, and SW620 were obtained from the American Type Culture Collection (ATCC).

3.1.2 Tissues and buffy-coats

All sampling was performed adhering to the local ethical board regulations and permission. Two adjacent normal liver tissue samples were taken from two patients with liver metastasis that underwent partial hepatectomy at the Department of Surgery, University Clinic of Schleswig-Holstein, Campus Lübeck, Germany. After routine histopathological examination, normal liver tissue adjacent to the malignant lesion was used for genomic DNA isolation and RNA isolation.

The retrospectively examined tissue samples of colorectal cancer (tumor tissue and adjacent tumor-cell free tissue) derived from 10 patients (detailed clinical data of these patients are listed in supplementary **Table 4**) undergoing curative surgery at the Department of Surgery, University Clinic of Schleswig-Holstein, Campus Luebeck, Germany. Buffy-coats were obtained from the Department of Immunology, University of Schleswig-Holstein, Campus Lübeck. All donors have been proved to be healthy before blood sampling.

3.2 Methods

3.2.1 Chemicals, kits, enzymes and buffers

All chemicals, kits, enzymes and buffers used in this study are itemized in the **Supplement** part.

3.2.2 Primers

All primers were synthesized and purified by MWG- Biotech. Detailed information is listed in Supplementary **Table 6**.

3.2.2.1 Primer design of bisulfite sequencing PCR

Design of primers for PCR amplification of bisulfite treated DNA is critical in the bisulfite sequencing procedure. As incomplete bisulfite modification of DNA can be a concern, primers should be designed to favor the amplification of fully bisulfite-reacted DNA from a mixture which may also contain some partially converted molecules. A number of rules were followed in primer designing: (1) Primers should be picked from a region that has enough numbers of non-CpG “C”s in the original sequence. (2) They should not contain any CpG dinucleotides within their sequence, to avoid discrimination against methylated or unmethylated DNA, unless methylation status of the CpG site within the primer region is already known. If a CpG dinucleotide within the primer sequence could not be avoided, a mismatch to both the methylated and unmethylated sequence should be incorporated into the primer at the C residue of the CpG dinucleotides. (3) Primers are approximately 25 to 30 bp in length. (4) They should have limited internal complementarities and limited complementary sequences between primer pairs. (5) Two primers should be of approximately equal annealing temperature (Clark et al., 1994; Li and Dahiya, 2002; Raizis et al., 1995).

3.2.3 Instruments and equipments

The instruments and equipments used in the current study are itemized in the **Supplement** part.

3.2.4 Cell culture and harvesting

All cell lines listed in *3.1.1* were maintained at 5% CO₂ in RPMI 1640 medium with 1% glutamine (Gibco/BRL, Eggenstein, Germany) and supplemented with 10% fetal bovine serum (Gibco/BRL) and 1% penicillin/streptomycin (Biochrom, Berlin, Germany).

Cultured cells were harvested according to a standard procedure: in brief, the adherent cells were washed, trypsin digested, suspended in PBS, and centrifuged at 1200 rpm for 5min. The obtained cell pellet was resuspended in PBS, counted and centrifuged again under the same conditions. Cell pellets were stored at -80°C prior isolation of genomic DNA and RNA.

3.2.5 Isolation of blood monocytes from buffy-coats

Blood monocytes were isolated from buffy-coats of healthy donors as described by Macey et al. (1995). Briefly, 30 ml blood, diluted with the same volume of $1\times$ PBS, was layered over 10 ml Ficoll (PAA Laboratory, Pasching, Austria). After 20 min centrifugation at 1600 rpm at 20°C , monocytes at the upper interface were collected, washed with PBS and preserved at 4°C .

3.2.6 Isolation of genomic DNA

Genomic DNA (gDNA) was isolated from cultured cells and monocytes (from buffy-coats of healthy donors) using Blood & Cell Culture DNA Midi Kit (Qiagen, Hilden, Germany), and from tissue samples using DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Concentration of gDNA was determined by reading $\text{OD}_{260\text{nm}}$ in a spectrophotometer. Purity of gDNA was determined by calculating the ratio of $\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}}$. Only gDNA that had a $\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}}$ ratio between 1.7-1.9 was used for subsequent analysis.

3.2.7 DNA agarose gel electrophoresis

Isolated gDNA, PCR products and purified plasmid DNA were analyzed by agarose gel electrophoresis. To obtain a 1% gel, 0.5 g agarose was dissolved in 50 ml of $1\times$ TAE buffer and melted in the microwave oven. DNA samples were loaded onto the gel (containing $0.5\ \mu\text{g}/\text{ml}$ ethidium bromide) and run in $1\times$ TAE at 80 V for 30-60 min.

3.2.8 mRNA expression analysis

To analyze the *pKi-67* transcription level, total RNA was extracted from cells and tissues (for RNA isolation, 5×10^6 cells or 20 mg frozen tissue was used) utilizing the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. An aliquot of 1 μ g RNA was used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. mRNA expression analysis for *pKi-67* and the reference gene *β 2-microglobulin* was carried out by semi-quantitative RT-PCR. The detailed conditions of the PCR are described in 3.2.11.3. The products were analyzed by electrophoresis on a 1% agarose gel and photographed afterwards.

3.2.9 Methylation-specific restriction-PCR-assay

The cleavage ability of methylation-sensitive endonuclease is inhibited by the methylation of their target sequence (Singer-Sam et al., 1990). We used methylation-sensitive restriction enzymes *HpaII*, *SacII* and *HaeII* (New England Biolabs, Ipswich, USA) to cut gDNA samples. *MspI*, the methylation-insensitive isoschizomer of *HpaII*, and an enzyme without recognition site in the target sequence, *EcorRI* (Pharmacia, Freiburg, Germany), were used as control. Restriction enzyme digestion was performed in a total volume of 30 μ l with 1.8 μ g substrate gDNA, using 40 U of each enzyme. After 6 h incubation at 37°C, additional 20 U of enzyme was added to ensure complete cleavage. Total incubation time was 16 h. A total of 100 ng digested DNA was then used in PCR to amplify a 301 bp target fragment which contains recognition sites of those methylation-sensitive restriction enzymes mentioned above (see **Figure 5** on the next page). When DNA was methylated at a specific recognition site, the corresponding methylation sensitive enzymes were not able to digest it and amplification took place. In case no methylation was present, DNA was digested and no product could be generated. The PCR conditions are described in 3.2.11.1. The PCR product was visualized by electrophoresis on a 1% agarose gel, stained with ethidium bromide and photographed.

```

11221 GTTTTTAATT TTTAGCAGTT TAAATCTTC TGGCAATGAG TAATGT TATA GTGTCCCAGG
11281 TGTTTGGTCC TTTAAGAAAA GGATAGCAGG ACGGGTTATC TCGGTGGGAG CGTGTTCAC
11341 CTCTGCCCTC CGCCAGCCGC CCTCG CCGGG GATGCACCCA GGTATTTTCC T CCGGATGCG
11401 TGAGTGGCTC GC CCGCGGAC ACGCCAGCCC CGCCCCGCGA GC CCGGCTTC CCCGCCCCCT
11461 CCCCGCCAC GCCCTGCGCT CCCTTCCTAT TGGTCCCATG CCGCGCTTTC CCGTTCAATC
11521 GC AGCGCTTA GCGCCAGAAT TT GAATCTTC GTTTTCGTTT GAATTGGGCG GCGCGCGCCGG
11581 GCTGGAAGAA GGAAGTGGAG GGCTGACGCT GCGGGCGGGC GGGAGGACTC GACTCGGTGG
11641 GAGCCGCTAG AGCCGGGCGC CCGGGGACGT AGCCTGTAGG GCCACCGGT CCCCGTCAGA
11701 GGCGGCGGCG GGAGCAGCGG GACTGCAGG CCGGGGTGCA GCGAACGCTA CCCCGCGGGC
11761 TGCGGCCCGG TGTGTGCGGA GCGTGGCGGG CGCAGCTTAC CCGGCGGAGG TGAGCGCGGC
11821 GCCGGCTCCT CCTGCGGCGG ACTTTGGGTG CGACTTGACG AGCGGTGGTT CGACAAGTGG
11881 CCTTGCGGGC CGGATCGTCC CAGGTGAGCT GCGGCCGGGA CTCCTGGGAG CTGTCCGGGG
11941 TCGAGGGCTG AGCCGCGGGG ACCCCCAGAG CTCTGCGGGG ACGGGCAGGG GACAGACCGC
12001 CGGCCTGGGC CCGACTCCTC CTGGGCTCTG GCGAGGGCGT CTCGGTGGAA GCTCCCAGGA
12061 GGCGCAGGCG CTGGCGACAA CACCCAGCT GCCGGACTTT GGGGCGCCGG GGGCTGCGGT
12121 CGGATCGTCC TGGGGTCCC GCTAGCTAGC TGCGGTGTGC GCTTCTGTGG CCGGGGATAG
12181 GTGAATTGGG CACGGGGCCC TTCTTAGCTC CTTTGCTATC AGAGTAACTC GCACCTCTTT
12241 TGCAGTGAA GAGTTGTAA TTTGCTTCTG GCCTTCCCCT ACGGATTATA CCTGGCCTTC

```

Figure 5 The CpG island in the *pKi-67* promoter region and the design of the methylation-specific restriction-PCR-assay. The CpG island is from 11288 to 12203 (according to GenBank gi:1944550). Sequences of primers used in the methylation-specific restriction-PCR-assay are highlighted with red background. Recognition sites of methylation-sensitive restriction enzymes (*HpaII*, *SacII*, *HaeII*) and methylation-insensitive isoschizomer *MspI* are indicated (*HpaII* & *MspI*: with boxes, *HaeII*: with underlines, *SacII*: with italic type and underlines). Recognition sequences of the enzymes are depicted as follows, *HpaII* & *MspI*: 5'-CCGG-3'; *HaeII*: 5'-RGCGCY-3'; *SacII*: 5'-CCGCGG-3' (single letter code: R=A or G, Y=C or T).

3.2.10 Bisulfite treatment

Bisulfite treatment of DNA leads to conversion of unmethylated cytosine residues to uracil but not to a change of methylated cytosine residues. Bisulfite treatment was performed as follows: two microgram of gDNA were diluted in 26 μ l TE buffer and incubated with 1 μ l restriction enzyme *Bam*HI (Promega, Mannheim, Germany) and 3 μ l RE 10 \times buffer overnight at 37°C. Digested DNA was denatured by adding 3.3 μ l freshly prepared 3 M NaOH and incubated at 42°C for 30 min. Afterwards, 33.3 μ l denatured DNA was added into a siliconized microcentrifuge tube together with 283 μ l 2M sodium metabisulfite and 17 μ l 10mM hydroquinone.

The 2M sodium metabisulfite solution was obtained by dissolving 3.8 g sodium metabisulfite (Aldrich, Steinheim, Germany) into 6 ml cold water, adjusting pH to 5.0 with 1 M NaOH, and finally adding water up to 10 ml. The 10 mM hydroquinone was prepared by dissolving 0.22 g hydroquinone (Sigma-Aldrich, Seelze, Germany) in 200 ml cold water. Solution preparation was carried out at 4°C in the dark by careful mixing. Bisulfite reaction mixture was overlaid with chill-out liquid wax (Biozym, Oldendorf, Germany) and incubated at 50°C for 12-14 h. The tube was covered with aluminum foil to protect the reaction against light during incubation. The aqueous phase was later recovered using the Wizard DNA clean-up system (Promega, Mannheim, Germany) following the manufacturer's guidelines. Purified DNA was mixed with 1M NaOH to a final concentration of 0.3 M and incubated for 20 min at 37°C to ensure complete desulfonation. The DNA sample was neutralized by adding 82.5 µl ammonium acetate (pH 7.0) before ethanol precipitation with 415 µl 100% ethanol. After centrifugation, the DNA pellet was washed with 70% ethanol, air dried, resuspended in 50 µl TE buffer, and kept at -20°C.

3.2.11 PCR amplification

PCR mixture was always assembled in a DNA-free environment. Aerosol resistant barrier pipette tips were used.

3.2.11.1 PCR amplification for methylation-specific restriction-PCR-assay

The following working steps were performed on ice, and components were added into a sterile 0.2 ml PCR cup: 40 µl dH₂O, 5 µl 10×PCR buffer without Mg²⁺, 1.5 mM MgCl₂, 1.5µl endonuclease digested DNA template (100 ng), 200 µM dNTPs (all Amersham-Biosciences, Little Chalfont, UK), 0.5µM of each primer, and 2.5 U *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany), total volume 50 µl. Primer sequences are as follows:

MSRPA forward: 5'-TATAGTGTCCCAGGTGTTTGGTC-3'

MSRPA reverse: 5'-CCAATTCAAACGAAAACGAAGATTC-3'

PCR reaction mixtures were incubated in a thermal cycler at 95°C for 3 min to completely denature the templates. Then, 35 cycles of “30 sec denature at 95°C, 30 sec anneal at 58°C and 20 sec extension at 72°C” were performed followed by a final incubation of 10 min at 72°C. The PCR products were analyzed by agarose gel electrophoresis.

3.2.11.2 PCR amplification for bisulfite converted DNA

The bisulfite treated DNA template was amplified by a two-stage nested or semi-nested PCR as shown in **Figure 6**, thus targeting three consecutive fragments (fragment I-III) of the *pKi-67* promoter. In the first PCR, 1.5 µl DNA was added into the reaction mixture. Then 1.5 µl of product from the first PCR was used as template in the next PCR with the same PCR reagents, except the specific primers that now contained the putative altered sequence of the sense strand due to bisulfite treatment (See supplementary **Table 6**). After 3 min denaturation at 95°C, both PCR reactions underwent 30 cycles (30 sec at 95°C, 30 sec at specific annealing temperature according to the different primers used (**Table 1**), and 45 sec and 30 sec at 72°C for the first and second reaction respectively). Finally, this was followed by 5 min incubation at 72°C. The PCR product was used for subsequent cloning and sequencing.

Table 1 Primers and annealing temperatures in bisulfite sequencing PCR

Amplified region	Primer set	Annealing Temperature	Product size
Fragment I	PF1L+F2R F1L+F1R	54°C 59°C	326 bp
Fragment II	F1L+F3R F2L+F2R	59°C 59°C	361 bp
Fragment III	F2L+F3R F3L+F3R	58°C 55°C	283 bp

3.2.11.3 Semi-quantitative RT-PCR

A total of 1.5 µl reverse transcribed cDNA was used as template for semi-quantitative RT-PCR. The PCR reaction mixture was prepared as described in 3.2.11.1. For *pKi-67* mRNA analysis, primer “*Ki67 forward*” and “*Ki-67 reverse*” were used. Primer “*B2M forward*” and “*B2M reverse*” were used to examine reference gene *β2-microglobulin* (primer sequences are listed in supplementary **Table 6**) mRNA expression. The RT-PCR assay was performed under following cycling conditions: the mixture was incubated at 95°C for 3 min to completely denature the template. Then, 35 cycles of “30 sec denature at 95°C, 30 sec anneal at 49°C (*pKi-67*) or 53°C (*β2-microglobulin*) and 20 sec extension at 72°C” were performed followed by a final incubation of 5 min at 72°C. PCR products were analyzed by electrophoresis on a 1% agarose gel and ethidium bromide staining.

3.2.12 Cloning of the PCR amplified fragment

The PCR amplified fragment was cloned into a pCR4-TOPO vector with the TOPO TA Cloning Kit (Invitrogen, Paisley, UK). Cloning reaction used 4 µl of PCR product according to the user’s manual. Then, One Shot TOP10 chemically competent *E. coli* was transformed by 2 µl pCR4-TOPO construct, spread on a pre-warmed LB-agar plate containing kanamycin (50 µg/ml), and incubated at 37°C overnight. A single colony was picked, inoculated to a new LB-agar plate, and incubated continuously for 24 h. An independent clone of transformed bacteria was then inoculated to a tube containing 5 ml LB medium and 250 µg kanamycin and incubated in a 37°C water bath overnight with vigorous shaking to culture the bacteria.

3.2.13 Plasmid DNA isolation

A total of 5 ml cultured bacteria were used to isolate plasmid DNA with the NucleoSpin Plasmid Kit (Macherey-Nagel, Düren, Germany) according to the user manual. DNA concentration was determined by spectrophotometry.

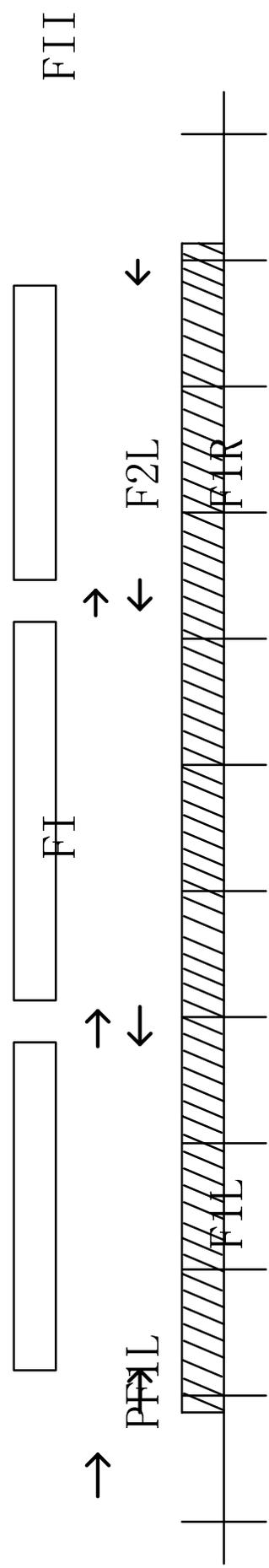


Figure 6 PCR amplification of the *pKi-67* promoter CpG island after bisulfite modification. The *pKi-67* promoter region is shown with its CpG island indicated by the shaded bar. The *pKi-67* CpG island is amplified by three consecutive fragments (I-III, marked by white boxes respectively) using nested or semi-nested PCR. Positions of primers are indicated with arrows. Primer PF1L, F1L, F2R and F1R are used for amplifying fragment I; F1L, F2L, F3R and F2R are for amplifying fragment II; F2L, F3L and F3R are for amplifying fragment III.

-600 -500 -400 -300 -200 -100 +1

3.2.14 Sequencing of plasmid DNA

3.2.14.1 Sequencing reaction

Cycle sequencing was performed with a fluorescent dye labeled primer “*M13 forward*” or “*M13 reverse*” (see supplementary **Table 6**) using the Thermo Sequenase kit RPN 2438 (Amersham Pharmacia, Little Chalfont, UK). A total of 13 μ l premix was prepared first containing 1 μ g purified plasmid DNA template, 1 μ l primer (*M13 forward* or *M13 reverse*, 1 μ g/ μ l), 0.5 μ l DMSO, and an appropriate volume of dH₂O. Reagents A, C, G, T were provided by the kit and placed into four sequencing tubes respectively (1 μ l each). Each reagent contained dATP, dCTP, 7-deaza-dGTP, dTTP, DNA polymerase, and the corresponding ddNTP (e.g., reagent A contains ddATP). Each of the four tubes was further supplied with 3 μ l premix. The reaction mixture was thoroughly mixed and overlaid with 20 μ l “chill-out liquid wax” before being applied to the thermal cycler. A special cycling reaction according to the following protocols was performed (**Table 2**). A total of 3 μ l stop solution was added after cycling.

Table 2 Cycling reaction for sequencing

Cycle number	Temperature	Time
1	95°C	1 min 45 sec
12	95°C	15 sec
	55°C	15 sec
	70°C	15 sec
13	95°C	20 sec
	55°C	20 sec
	70°C	20 sec
1	95°C	40 sec

3.2.14.2 Casting and gel electrophoresis

Sequencing gel solution was prepared by mixing the following components (**Table 3**): a gel sandwich (40 cm × 20 cm) was made by pouring gel solution into the slot between two glass plates with a 64-well comb at the top. Gel thickness was set to 0.25 mm. After one hour of polymerization, the gel was eletrophoresed in the LI-COR DNA Sequencer (LI-COR, Lincoln, NE) for 30 min (pre-run) before the templates (from 3.2.14.1 (1 µl/lane)) were loaded and run at the same conditions overnight.

Table 3 Composition of sequencing gel solution

Reagent	Dosage
RapidGel XL Sol 40% (Rockland, USA)	7.5 ml
Urea	21 g
10 × TBE buffer	5 ml
dH ₂ O	28 ml
DMSO	500 µl
TEMED	50 µl
10% APS fresh	350 µl

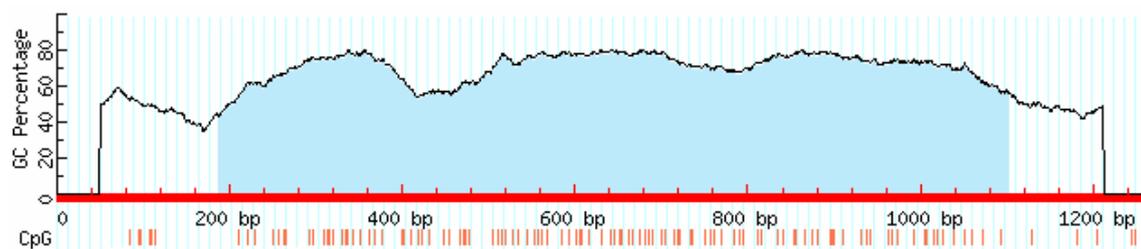
3.2.14.3 Sequence analysis

Data collection and image analysis was automatically done by the Base ImageIR software supplied with the LI-COR model 4000 DNA sequencer. The obtained DNA sequence was further analyzed by comparison to the published sequence of the *pKi-67* gene (GenBank sequence data base, accession number: gi 1944550) using a freely available software tool (www.seqtools.dk). To determine whether the *pKi-67* promoter CpG-rich fragment meets the criteria of a “CpG island” (Gardiner-Garden and Frommer, 1987), a freely accessible program “Methprimer” (at <http://itsa.ucsf.edu/~urolab/methprimer>) was used.

4. Results

4.1 Determination of the *pKi-67* promoter CpG island

In order to determine whether the *pKi-67* promoter CpG-rich fragment meets the criteria of a “CpG island” (Gardiner-Garden and Frommer, 1987), the gene’s promoter region sequence (from base 11101 to 12301, GenBank gi:1944550) was loaded into the program “Methprimer” (at <http://itsa.ucsf.edu/~urolab/methprimer>). The data indicated a typical CpG island in the *pKi-67* promoter region extending from 510 bases upstream of the transcriptional start site to 406 bases down stream of the transcriptional start site (from base 11288 to 12203, GenBank gi:1944550). It showed a G/C content above 50% and a ratio of observed versus expected frequency of CpG dinucleotides above 0.6, therefore meeting the formal criteria of a “CpG island” (**Figure 7**).



```
CpG island prediction results:
(Criteria used: Island size > 100, GC Percent > 50.0, Obs/Exp > 0.6)
1 CpG island(s) were found in your sequence
  Size      (Start - End)
Island 1   916 bp      (187 - 1102)
```

Figure 7 The *pKi-67* promoter CpG island prediction by the “Methprimer” program. The horizontal axis represents the sequence of the *pKi-67* promoter region from 5’-end (left) to 3’-end (right). The G/C content values are indicated on the vertical axis. The range of the CpG island is marked with blue color.

4.2 *pKi-67* mRNA expression in normal and malignant cells

To examine whether DNA methylation modification of the promoter CpG island is a potential epigenetic transcription regulation of the *pKi-67* gene, we first performed semi-quantitative RT-PCR to analyze *pKi-67* mRNA level in a set of samples from normal blood monocytes, normal liver tissue and colon cancer cell lines (SW480 and DLD1).

According to this analysis, tumor cells had distinctively higher *pKi-67* mRNA levels than normal blood monocyte and normal liver tissue (**Figure 8**).

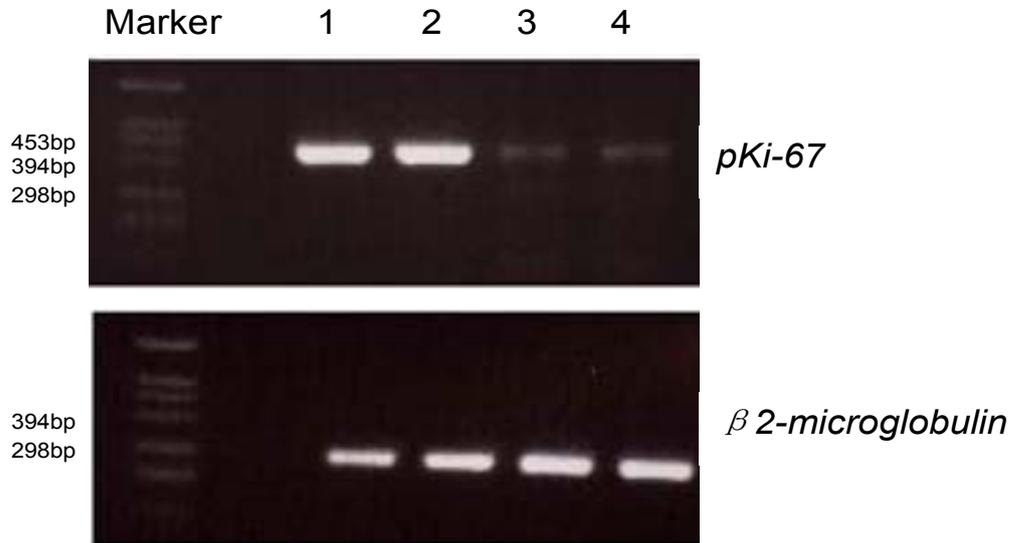


Figure 8 *pKi-67* mRNA expression analysis. RNA extracted from colon cancer cell SW480 and DLD1 (1&2), blood monocytes of healthy donor (3), and normal tissue of liver (4). An aliquot of 1 μ g RNA was reverse transcribed to cDNA. PCR for *pKi-67* cDNA and β 2-microglobulin cDNA (served as reference) were performed. PCR products were analyzed by electrophoresis on 1% agarose gel and photographed. The *pKi-67* mRNA was expressed distinctively higher in cancer cells than in normal blood monocytes or normal liver tissue while the reference gene β 2-microglobulin was expressed quite similarly.

4.3 Methylation status of the *pKi-67* promoter CpG island

4.3.1 Results of the methylation-specific restriction-PCR-assay

The data above show that there is a remarkable difference of the *pKi-67* mRNA transcription level between malignant cells and normal somatic cells. Whether promoter methylation is involved in the transcription regulation of this gene needs to be clarified. We assessed the methylation status of the *pKi-67* promoter CpG island in a diverse set of cells and tissues using methylation-specific restriction-PCR-assay. As shown in **Figure 5**, the target fragment for this assay is indicated by the highlighted primers used in PCR. Recognition sites of different methylation-sensitive restriction enzymes *Hpa*II (3 sites), *Sac*II (1 site), *Hae*II (2 sites) and the methylation-insensitive isoschizomer of *Hpa*II:

MspI (3 sites) are also indicated. Genomic DNA samples were isolated from blood monocytes of healthy donors (3 cases), normal colon mucosa (10 cases), normal liver tissues (2 cases), colon cancer cell lines (SW480, DLD1, SW620), and tumor tissues of ten colorectal cancer patients. Each sample was digested in parallel with *HpaII*, *SacII*, *HaeII*, *MspI* and *EcorRI*. In contrast to all others, *EcorRI* does not have a recognition site in the *pKi-67* promoter and therefore served as a control. In the subsequent PCR reactions, all kinds of *EcorRI* digested gDNA samples were successfully amplified with a product of 301 bp which was consistent with the positions of the two primers. In contrast, amplifications of *HpaII*, *SacII*, *HaeII* and *MspI* digested gDNA samples of all sources did not generate any specific PCR product. To verify the effectiveness of this procedure, a CpGenome Universal Methylated DNA (CHEMICON, Chandlers Ford, UK) was used as a methylation-positive control for the methylation-specific restriction-PCR-assay. The methylation-positive control DNA was amplified successfully after *HpaII* or *EcorRI* digestion and generated the same 301 bp product. No product could be seen after PCR amplification of *MspI* treated universal methylated DNA, as we expected, as this enzyme cleaves the target sequence without consideration of its methylation status (**Figure 9**). These results indicated that the restriction sites of *HpaII*, *SacII* and *HaeII* were unmethylated in the examined gDNA samples from normal blood monocytes, normal colon and liver tissue, colon cancer cell lines, and colorectal cancer tissue.

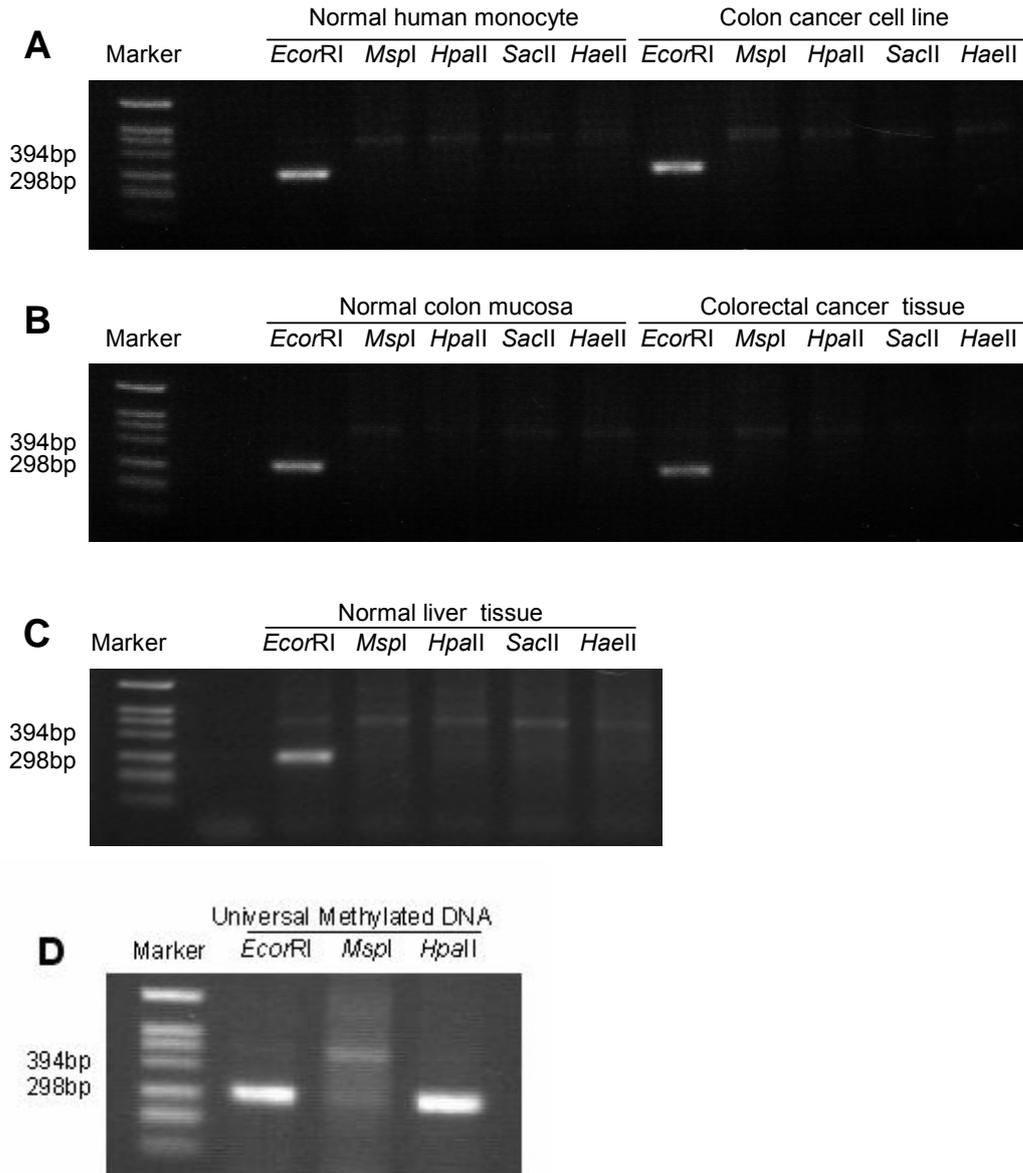


Figure 9 Results of methylation-specific restriction-PCR-assay. Human genomic DNA was extracted from different cell lines or tissues as indicated, digested with *Ecor*RI (without recognition site), *Msp*I (methylation-insensitive isoschizomer of *Hpa*II) and *Hpa*II, *Sac*II, *Hae*III (methylation-sensitive) in parallel, and PCR amplified with primers indicated in **Figure 5**. All of the *Ecor*RI digested gDNA samples were amplified successfully (product size 301 bp), serving as control. No product could be detected after PCR amplification of *Msp*I digested DNA, because this enzyme cleaves the target sequence without consideration of its methylation status. Amplification of *Hpa*II, *Sac*II, *Hae*III digested gDNA samples from normal blood monocytes, normal colon mucosa, normal liver tissues, colon cancer cell lines (SW480, DLD1, SW620), and colorectal cancer tissues did not generate specific PCR products (**A** to **C**), suggesting that the restriction sites of *Hpa*II, *Sac*II, *Hae*III in these gDNA samples are uniformly unmethylated. A CpGenome Universal Methylated DNA was used as a methylation-positive control for this procedure. It was positively amplified after *Hpa*II digestion generating the same 301 bp product (**D**).

4.3.2 Results of the bisulfite sequencing assay

As the methylation-specific restriction-PCR-assay can only detect CpG methylation at specific recognition sites of methylation-sensitive restriction enzymes, the information obtained from this assay was limited. Thus, bisulfite sequencing assay was performed to obtain an exact methylation map of all the CpG dinucleotides within the *pKi-67* promoter CpG island.

4.3.2.1 Optimization of bisulfite sequencing procedure

We first followed the classical protocol of bisulfite treatment described by Frommer et al. (1992) using a pair of primers spanning the whole CpG island (more than 900bp) to amplify the bisulfite modified DNA. However, we did not get any specific amplification product after the PCR reaction which could be caused by: (1) low efficiency of the bisulfite treatment which did not produce enough converted DNA template for PCR reaction, (2) serious loss of template DNA during the bisulfite reaction and subsequent purification steps. In order to solve this problem of incomplete bisulfite conversion, we tested to increase bisulfite incubation times (to 18, 20, and 24 hours respectively), however without obvious effect. Afterwards, bisulfite modification procedure was performed in a thermocycler for 5 h with repeated cycles of heating to 95°C every 15 min and with an additional digestion of gDNA before bisulfite treatment as suggested by Rein et al. (1997). Although the authors claimed a high conversion rate of substrate DNA (over 95%) combined with limited DNA degradation, it did not work in our setup. Therefore, the degradation of template DNA seemed to be the major problem in our experiment. Indeed, some authors have pointed out that a product size greater than 300 bp would be difficult to amplify from bisulfite modified DNA because these DNA would be fragmented after the bisulfite treatment (Li and Dahiya, 2002). Thus, we divided the target CpG island sequence into three consecutive fragments with each size around 300 bp (as shown in **Figure 6**), and redesigned three pairs of primers (*F1L*, *F1R*, *F2L*, *F2R*, *F3L*, *F3R*) to amplify them. However, we still did not get any specific amplification product after standard PCR.

We then tried to improve the sensitivity of the PCR reaction: nested/semi-nested PCRs were performed using different combinations of the existing primers (**Table 1**), resulting in a dramatic improvement: all three fragments were amplified successfully and competent for subsequent cloning and sequencing reactions (see **Figure 10**).

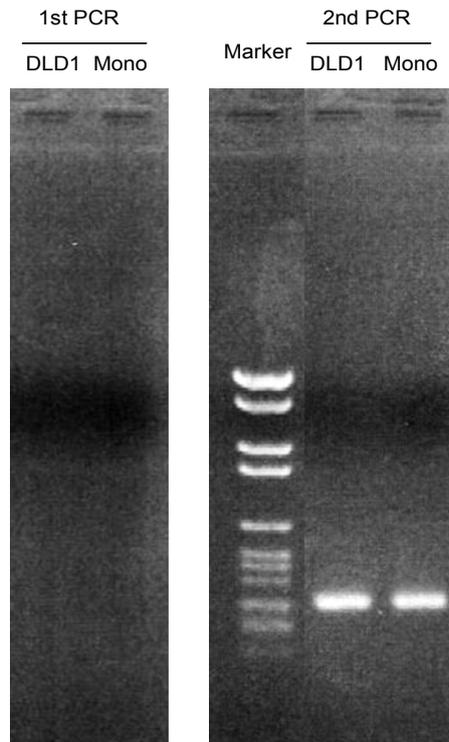


Figure 10 Bisulfite modified DNA amplified by nested PCR. Genomic DNA samples (from colon cancer cell line DLD1 and normal human monocytes) were bisulfite treated as described in 3.2.9 and underwent nested PCR to amplify fragment I (**Table 1**). After the first PCR with the primer set of *PF1L* and *F2R*, there was no visible product. A dramatic progress was obtained after the second PCR. The target fragment was successfully amplified for both of the samples with sharp bands on the agarose gel.

4.3.2.2 Results of bisulfite sequencing

Genomic DNA samples from blood monocytes of healthy donors (3 cases), normal colon mucosa (2 cases), normal liver tissues (2 cases), colon cancer cell lines (SW480, DLD1), and tumor tissues of two colorectal cancer patients (randomly selected from the 10 patients mentioned in 4.3.1) were included in the bisulfite sequencing assay.

After sodium bisulfite treatment, all cytosines in the DNA template were converted to uracils except the methylated ones. In the following PCR amplification step, all uracil (formerly cytosine) and thymine residues were amplified as thymines, while unconverted methylated cytosines were amplified as cytosines. Subsequent cloning and sequencing of the PCR product provided methylation maps of single DNA strands from individual DNA molecules in the original gDNA sample. As illustrated in **Figure 11**, two types of sequences could be seen when reading the sequencing gel. The first type lost all the cytosines (replaced by thymines) only with exception at the position of m⁵C (compare with the sense strand). The next type lost all the guanine residues (replaced by adenines) except when it was opposite to m⁵C (compared with the anti-sense strand).

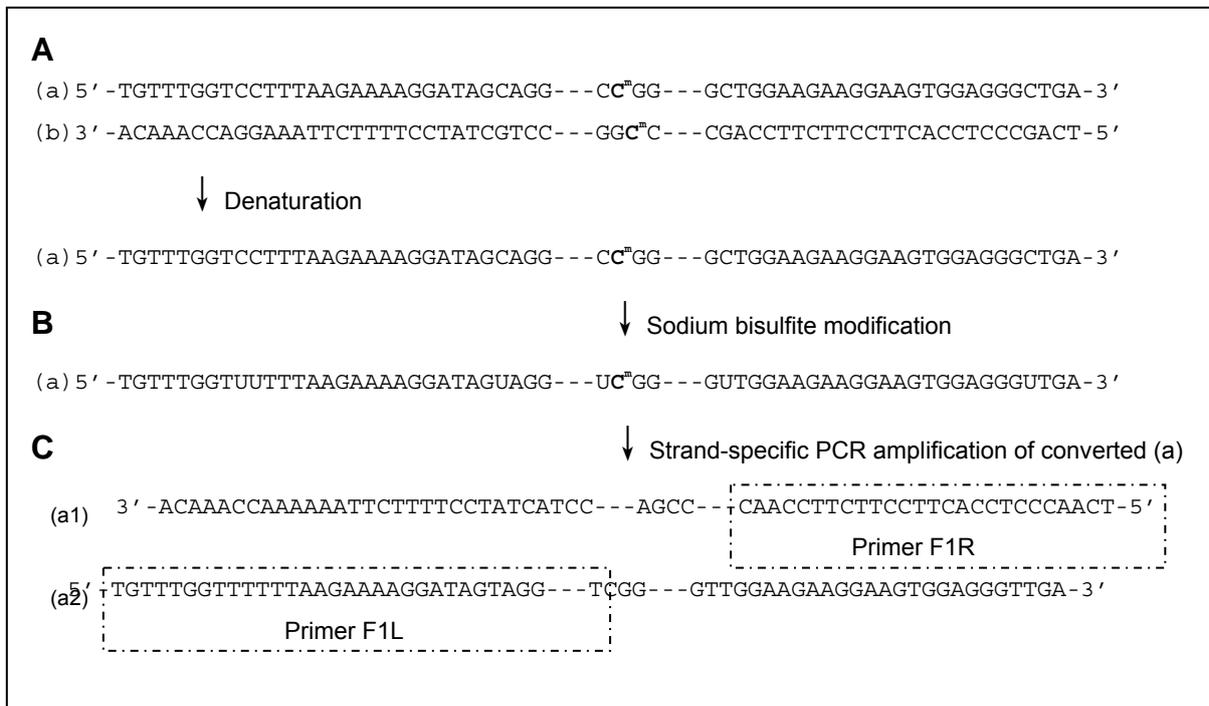


Figure 11 Amplification of a sequence in the promoter region of the *pKi-67* gene after bisulfite treatment of genomic DNA. (A) Sequence before bisulfite treatment. Methylated cytosine is marked as c^m. (B) Bisulfite converted sequences are shown (all the cytosines were converted to uracils except the methylated ones). (C) Strand-specific primers for the sense strand are indicated. Uracil (formerly cytosine) and thymine residues were amplified as thymines in the PCR amplification, while unconverted methylated cytosines were still amplified as cytosines. Two types of sequences are obtained after PCR. Strand (a2) loses all the cytosines (replaced by thymines) only with exception at the position of m⁵C (compare with the sense strand). Strand (a1) loses all the guanine residues (replaced by adenines) except when it is opposite to m⁵C (compare with the anti-sense strand).

At least eight clones of each PCR product sample were sequenced ensuring analysis of both alleles (the probability for “missing” one allele would be less than 1/256 under this circumstance). One hundred and five CpG sites in the *pKi-67* promoter, extending from 510 nucleotide bases upstream of transcriptional start site to 416 bases downstream of transcriptional start site, were examined for methylation. Analysis of all sequences (including gDNA samples from all sources mentioned above) revealed a universal unmethylation pattern of the *pKi-67* promoter. There was not a single sequence possessing more than four methylated CpG dinucleotides (percentage of methylated CpG motifs is less than 4%). A sequence with CpG methylation less than 5% is regarded as unmethylated (Toyota et al., 2000). As described in **Figure 12**, the methylated CpG sites are distributed in a sporadic pattern and no site-specific or region-specific methylation event was found either in normal or in tumor cells (tissues). No single CpG site has been detected to be methylated in more than two clones in each sample. The CpGenome Universal Methylated DNA was again used as a methylation-positive control. In this control sample, all cytosine residues were converted to thymines after bisulfite modification, except those methylated cytosines within CpG dinucleotides kept intact (**Figure 13**). Therefore the effectiveness of the procedure was verified.

A

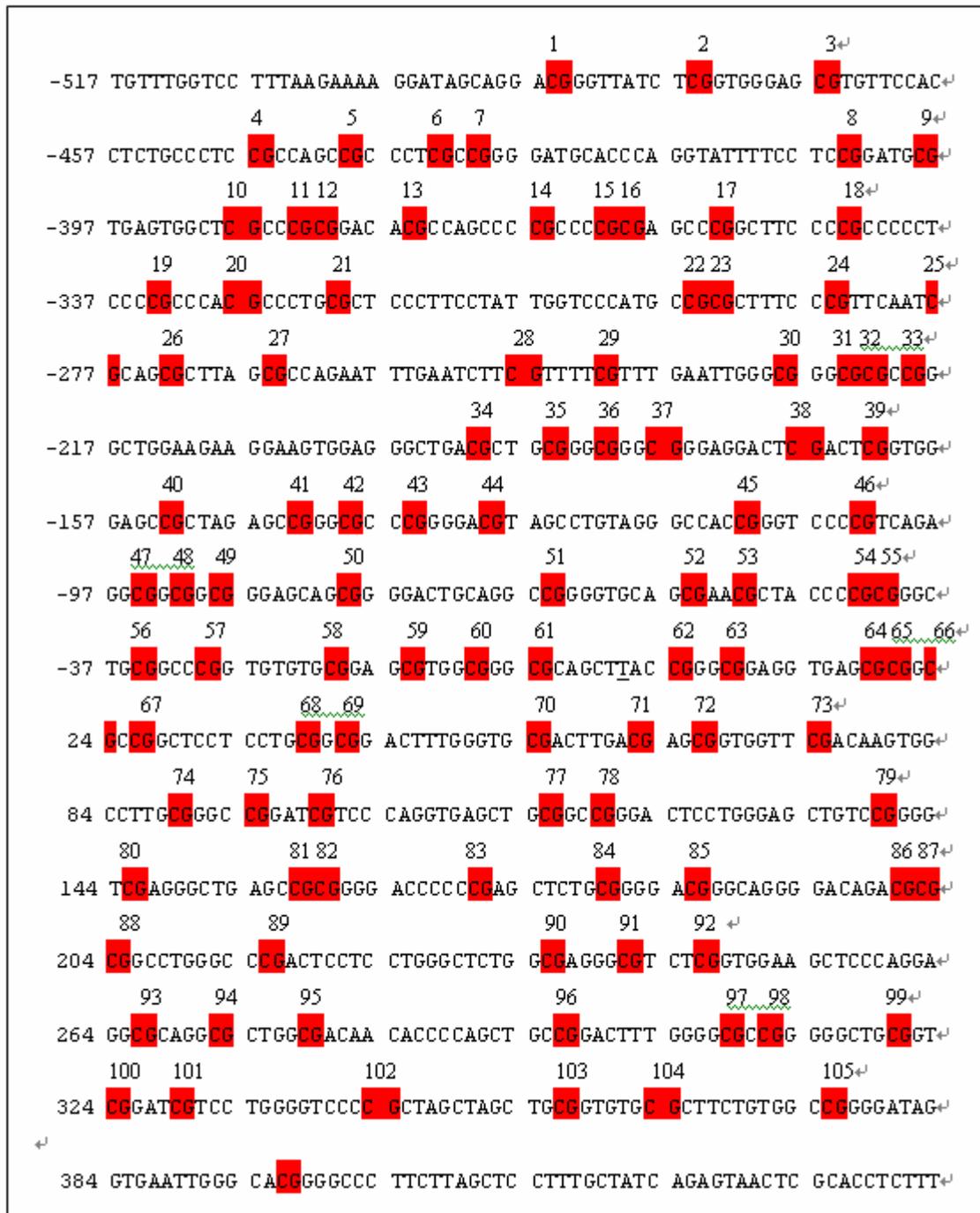


Figure 12 Methylation pattern of the *pKi-67* promoter region. (A) One hundred and five CpG sites in the *pKi-67* promoter, extending from 510 nucleotide bases upstream of transcriptional start site to 416 bases downstream of transcriptional start site, were examined for methylation status using bisulfite sequencing assay. Each CpG dinucleotide is highlighted with red color and numbered.

B-FIII

Sample	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105		
MNC-1	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
MNC-2	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○
MNC-3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
N168	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
N199	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
NL-1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
NL-2	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
SW480	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
DLD1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
T168	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
T199	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○

Figure 12 Methylation pattern of the pKf-67 promoter region. (B) Genomic DNA samples from blood monocytes of healthy donors (MNC1-3), normal colon mucosa (N168, N199), normal liver tissue (NL1, NL2), colon cancer cell lines (SW480, DLD1), and tumor tissues of colorectal carcinoma patients (T168, T199) were included. Methylation status of CpG sites are indicated as follows: white circles mean: unmethylated clone (from eight) at this site, gray circles: one methylated clone and black circles: two methylated clones. No single CpG site has been detected to be methylated in more than two clones of each sample.

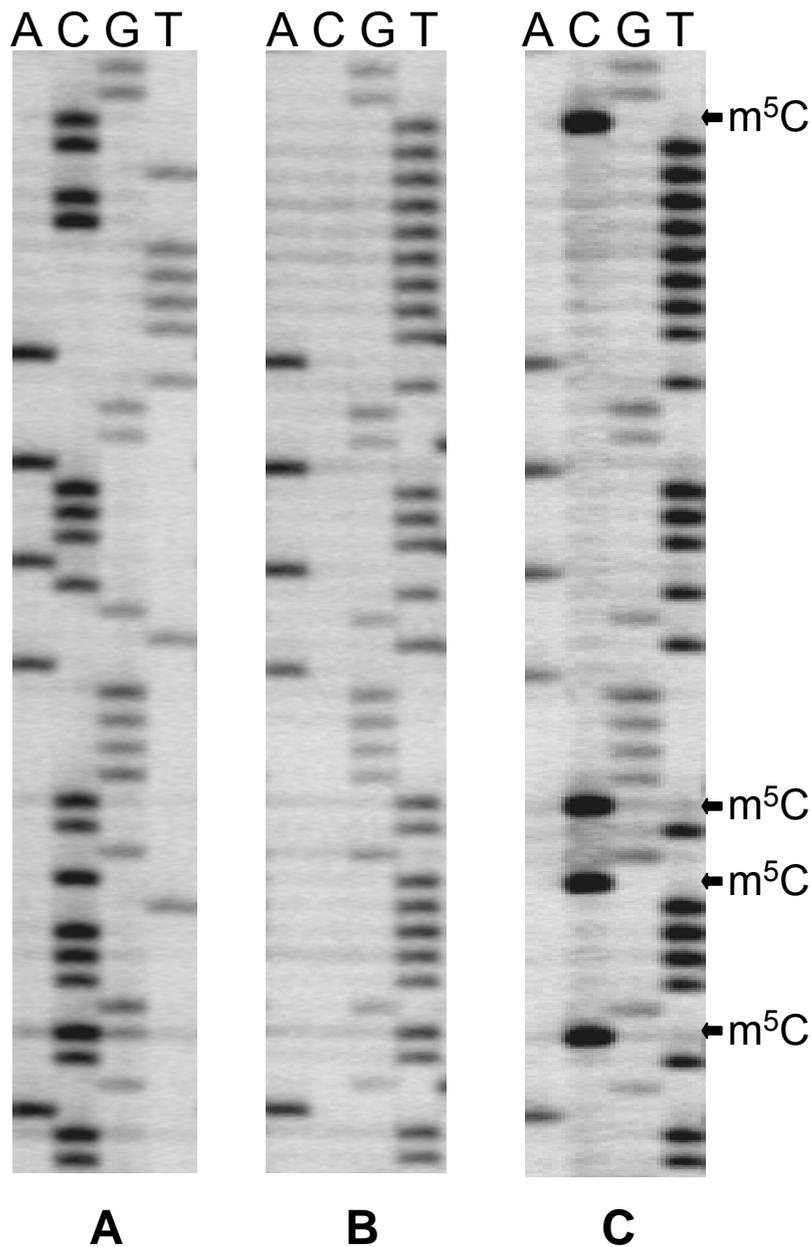


Figure 13 Sequence of the *pKi-67* CpG island in the original template DNA, bisulfite converted template DNA and bisulfite converted “Universal Methylated DNA”. DNA samples with or without bisulfite modification were cloned and sequenced. **(A)** Original sequence of DNA sample without bisulfite treatment. **(B)** Sequence of bisulfite converted template DNA. All cytosines are replaced by thymines including the cytosines within CpG dinucleotides indicating that these CpG sites are all unmethylated. **(C)** Sequence of bisulfite converted “Universal Methylated DNA”. All cytosines have been converted to thymines except at four CpG sites that were known to be methylated and used as a control.

4.3.3 Results of the two alternative methods are consistent

In this work, we used a combination of two different methods for detecting the methylation status of the *pKi-67* promoter CpG island. First, we used the methylation-specific restriction-PCR-assay to check methylation status of six specific CpG sites (at recognition sites of methylation-sensitive restriction enzymes as shown in **Figure 5**) within the *pKi-67* promoter region. Subsequently, methylation pattern of the whole promoter CpG island was mapped using the bisulfite sequencing method. A consistency between the results of the two different procedures was observed. We did not encounter any “imbalanced” methylation pattern: while the CpG sites at the recognition sites of methylation-sensitive endonucleases are unmethylated, quite many methylated CpGs could be found at other regions of the CpG island.

The methylation status of the *pKi-67* promoter in colorectal cancer and normal colon mucosa (10 cases each) samples were analyzed by the methylation-specific restriction-PCR-assay only. No methylated CpG was found in this procedure. Later, four DNA samples (2 cases from colorectal cancer and 2 cases from normal colon mucosa) were randomly selected, and underwent bisulfite sequencing assay to verify the correctness and representativeness of the methylation-specific restriction-PCR-assay. In agreement with the former results, a universal unmethylation pattern was detected.

In conclusion, the two methods revealed independently of each other a universal unmethylation status of the *pKi-67* promoter.

5. Discussion

5.1 DNA methylation: an important mechanism of epigenetic regulation

DNA methylation (5-position methylation of cytosine within the symmetric dinucleotides CpG) is an important epigenetic modification of the eukaryotic genome affecting various cellular processes. In normal healthy cells, methylated CpGs are widely spread throughout the whole genome, while unmethylated CpGs are primarily confined to those “CpG islands” which usually occur at promoter and first exon regions of protein encoding genes. Specific proteins that bind to CpG islands and protect them from *de novo* methylation were postulated to be involved in this phenomenon (Turker, 1999).

Physiologically, DNA methylation is associated with a compact chromatin structure (Bird, 2002; Bird and Wolffe, 1999; Ng et al., 1999), chromosomal stabilization (Hsieh and Fire, 2000), X-chromosome inactivation (Avner and Heard, 2001), and genomic imprinting of specific genes (Reik and Walter, 2001). DNA methylation has been shown to play an important role in regulation of gene expression during embryogenesis and further cell differentiation (Bird, 2002; Li, 2002; Thomassin et al., 2001).

Even though hypermethylation of a gene’s promoter is known to cause inhibition of its transcription, the precise mechanism accounting for this inhibition is still not entirely clear. One explanation could be that methylation of certain promoter DNA sequences suppresses gene transcription by directly interfering with the binding of transcription factors (Comb and Goodman, 1990; Huntriss et al., 1997). However, this is unlikely to be a prevalent mechanism for transcriptional silencing, since most transcription factors do not have CpG dinucleotides within their binding sites. Furthermore, this direct interference model cannot account for the wide range of biological phenomena that rely on methylation for the global silencing of large domains or even entire chromosomes, such as X inactivation in females (Robertson, 2001). An alternative model for methylation-dependent gene silencing is that the hypermethylated DNA sequence causes the binding of a family of methyl-CpG-binding proteins (MeCP2 MBD1, MBD2, and MBD3).

These proteins would then recruit a protein complex to the local chromatin containing a histone deacetylase and histone methylases (Fuks et al., 2003; Nan et al., 1998). Modification of histones (histone deacetylation, histone H3 lysine 9 methylation and binding of chromatin regulatory proteins such as HP1 and others) will lead to a conversion of chromatin structure from a transcriptionally competent pattern into a closed pattern that can no longer be accessed by the basal transcriptional machinery (Bird, 2002; Kondo et al., 2003).

5.1.1 Aberrant DNA methylation in cancer

Aberrant DNA methylation is now recognized as a common and important event in cancer development (Kondo and Issa, 2004). It can be generally summarized as genome wide hypomethylation and regional hypermethylation, especially at selected gene-associated CpG islands that are normally unmethylated (Baylin and Herman, 2000; Jones and Laird, 1999).

5.1.1.1 Hypomethylation

In the physiologically state, repetitive and parasitic elements (such as transposons and retroviruses) are heavily methylated in normal cells. However in cancers, an extensive change into hypomethylation occurs to these elements and is supposed to cause increased genomic instability (Robertson, 2001). De-repression of satellite sequences by hypomethylation can result in non-homologous recombination and chromosomal alterations (Scarano et al., 2005). An example for hypomethylation related chromosomal rearrangements in tumors is the rearrangement in the pericentromeric heterochromatin of chromosome 1 and 16, which is usually associated with Wilm's tumor (Qu et al., 1999). Hypomethylation has also been implicated in activation of specific genes in cancer. As mentioned in the preceding paragraph, CpG islands are usually unmethylated in human somatic cells. However, some of them seem to be methylated (Strichman-Almashanu et al., 2002). Examples of hypomethylation associated gene activation are *HRAS* (Feinberg and Vogelstein, 1983) and *MYC* (Ghazi et al., 1992), which are two important examples of oncogenes involved in the onset of cellular transformation and/or in tumor progression.

5.1.1.2 Hypermethylation

The most emphasized effect of aberrant DNA methylation in tumorigenesis is the inactivation of tumor suppressor genes by promoter region hypermethylation (Jones, 2002). These genes, including DNA repair genes (e.g. *MLH1*, *GSTP1*, *MGMT*), cell cycle regulation genes (e.g. *p16*, *p15*, *APC*), genes related to apoptosis (e.g. *p73*, *DAPK1*) and signal transduction (e.g. *E-cadherin*) normally possess in their promoter regions unmethylated CpG islands in human somatic cells. On the contrary in neoplastic cells, these CpG islands are found to be hypermethylated (Esteller et al., 2001; Robertson, 2001). The expression of the corresponding gene is silenced and the silencing can be partially relieved by demethylation of the promoter region (Baylin and Herman, 2000; Jones and Laird, 1999). This epigenetic event is somatically heritable and an increased density of methylated CpG sites within a promoter region can accumulate over time during tumorigenesis (Jones and Baylin, 2002). The hypermethylation of promoter CpG islands may inactivate both alleles of a tumor suppressor gene or may act in concert with classical genetic mechanisms such as point mutations or deletions. It may play a causal role in promoting tumor development (Worm and Guldborg, 2002). It has been noted that gene promoter hypermethylation in cancer showed significant tissue specificity. For example, *MLH1* is hypermethylated in colorectal (Kane et al., 1997) and gastric carcinomas (Toyota et al., 1999) but is infrequently methylated in esophageal (Eads et al., 2001) and hepatocellular carcinomas (Shen et al., 2002).

5.1.1.3 Clinical implications of DNA methylation

Aberrant DNA methylation in cancer has been proposed for early diagnosis, determination of prognosis, and as potential therapeutic targets (Herman and Baylin, 2003). For analysis of the methylation status, tumor specific DNA can be isolated from blood (based on the assumption that tumor cell DNA is shed into the blood stream) and other body fluids such as, sputum, urine, and breast duct fluid (Sidransky, 2002). For example, Palmisano et al. (2000) showed that aberrant hypermethylation of the *p16* and/or *MGMT* promoters was detected in tumor and sputum DNA from patients with lung cancer in 100%.

Such changes of methylation could even be detected in sputum samples up to 3 years prior to clinical diagnosis. Recently, detection of aberrant methylation (promoter hypermethylation of *SFRP2*, *HPPI1*, and *MGMT*) in fecal DNA was used as a molecular screening tool for colorectal cancer. The result indicated a 93.7% sensitivity and a 77.1% specificity (Huang et al., 2007). An aberrant methylation pattern has also been proposed as a prognostic marker for colorectal cancer: simultaneous hypermethylation of multiple genes seems to reflect an adverse prognostic event, that is potentially independent of classical prognostic markers (Toyota et al., 1999).

Epigenetic changes do not interfere with the information content of the affected genes and are potentially reversible (Lubbert, 2000). In this regard, the suppressed activity of tumor suppressor genes by epigenetic mechanism may be recovered at two different levels: by inhibition of DNA methylation and by inhibition of histone deacetylation. Potent inhibitors of DNA methylation or histone deacetylase are available and they can modulate gene transcription *in vitro* and *in vivo* at non-toxic concentrations. An inhibitor of DNA methylation, 5-aza-2'-deoxycytidine, has been widely used as a demethylating agent *in vitro*, and is used clinically in the treatment of acute leukemias and myelodysplasia (Lubbert, 2000). Likewise, cell culture experiments have shown that histone deacetylase inhibitors (e.g. trichostatin A) can reactivate a range of methylation silenced genes, and several of these agents are now in clinical trials (Marks et al., 2001). However, a major drawback of drugs targeting the DNA methylation or histone acetylation machineries is that they lack essential specificity. Concomitant activation of genes that are normally silenced, but contribute to cellular immortalization and tumor progression in the activated state, may significantly compromise the beneficial effects of these drugs (Worm and Guldborg, 2002). Future research will be directed at developing more refined strategies to reactivate specific genes.

5.2 Methylation status of the *pKi-67* promoter CpG island

5.2.1 Methylation status of the *pKi-67* promoter in human somatic cells

Within the scope of this thesis it was shown that a “CpG-rich region” in the *pKi-67* promoter region fulfills the formal criteria of a CpG island (Gardiner-Garden and Frommer, 1987). In the human genome, CpG islands within the promoter region of protein encoding genes are usually unmethylated (Yamashita et al., 2005). Nevertheless, there are a few exceptions: it has been suggested that some oncogenes and tumor promoting genes have their associating CpG islands hypermethylated in normal somatic cells and therefore expression of these genes is silenced. Examples of such genes are: *HRAS* (Feinberg and Vogelstein, 1983), *MYC* (Ghazi et al., 1992), *Xmrk* (Altschmied et al., 1997), *HOX11/TLX1* (Watt et al., 2000), and *u-PA/PLAU* (Pakneshan et al., 2004). A demethylation of these genes has been observed in different types of carcinomas and was associated with up-regulation of gene expression. Recently, tissue-specific methylation differences in human somatic cells have also been reported. One example is the methylation of the *maspin/SERPINB5* promoter. In normal cells expressing *SERPINB5*, the promoter CpG island is unmethylated and the promoter region has an accessible chromatin structure. By contrast, normal cells that do not express *SERPINB5* have a completely methylated promoter, which forms an inaccessible chromatin structure for gene transcription. The transcriptional repression could be relieved by inhibition of DNA methylation (Futscher et al., 2002).

In order to investigate the methylation status of the *pKi-67* promoter CpG island in normal human somatic cells, we first used gDNA samples from healthy donors' blood monocytes. Both, the methylation-specific restriction-PCR-assay and the bisulfite sequencing assay indicated that the CpG island of the *pKi-67* promoter was uniformly unmethylated in human blood monocytes. In order to investigate whether there is a different methylation pattern of the *pKi-67* promoter CpG island in other human tissues, we then tested normal tissue from liver (2 samples) and colon mucosa (10 samples). As promoter hypermethylation is usually associated with repression of gene expression (Scarano et al., 2005), it is more likely to find a hypermethylated *pKi-67* promoter in the tissues that have

very low or even no expression of this gene. According to previous literature, the Ki-67 labeling indices varied from 0.1% (crypt base) to 27.7% (luminal surface) in normal colon tissue (Kikuchi et al., 1997). In normal liver tissue, Ki-67 is almost not expressed (Ki-67 indices between 0% - 0.5%) (Rudi et al., 1995). Semi-quantitative RT-PCR analysis of our samples also demonstrated a relatively low *pKi-67* mRNA level in normal liver tissue compared to the reference gene *β2-microglobulin*. We tested gDNA samples from normal tissue of liver and colon mucosa with the methylation-specific restriction-PCR-assay as well as the bisulfite sequencing assay. However, we did not find any significant *pKi-67* promoter hypermethylation event in these two tissue types. It is therefore reasonable to assume that the *pKi-67* promoter CpG islands are essentially unmethylated in all kinds of human tissues.

It has been a general assumption that the Ki-67 protein is present in all proliferating cells (G₁, S, G₂, and mitosis), but absent from quiescent cells (G₀) (Scholzen and Gerdes, 2000). A recent article showed that despite the low expression of *pKi-67* in non-proliferating cells, the protein can nevertheless be detected in these cells using a very sensitive immunofluorescence technique. Further more, the Ki-67 protein is located at sites linked to rRNA transcription both in quiescent and proliferating cells, indicating a role of the Ki-67 protein in the early steps of rRNA synthesis (Bullwinkel et al., 2006). This finding suggested that the Ki-67 protein is involved in basic cell functions. One can hypothesize that this protein is essential not only for the proliferating cells but also for quiescent cells although a quite low amount respective expression level could be sufficient for the quiescent cells. Since the half life of the Ki-67 protein is short (only 90 min) (Heidebrecht et al., 1996), it is reasonable even for quiescent cells to maintain a certain degree of *pKi-67* mRNA transcription to ensure the minimal Ki-67 protein amount that is necessary for rRNA synthesis. This hypothesis seems to be consistent to our results: The *pKi-67* promoter is uniformly unmethylated in various tissues, thus allowing gene transcription. This phenomenon is also observed in tissues expressing very low level of Ki-67 protein (such as liver).

5.2.2 Methylation status of the *pKi-67* promoter in cancer

Although infrequent, there is evidence that some genes (such as *COX2* and *TERT*) whose expression should favor the neoplastic process have their promoters “oddly” hypermethylated in colorectal cancer and some other tumors with or without gene down regulation (Devereux et al., 1999; Toyota et al., 2000). To rule out the possibility that there might be a different methylation pattern of the *pKi-67* promoter CpG island in cancer as compared to normal somatic cells, we then assessed three colon cancer cell lines (SW480, DLD1, SW620) and ten tumor tissues from colorectal cancer patients with tumor stages ranging from Dukes’ grade A to C. The two methods (methylation-specific restriction-PCR-assay and the bisulfite sequencing assay) consistently indicated that the *pKi-67* promoter CpG islands were unmethylated in colon cancer cell lines as well as in colorectal cancer tissues. This result is surely in accordance with the high *pKi-67* mRNA transcription level in colorectal cancer. Thus, the “odd” promoter hypermethylation in cancer that has been observed for *COX2* and *TERT* does not seem to apply for *pKi-67*.

5.2.3 DNA methylation and the regulation of *pKi-67* transcription

Our semi-quantitative RT-PCR analysis revealed that the *pKi-67* mRNA was expressed distinctively higher in tumor cells (SW480, DLD1) than in normal somatic cells (blood monocyte and normal liver tissue) (**Figure 8**). However, both the malignant and normal cells share the same methylation pattern in their *pKi-67* promoter CpG islands — all of them are unmethylated. Therefore, we conclude that the promoter DNA methylation mechanism is not involved in the regulation of *pKi-67* transcription, in particular in colon cancer. The sharp difference of *pKi-67* transcription level between normal and cancer tissue is most likely due to some other methylation-independent regulating mechanisms, e.g., gene amplification, differently expressed transcription factors or altered sequences within the gene’s promoter.

5.3 Technical problems

5.3.1 Technical problems in the methylation-specific restriction-PCR-assay

The methylation-specific restriction-PCR-assay is a frequently applied method for DNA methylation analysis because of its relative simplicity, low cost, and ease of result interpretation (Oakeley, 1999). It is extremely beneficial for projects that require to process large number of samples. Hereby, the polymerase chain reaction allows very high sensitivity with a minimum detection rate of 0.1% of methylated sequences in 10 ng of the initial DNA (Singer-Sam et al., 1990).

Nevertheless, the methylation-specific restriction-PCR-assay has some immanent disadvantages. It can only detect a limited number of potential methylation sites which are contained in the recognition sequences of methylation-sensitive endonucleases (Oakeley, 1999). Another technical problem of this approach is a possible artifact caused by incomplete digestion of a DNA sample (Oakeley, 1999). This problem generally results from impurity of the DNA sample and can be corrected by a re-purification before digestion. Careful extraction of gDNA in the presence of proteinase K and SDS followed by phenol extraction and extensive dialysis (no ethanol precipitation) usually results in complete DNA cleavage. If not, addition of 1 mM spermidine often alleviates the problem (Rein et al., 1997).

In order to make sure whether or not we encountered the problem of incomplete digestion of template DNA in our experiment, a methylation-positive control (a CpG Universal Methylated DNA) was used in the methylation-specific restriction-PCR-assay. The methylation-positive control DNA was amplified successfully after *HpaII* (methylation-sensitive enzyme) digestion. When treated with a methylation-insensitive isoschizomer of *HpaII*: *MspI*, no product could be seen after PCR amplification verifying a complete template digestion in our procedure (**Figure 9D**).

5.3.2 Technical problems in the bisulfite sequencing assay

The bisulfite sequencing assay can provide an exact display of every methylated cytosine in the sequence of interest.

It is extremely useful to detect a site-specific or region-specific methylation. However, the bisulfite sequencing assay is often technically demanding and may be prone to several pitfalls as discussed below.

5.3.2.1 Incomplete conversion of template DNA

First, the bisulfite conversion of cytosine to uracil requires the DNA substrate to be single-stranded, while in double-stranded DNA the conversion rate decreases to less than 0.1% (Hayatsu, 1976; Shortle and Nathans, 1978). Therefore, it is important to make the DNA template completely denatured prior to bisulfite treatment and to keep the single DNA strands dissociated throughout the process of incubation. As reported by Frommer et al. (1992), DNA was alkaline denatured and treated with 3.1 M sodium bisulfite (pH 5.0) at 55°C for 16 h. However, the high salt concentration of the bisulfite reaction leaves the single-stranded DNA in a particularly unfavorable conformation, and virtually leads to DNA renaturation. Obviously, this would increase the risk of incomplete conversion of cytosine to uracil. Various attempts have been tried to reduce strand annealing. One possibility is digestion of gDNA into small pieces (with average length of ~1 kb) before bisulfite treatment. Then DNA is denatured and incubated with sodium bisulfite for 5 h only with repeated cycles of heating to 95°C (Rein et al., 1997). This approach might be helpful in reducing DNA renaturation, but suffers from another problem: DNA is very prone to acid-catalyzed depurination. Repeated heating to 95°C combined with an acidic reaction condition can also cause severe degradation of the DNA sample by catalyzing DNA depurination as discussed later. Other solutions to protect DNA against renaturation such as diluting the initial DNA template or incubating DNA with bisulfite at a temperature of 0°C for 24 h were also reported (Feil et al., 1994). Olek et al., (1996) introduced an improved modification of the bisulfite sequencing procedure in which the bisulfite treatment and subsequent PCR steps are performed on material embedded into agarose beads. This ensures optimal bisulfite reactivity by maintaining the DNA in single stranded form and efficiently prevents loss of DNA during the experimental procedure.

5.3.2.2 Template degradation due to DNA depurination

Long time bisulfite treatment in an acidic condition (pH 5.0) can cause template DNA to be significantly degraded, mainly as a result of DNA depurination. This excessive depurination limits the sensitivity of subsequent PCR by reducing the number of full-length molecules that can be amplified (Oakeley, 1999). This risk of DNA degradation can be reduced by changing bisulfite reaction parameters. Such a modified protocol was reported using higher bisulfite concentration (5 M bisulfite), lower incubation temperature (50°C), and reduced reaction time (only 4 h) (Raizis et al., 1995). Using agarose beads is another accepted alternative method to prevent template degradation (Olek et al., 1996). It is of additional advantage to improve the sensitivity of PCR reaction by performing a nested PCR for amplification of a bisulfite treated DNA sample (Clark et al., 1994).

In our bisulfite sequencing experiment, the degradation of template DNA emerged to be the major problem. It was solved by the following attempts: (1) DNA template was treated with sodium bisulfite at a lower temperature (50°C) and with shortened time duration (12-14 hours) as compared to the classical procedure of Frommer et al., (1992). (2) The sequence of interest was divided into three consecutive fragments, each size about 300 bp for the following PCR and cloning. (3) After bisulfite modification, Nested/semi-nested PCR was run to improve the efficiency of amplification.

5.4 Future perspectives

As we addressed all possible pitfalls of the utilized DNA methylation assay procedures, our data clearly demonstrate that the *pKi-67* promoter CpG islands are unmethylated in both normal and malignant cells. The sharp difference of *pKi-67* transcription level between normal and cancer cell (tissue) is most likely due to some other methylation-independent regulating mechanisms. Future investigations about the *pKi-67* gene's expression regulation should focus on determination of transcription factor binding sites in the promoter sequence and analysis of upstream signal transduction pathways. A better understanding of gene-regulation of proteins involved in cell cycle maintaining and/or regulation could enable development of new therapeutic approaches for cancer diseases.

6. Summary

The expression of the human Ki-67 protein is strictly associated with cell proliferation. It is expressed in all active phases (G₁, S, G₂ and mitosis) of proliferating cells, but absent from quiescent cells (G₀). This fact makes the Ki-67 protein a widely used marker to assess proliferation activity in a given cell population. The *pKi-67* gene is expressed distinctively stronger in malignant tumors than in normal tissues at both the mRNA and protein level. However, little is known about the regulating mechanism of this gene's transcription. The sequence information of the *pKi-67* gene's 5' promoter region shows a large CpG dinucleotide rich fragment (a CpG island). CpG-rich regions are related to DNA methylation modification. This project therefore aimed at analyzing the methylation status of the *pKi-67* promoter CpG island in normal cells and neoplastic cells, and investigated whether promoter methylation was involved in the regulation of *pKi-67* mRNA transcription.

Semi-quantitative RT-PCR demonstrated that the *pKi-67* mRNA was expressed higher in tumor cells than in normal somatic cells. Genomic DNA samples were tested by methylation-specific restriction-PCR-assay and bisulfite sequencing to determine the methylation status of their *pKi-67* promoter CpG islands. Results showed that in normal cells of human blood (blood monocytes), colon mucosa and liver (all exhibit a relative low *pKi-67* expression), the promoter CpG islands of the *pKi-67* gene were uniformly unmethylated. Further more, the *pKi-67* promoter CpG islands were found to be unmethylated in the neoplastic cells (colon cancer cell lines of SW480, DLD1, SW620, and samples of primary colorectal cancer).

It is therefore reasonable to assume that the *pKi-67* promoter CpG islands are essentially unmethylated in all kinds of human somatic cells. The methylation status of this CpG island is not changed in carcinomas although the transcription level of *pKi-67* mRNA is distinctively up-regulated.

Therefore, the promoter DNA methylation mechanism does not seem to be involved in the regulation of *pKi-67* transcription. Thus, alternative mechanisms must be responsible for the different *pKi-67* transcription levels between malignant and normal cells, e.g., differently expressed transcription factors, altered promoter sequences or gene amplifications. Future investigations about the *pKi-67* gene's expression regulation should focus on determination of transcription factor binding sites in the promoter sequence and analysis of upstream signal transduction pathways.

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8. Abbreviations

A	ampere
APS	ammonium persulfate
bp	base pairs
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddNTP	2', 3'-dideoxynucleotide 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dH ₂ O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratories
h	hour
gDNA	genomic DNA
kD	kilo Dalton
LB	Luria-Bertani
M	molar
min	minute
mRNA	messenger ribonucleic acid
NF κ B	Nuclear Factor kappa B
ng	nanogramme
nm	nanometer
OD	optical density
PBS	phosphate buffered saline

PCR polymerase chain reaction
RNA..... ribonucleic acid
rpm..... rotations per minute
sec second
TAE Tris-acetate-EDTA buffer
TBE..... Tris-boric acid-EDTA buffer
TEMED tetramethyl ethylenediamine
Tris..... trishydroxymethylaminomethane
Taq..... *Thermophilus aquatus*
V volt

9. Supplement

9.1 Clinical data

Table 4 Clinical data of colorectal cancer patients in this study

Sample No.	Age	Gender	TNM	UICC grade
1	63	Male	T ₃ N ₁ M ₀	IIIB
2	73	Male	T ₃ N ₂ M ₀	IIIC
3	74	Female	T ₃ N ₀ M ₀	IIA
4	70	Male	T ₂ N ₀ M ₀	I
5	57	Male	T ₃ N ₀ M ₀	IIA
6	58	Male	T ₃ N ₀ M ₀	IIA
7	85	Female	T ₃ N ₀ M ₀	IIA
8	58	Male	T ₂ N ₀ M ₀	I
9	83	Female	T ₃ N ₁ M ₁	IV
10	77	Female	T ₃ N ₀ M ₀	IIA

Table 5 Samples used in different tests

Test	Samples
<i>pKi-67</i> mRNA expression test	<p>Normal blood monocytes (n = 1), Normal liver tissue (n = 1) colon cancer cell lines (SW480, DLD1) Normal blood monocytes (n = 3)</p>
Methylation-specific restriction-PCR-assay	<p>Normal colon mucosa (n = 10), Normal liver tissues (n = 2) Colon cancer cell lines (SW480, DLD1, SW620), Colorectal cancer tissues (n = 10) Normal blood monocytes (n = 3) Normal colon mucosa (n = 2)</p>
Bisulfite sequencing assay	<p>Normal liver tissues (n = 2) Colon cancer cell lines (SW480, DLD1) Colorectal cancer tissues (n = 2)</p>

9.2 Chemicals and Kits

Acetic acid	Merck (Darmstadt, Germany) (K 17991588)
Agarose	Invitrogen (Paisley, UK) (15510-027)
Ammonium acetate	Merck (Darmstadt, Germany) (101115-1000) (24861-4)
Blood & Cell Culture DNA Midi Kit	Qiagen (Hilden, Germany) (13343)
Chill-out liquid wax	Biozym (Oldendorf, Germany) (999908)
DMSO	Merck (Darmstadt, Germany) (102952-100)
DNA Polymerization Mix	Amersham-Biosciences (Little Chalfont, UK) (27-2094-01)
DNA molecular weight marker VI	Roche Applied Science (Mannheim, Germany) (1062-590)
DNeasy Tissue Kit	Qiagen (Hilden, Germany) (69504)
EDTA	Merck (Darmstadt, Germany) (1-12029-1000)
Ethanol	Mallinckrodt Baker (Deventer, Netherland) (8006)
Ethidium bromide	Roth (Karlsruhe, Germany) (22181)
Ficoll	PAA (Pasching, Austria)

	(J 15-004)
Hydroquinone	Sigma-Aldrich (Seelze, Germany) (15616)
Isopropanol	Merck (Darmstadt, Germany) (818766)
Kanamycin	Sigma (Deisenhofen, Germany) (K 4000)
NucleoSpin Plasmid Kit	Macherey-Nagel (Düren, Germany)(740588250)
1 × PBS	Gibco/BRL (Eggenstein, Germany) (20012-019)
QuantiTect Reverse Transcription Kit	Qiagen (Hilden, Germany) (205311)
Rapid Gel XL Sol.	USB (Cleveland, USA) (US 75863)
RNeasy Plus Mini Kit	Qiagen (Hilden, Germany) (74134)
Sodium metabisulfite	Aldrich (Steinheim, Germany) (161519)
Sodium hydroxide	Merck (Darmstadt, Germany) (1-06482-1000)
Trypsin	PAA (Pasching, Austria) (L 11-002)
Thermo Sequenase kit RPN 2438	Amersham-Biosciences (Little Chalfont, UK) (RPN 2438)
<i>Hpa</i> II	New England Biolabs (Ipswich, UK) (R0171S)
<i>Msp</i> I	New England Biolabs (Ipswich, UK) (R0106S)

*Sac*II

New England Biolabs (Ipswich, UK)
(R0157S)

Taq DNA Polymeras

Invitrogen (Paisley, UK)
(10342-020)

9.3 Markers for nucleic acids

DNA Molecular Weight Marker VI

Roche (Mannheim, Germany)
(1062-590)

9.4 Primers

Table 6 Sequence information of primers used in the current study

Primer name	Sequence	Location
MSRPA forward	5' -TAT AGT GTC CCA GGT GTT TGG TC-3'	11267-11289 *
MSRPA reverse	5' -CCA ATT CAA ACG AAA ACG AAG ATT C-3'	11533-11557 *
PF1L	5' -TTT TTA <u>GTA</u> GTT TTA AAT <u>TTT</u> <u>TTG</u> <u>GTA</u> ATG-3'	11229-11258 *
F1L	5' -TGT TTG <u>GTT</u> <u>TTT</u> TAA GAA AAG GAT <u>AGT</u> AGG-3'	11281-11310 *
F1R	5' -TCA <u>ACC</u> CTC CAC TTC CTT CTT CCA <u>AC</u> -3'	11581-11606 *
F2L	5' -GG <u>T</u> TGG AAG AAG GAA GTG GAG <u>GGT</u> T-3'	11580-11604 *
F2R	5' -ACC CC <u>T</u> <u>AAC</u> <u>AAC</u> TCC CAA <u>AAA</u> TCC-3'	11918-11941 *
F3L	5' -G <u>TA</u> GGG <u>ATT</u> <u>TTT</u> GGG <u>AGT</u> TGT <u>T</u> -3'	11914-11935 *
F3R	5' -CCC C <u>TT</u> <u>ACC</u> CAA TTC ACC TAT C-3'	12176-12197 *
M13 forward	5' -TGT AAA ACG ACG GCC AGT-3'	pCR4-TOPO vector
M13 reverse	5' -CAG GAA ACA GCT ATG ACC-3'	pCR4-TOPO vector
Ki67 forward	5' -CCA AAG CAA ACA CCT GAC-3'	9218-9235 #
Ki67 reverse	5' -AGG GAC CGA GTC TTG TAA T-3'	9616-9634 #
B2M forward	5' -ATC CAG CGT ACT CCA AAG ATT-3'	121-141 †
B2M reverse	5' -CAT GTC TCG ATC CCA CTT AAC TAT-3'	394-417 †

* according to GenBank data base gi 1944550; # gi 103472004; †gi 37704380

Primers used for amplification of bisulfite treated DNA samples are: PF1L, F1L, F1R, F2L, F2R, F3L, and F3R. In forward primers, all the non-CpG “C”s in the original sequence are replaced by “T”s, while in reverse primers, all the non-CpG “G”s are replaced by “A”s (underlined). When a CpG dinucleotide was included in the primer sequence, a mismatch to both the methylated and unmethylated sequence was incorporated into the primer at the C residue of the CpG dinucleotide for forward primer and at the G residue of the CpG dinucleotide for reverse primer (with boxes).

9.5 Buffers and stock solutions

TE buffer (per liter):

5 ml 2 M Tris (pH 8.0), 2ml 0.5 M EDTA, pH 8.0

50× TAE buffer (per liter):

242 g Tris base, 57.1 g glacial acetic acid, 100 ml 0.5 M EDTA, pH 8.0

10× TBE (per liter):

108 g Tris base, 55 g boric acid, 40 ml 0.5 M EDTA, pH 8.0

9.6 Instruments and equipments

Biofuge 22R Centrifuge	Heraeus (Hanau, Germany)
Biosphere filtered pipette tips	SARSTEDT (Nümbrecht, Germany)
Electrophoresis power supply E443	CONSORT (Turnhout, Germany)
Equipment for digital photo documentation	MWG Biotech (Ebersberg, Germany)
Incubator Kelvitron	Heraeus (Hanau, Germany)
Lab scale PC 400	Mettler-Toledo (Giessen, Germany)
Model 4300L DNA sequencer	LI-COR (Lincoln, USA)
Megafuge 1.0R Centrifuge	Heraeus (Hanau, Germany)
Microscope Axiovert 10	Zeiss (Jena, Germany)
Multiply PCR cups	SARSTEDT (Nümbrecht, Germany)
NanoDrop ND-1000 spectrophotometer	NanoDrop Technologies (Wilmington, USA)
pH-Meter Toledo 320	Mettler-Toledo (Giessen, Germany)
Primus 96 ^{plus} Thermal cycler	MWG-Biotech (Ebersberg, Germany)
Thermomixer compact	Eppendorf (Hamburg, Germany)
UV Transluminator	Fröbel (Wasserburg am Bodensee, Germany)
Vortex-genie 2	Scientific Industries (Bohemia, USA)
Water bath	GFL (Burgwedel, Germany)

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