DNA methylation in the placenta and in cancer
with special reference to folate transporting genes

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Abstract

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DNA methylation is an epigenetic mechanism that regulates the gene transcription. Folate is used in cellular synthesis of methyl groups, nucleic acids and amino acids. In complex diseases like cancer and neural tube defects (NTD), various genetic and epigenetic alterations can be found that disrupt the normal cell function. The main goals of this thesis were to analyze whether the genes responsible for the folate transport (*FOLR1, PCFT,* and *RFC1*) could be regulated by DNA methylation in placenta, blood leukocytes and colorectal cancer. We also addressed the genome-wide DNA methylation changes in colorectal cancer and cervical cancer.

We found that changes in the methylated fraction of the *RFC1* gene were dependent on the *RFC1* 80G>A polymorphism in placental specimens with NTDs and blood leukocytes from subjects with high homocysteine (Paper I). In colorectal cancer, the greatest difference in DNA methylation was observed in the *RFC1* gene and was related to a lower protein expression (Paper II).

In Paper III and IV we studied the DNA methylated fraction using a high-density array. Paper III was focused on genes in the DNA repair pathway and frequently mutated in colorectal cancer. We found that aberrant methylation in the DNA mismatch repair genes was not a frequent event in colorectal cancer and we identified five candidate biomarker genes in colorectal cancer, among them the *GPC6* and *DCLRE1C* genes. In Paper IV, we found hypomethylation of genes involved in the immune system in cervical cancer specimens compared to healthy cervical scrapes and we identified twenty four candidate genes for further evaluation of clinical value.

In conclusion, the work of this thesis filled a relevant knowledge gap regarding the role of differential methylation of the folate transport genes in NTD and colorectal cancer. This thesis work also provided insights into the functional role of DNA methylation in cancer specific pathways and identified potential novel biomarker genes.

Keywords: DNA methylation, CRC, placenta, cervix, leukocytes, T-DMRs, folate, array, expression

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Sammanfattning

DNA metylering är en epigenetisk mekanism som styr genuttrycket. Folat är viktig för många reaktioner i cellen bland annat för produktion av metylgrupper som används vid DNA metylering. Komplexa sjukdomar som till exempel cancer och ryggmärgsbråck orsakas förmodligen av flera genetiska och epigenetiska rubbningar av normala cellfunktioner. Syftet med denna avhandling var att studera DNA metyleringsmönstret i gener som transporterar folat (*FOLR1*, *PCFT*, och *RFC1*) i placenta, vita blodceller och colorektalcancer. Det andra målet var att studera globala förändringar i DNA metylering i colorektalcancer och cervixcancer.

I de två första studierna analyserade vi DNA metyleringsmönstret i folattransportgenerna i vita blodceller från individer med lågt och högt homocystein (användes som uppskattning på folat status), placentavävnad från normalt utvecklade foster och de med ryggmärgsbråck samt colorektalcancer och frisk colon vävnad. Vi fann att skillnader i *RFC1* genen, i placentavävnad från foster med ryggmärgsbråck och vita blodceller från individer med högt homocystein, berodde på vilken genotyp individerna hade vid polymorfin *RFC1*80G<A. I colorektalcancer fann vi att *RFC1* genen var mer metylerad och hade ett lägre uttryck av RFC1 proteinet jämfört med frisk colon vävnad.

Vi analyserade DNA metyleringsmönstret med hel-genom array teknik i colorektalcancer och cervixcancer. Studie III var inriktad på specifika gener i DNA reparationsmekanismen och gener som är ofta muterade i colorektalcancer. Resultaten visade att det fanns få skillnader i metylering mellan colorektalcancer och frisk vävnad i gener som tillhör DNA reparations mekanismen. Vi identifierade skillnader i metylering i fem gener som kan vara av betydelse för colorectalcancer utvecklingen. Den sista studien i avhandlingen analyserade förändringar i cervixcancer vävnad jämfört med premalign vävnad och normal cervixvävnad. Vi fann att gener som tillhör immunsystemet var ofta hypometylerade i cervixcancer. Dessutom identifierade vi 24 kandidatgener som kan vara av betydelse för utveckling av cervixcancer.

Arbeten i denna avhandling har belyst vilken roll DNA metyleringsmönster har i gener som transporterar folat vid placentavävnad från födslar med ryggmärgsbråck, och även identifierat nya potentiella biomarkör gener i colorektalcancer och cervixcancer.

List of publications

This thesis is based on the following papers and will be referred in the text by their Roman numerals:

I. Farkas SA, Böttiger AK, Isaksson HS, Finnell RH, Ren A, Nilsson TK. Epigenetic alterations in folate transport genes in placental tissue from fetuses with neural tube defects and in leukocytes from subjects with hyperhomocysteinemia. Epigenetics. 2013;8(3):303-16.

II. Farkas SA, Befkadu R, Hahn-Strömberg V, Nilsson TK. DNA methylation and expression of the folate transporting genes in colorectal cancer. *Manuscript*.

III. Farkas SA, Vymetalkova V, Vodičkova V, Vodička P, Nilsson TK. DNA methylation changes in genes frequently mutated in colorectal cancer and in the DNA repair and Wnt/β-catenin signaling pathway genes. *Submitted*.

IV. Farkas SA, Milutin-Gašperov N, Grce M, Nilsson TK. Genome-wide DNA methylation assay reveals novel candidate biomarker genes in cervical cancer. Epigenetics. 2013;8(11):1213-25.

List of abbreviations

ATP	adenosine tri phosphate
CGI	CpG island
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CpG	cytosine phosphate guanine
CRC	colorectal cancer
DNA	deoxyribonucleic acid
Dnmt	DNA methyltransferase
FFPE	formalin fixed paraffin embedded
FOLR1	folate receptor 1
HPV	Human papillomavirus
IHC	immunohistochemistry
MSI	microsatellite instability
NTD	neural tube defect
PCFT	proton-coupled folate transporter
RFC1	reduced folate carrier
RNA	ribonucleic acid
RT-PCR	real time polymerase chain reaction
SAM	S-adenosylmethionine
SNP	single nucleotide polymorphism
T-DMR	tissue-specific differential methylated region

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Introduction

Every cell in our body contains the same genetic material in the form of deoxyribonucleic acid (DNA). The genetic information is contained in the sequence of the nucleotides adenine (A), guanine (G), cytosine (C), and thymine (T). The genes are turned on (expressed) in various combinations and give rise to the set of proteins that are specific for the shape of the cell and its function. Expressed genes are first transcribed into ribonucleic acid (RNA) and then translated to proteins.

In 1942, Conrad Hal Wadding introduced the term epigenetics and defined it as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" (1). Today, the definition is somewhat modified: ''An epigenetic trait is a stable heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence.' (2).

Epigenetic mechanisms can be divided into three major categories: DNA methylation, histone modifications, and non-coding RNAs (3), see Figure 1. This thesis focuses on the DNA methylation changes that occur in cell types of various origins and therefore a comprehensive introduction of how this mechanism regulates gene expression is given.

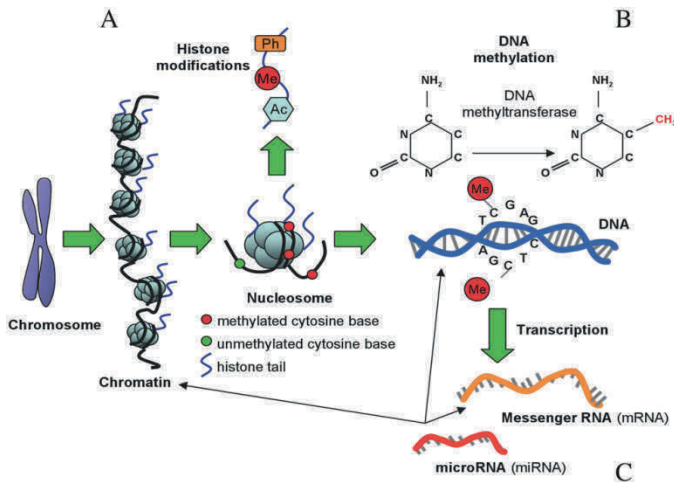

Figure 1 *The epigenetic mechanisms. The nucleosome is composed of the DNA helix wrapped around histones. The tail of the histone can have various modifications such as methylation (Me), acetylation (Ac) or phosphorylation (Ph, fig A). The cytosine nucleotide in the DNA sequence can be methylated or unmethylated and is called DNA methylation (B). The RNA molecules can be in the form of microRNA and affect the gene expression by binding to the DNA or affect the mRNA in various ways (C), reprinted from (4).*

The DNA helix is wrapped around proteins called histones. There are many types of histone modifications that can take place such as methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, and glycosylation (5, 6). These modifications result in chromatin remodeling that affect the compactness of the chromosome and attracts various proteins that regulate downstream events such as transcription (5, 6).

About 90% of the genome is transcribed into ribonucleic acid (RNA) but only ~2% of these are mRNAs that code for a protein (7). The rest of the transcripts can be categorized as various short RNA molecules such as micro RNAs (miRNAs, Figure 1C) (7). They all have a regulatory role in gene expression either at a transcriptional or post-translational level by blocking the transcription or inducing cleavage of a target mRNA.

DNA methylation

DNA methylation is a covalent modification that occurs on the cytosine nucleotide (Figure 1B). The methylation pattern in vertebrates occurs in the context of a cytosine next to a guanine (CpG) while in other eukaryotic phyla such as plants and fungi, methylation of cytosine next to other nucleotides (for example CT, CTG) is common (8). Methylated DNA regions are associated with unexpressed genes while the unmethylated DNA regions are associated with expressed genes (9-11).

Nucleotide regions that are rich in CpG sites are called CpG islands (CGI) (12, 13). There are approximately 25,000 CGIs distributed in the human genome and approximately 60% are associated with a gene promoter region (12, 13). Gene promoter regions are DNA sequences that can bind proteins (transcription factors) that either activate or inhibit gene expression.

There are six DNA methyltransferase (Dnmt) enzymes involved in the methylation reactions of mammalian DNA: Dnmt1, Dnmt2, Dnmt3, Dnmt3a, Dnmt3b, and Dnmt3L (10, 11). The methyl group from S-adenosylmethionine (SAM) is used as a donor in the methylation reactions. It is synthesized from folate in the one carbon metabolism, Figure 2 (14).

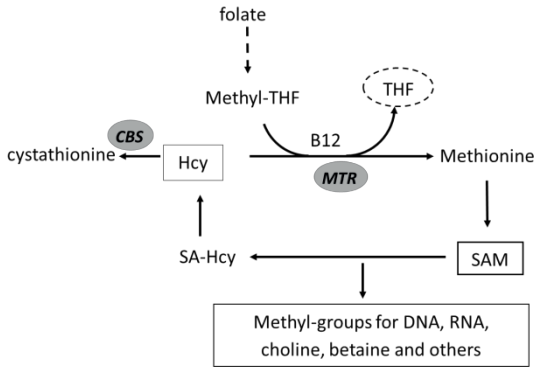

Figure 2. *Methyl donor S-adenosylmethionine (SAM) is generated in the one carbon metabolism. Folate is active in the forms of di- and tetrahydrofolates (DHF and THF), which transfer one carbon groups. Homocysteine can be converted to cystathionine or methionine which is a precursor molecule to SAM that acts as a methyl donor to a variety of cellular molecules including nucleic acids and proteins. The enzymes that catalyze the reactions are: MTR, methionine reductase and CBS, cystathione synthase. Modified from Breimer & Nilsson (15).*

Dnmt enzymes are regulated by other cellular proteins which may promote or inhibit their function, but also signal which parts of the DNA that should be methylated (11). The maintenance of the DNA methylation pattern from one cell to another is sustained by the Dnmt1 enzyme. It is located at the DNA replication site and methylates the newly synthesized DNA strand using the old one as template (10, 11). The Dnmt3 family members function primarily to establish *de novo* methylation, establishing new DNA methylation patterns. The function of the enzyme Dnmt2 is still not clear but it is suggested to be involved in the methylation reactions of RNA (10, 11).

DNA methylation is often studied by comparing two tissues, or comparing healthy and diseased cells. The terms hypo- or hypermethylation are used and mean that a DNA region is either less (hypo) or more (hyper) methylated compared to another one.

Methylation profile of healthy tissues

Profiling of the "healthy" human genome revealed several key features of the DNA methylated fraction of genes and CGIs. Genes have a structure composed of a 5' region that often involves promoter sequences, a gene-body region that includes introns and exons, and a 3' region that codes for the regulatory regions that influence post-transcriptional gene expression.

Because promoters are often localized in the vicinity of the 5' part makes this gene region interesting to study. Higher density of methylation at the promoter regions were associated with a lower gene expression (16-18), and a higher methylated fraction in gene-body regions was found in highly expressed genes (17, 19), suggesting a functional role for gene-body methylation. Most of the CpG islands in the 5' gene regions were found to be unmethylated (18). One study showed that DNA methylation of neighboring CpG sites at shorter distances are similar, suggesting co-methylation (18). From a chromosomal point of view, subtelomeric areas are more frequently methylated than other regions of the chromosome (16). Many repetitive elements are found in the subtelomere and are in this way silenced. Furthermore, changes in DNA methylation can also be observed between individuals (20), different age groups (21), sex (22), and populations (23).

The gene regions that display variation in DNA methylated fraction between tissues are termed tissue-specific DNA methylated regions (T-DMRs) and can be used to identify functionally important genes. The regions harboring T-DMRs were found to be prominently outside the CGIs (18, 24).

Placental tissues

Gene expressions in placental tissue play an important role in the development of the fetus. In particular, the imprinted genes in placenta have been given a lot of attention. Genomic imprinting means that the expression occurs from one allele, either of the maternal or paternal side of origin, while the inactive allele is silenced by one of the epigenetic mechanisms, usually DNA methylation (25). Incorrectly imprinted genes in placental tissue have been associated with very severe developmental disorders in the fetus because many of the imprinted genes in placenta regulate traits such as growth (26, 27). Furthermore, the placental epigenetic profile can be affected by environmental exposures such as diet. A striking example of how diet may affect the gene methylation is the agouti *Avy* mouse Dams fed with supplements of folic acid increased the methylation of the Avy CpG locus and the offspring showed a change in coat color that correlated to the methylation change (28).

DNA methylation in cancer

Thirty years ago, the first epigenetic change observed in cancer was loss of DNA methylation (29). Global DNA hypomethylation has been detected in several cancer types such as colorectal cancer (30), breast cancer (31),

and hepatoma (32). Hypomethylation has been located to the intragenic regions and has been associated with a higher expression of repetitive DNA sequences (31). It has also been suggested that hypomethylation contributes to the genomic instability (33), a frequent event in many cancers.

Hypermethylation in cancers has been suggested to occur in a high frequency at the CGIs in the gene promoter regions (34, 35). Therefore much of attention has been given to the hypermethylation events in cancer, and many methods have been developed to target mainly methylation in the CGIs. In the last few years, with the development of high-throughput technologies, the importance of the flanking CGI regions has been highlighted (24, 36-39).

Colorectal cancer

Colorectal cancer (CRC) is one of the three most commonly diagnosed cancers amongst both males and females (40) with the highest incidences in developed countries. CRCs can be divided into hereditary and sporadic cancer forms (>85% of all CRCs). However, only 5% of the hereditary CRCs have a gene mutation linked to the syndrome (41). In sporadic CRC, several molecular pathways have been suggested as the determinants of the progression to cancer (Figure 3). Molecular diagnostics have classified tumors in relation to genetic instability, CpG island methylation phenotype (CIMP), and the underlying precursor lesion (42, 43).

Tumors with genetic instability can be either microsatellite stable (MSS) with chromosomal instabilities (CIN) and microsatellite instable (MSI) with mutations in microsatellite regions due to underlying mutations in DNA mismatch repair genes (42, 43).

The tumors originating from the CIN pathway are suggested to arise from an adenoma, and due to mutations in oncogenes such as *KRAS* or tumor suppressor genes *p53* and *APC* (Figure 3a) (42, 43). The tumors originating from MSI are also suggested to arise from an adenoma but are due to mutations in one of the DNA mismatch repair genes and this leads to other gene mutations that affects the cell regulation mechanisms, such the TGF-β gene (Figure 3b) (42, 43). The third group of tumors are suggested to originate from a different precursor lesion called the serrated adenoma (Figure 3c) and these tumors are suggested to be driven by DNA methylation changes and inactivation of the *MLH1* gene and therefore the pathway is called CpG island methylator phenotype (42, 43). The CIMP pathway can be divided into following subgroups: cancers with a *BRAF* mutations and

methylation in many markers (CIMP-high) and cancers with *KRAS* mutations and methylation in few gene markers (CIMP-low) (42, 43).

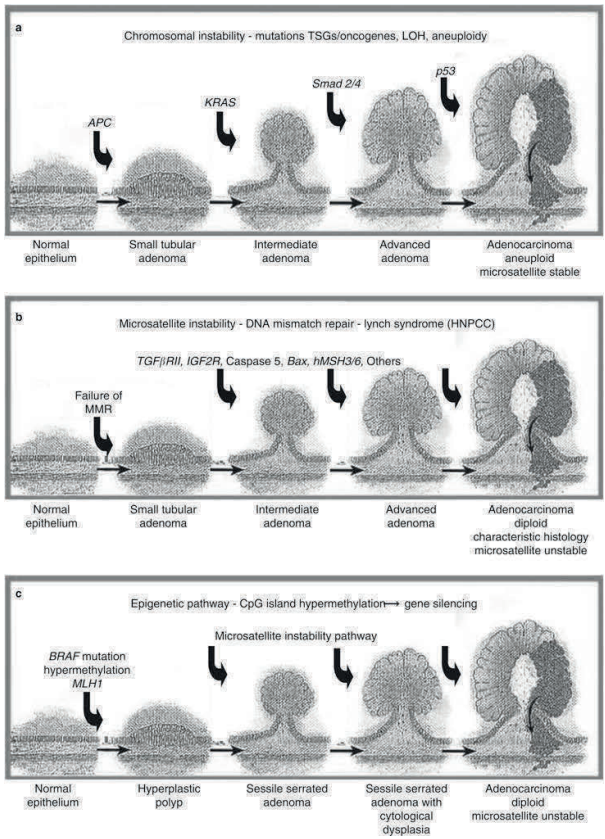

Figure 3. *Molecular pathways of the adenoma-carcinoma progression. a) The chromosomal instability (CIN) pathway includes mutations in many different genes such as in the adenomatous polyposis coli (APC) gene. b) The microsatellite instability (MSI) pathway is suggested to originate from mutations in one of the DNA mismatch repair genes. c) The CpG island methylator phenotype (CIMP) pathway is suggested to be driven by hypermethylation and inactivation of the MLH1 gene. Reprinted by permission from Macmillan Publishers Ltd: The American College of Gastroenterology (43), copyright 2011.*

Several microarray based studies have made attempts to investigate the association between CIN and CIMP and to define CIMP genes. They show that probably several CIMP groups exist (44-49). However, a clear definition of CIMP gene panel is still missing.

CRCs can be staged according to the tumor (T), node (N) and metastasis (M) system and describes the intestinal wall and peritoneal (pT1–pT4) penetration of the cancer, the number of affected regional lymph nodes (pN0–pN2), and occurrence of distant metastasis (pM)(50). The tumor stage and invasiveness are very strong prognostic predictors (42) although predicting the invasiveness of the tumor based on morphology can sometimes be difficult if structures such as veins and nerves are not visible (50). Molecular markers such as DNA methylation can be useful to refine the prognostic prediction (50). There are many potential DNA methylation biomarkers identified but few have been validated enough to be used in clinical practice, one gene that is used in diagnostic tests is the *SEPT9* gene (51-54).

Cervical cancer

Cervical cancer is the third most common cancer in women after breast cancer and CRC (40). Approximately 85% of all cases are found in the developing countries due, most probably to the lack of screening for precancerous stages and human papilloma virus (HPV) (40). Precancerous stages have been associated with cervical intraepithelial lesions (CIN) stages 1-3, but not all cases develop cervical cancer (55, 56). The combination of CIN and a persistent HPV infection seems to be the causative agents (55, 56).

The HPV infection starts at the basal cells due to micro-ruptures in the mucosa. The infected cells have a low expression of the HPV proteins (E) that stimulate the basal cells to divide. When the infection reaches to the upper zones of the epidermal layer the expression of viral proteins are increased, see Figure 3 (57, 58).

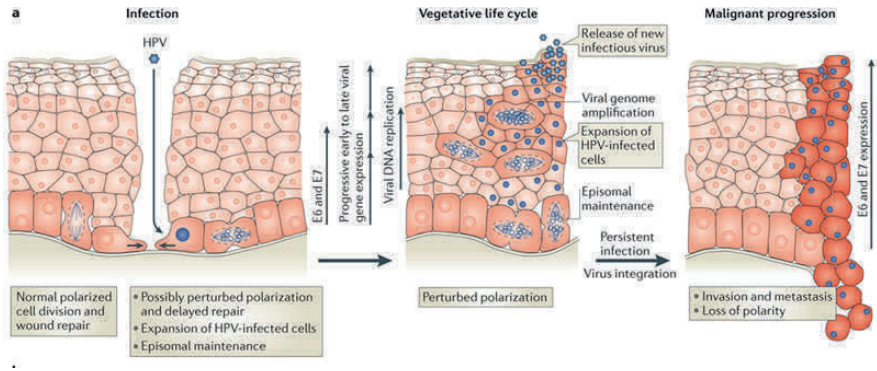

Figure 4. *The human papilloma virus (HPV) infects the basal cell layer and expresses the HPV proteins (E) at a low level. The cells divide and the HPV DNA replicates. When the cells in the middle layer are affected, the HPV protein expression also increases. The persistent infection disrupts the host cell mechanisms such as apoptosis and this process may lead to cancer progression. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (59), copyright 2012.*

The HPV infection is cleared in about 90% of the cases with both innate immunity (non-specific) and adaptive immunity (specifically towards HPV proteins) (60). It has been shown that persistent infection affects host cell apoptosis, cell cycle control, cell adhesion and DNA repair mechanisms that can lead to cancer initiation. Integration of the viral DNA into the host's has been correlated with the progression of cervical lesions to cancer (57, 58). There is a need for molecular biomarkers that can more accurately distinguish between tissues in the pre-malignant stage that will progress to cancer and those that will not.

There are many studies of DNA methylation in viral and host gene DNA (61, 62) but no biomarker has yet been established for molecular diagnostics. Most of the studies on the host gene methylation were performed on selected gene panels. Lendvai et al (63) used a more extended approach and comprehensively analyzed the differentially methylated regions in samples with CIN3 compared to normal cervical scrapes using a method based on precipitation of the methylated fraction and sequencing. They found hypermethylated regions in CGIs and hypomethylation outside CGIs and identified possible candidate biomarkers (63). More genome-wide DNA methylation studies analyzing cervical cancers are needed to identify genes useful to predict the outcome in cervical cancer.

Folate and the folate transporting genes

Folate is a water soluble B vitamin that can be found in leafy vegetables and in dairy products, which is a main source of folate in the Nordic countries. Folate is used as a one carbon donor in cellular biosynthesis of nucleic acids, amino acids, and methyl groups (14) as a part of the one carbon metabolism. Folate that comes from the diet is usually in the polyglutamate form (Figure 5) and is converted to monoglutamate by the enzyme glutamate carboxypeptidase present on the surface of the mucosal epithelium in the small intestine. The monoglutamate form is then converted to di- and tetrahydrofolate and this form is participating in the one carbon transfer reactions.

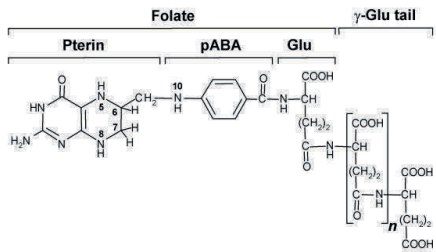

Figure 5. *Natural folates have up to eight glutamate molecules attached (γ-Glu tail). Reprinted from (64).*

Uptake of folates

Dietary folates are absorbed in the small intestine primarily by the proton coupled folate transporter (PCFT), the reduced folate carrier (RFC1) is also expressed in the small intestine but not functional due to low pH (65, 66), Table 1. Folate can be stored in the cells in the form of polyglutamate in the cytosol or mitochondria (14). Transport into peripheral tissues occurs mainly through the RFC1 protein and folate receptors (FR) (65), see Table 1. The placental tissue expresses the FRα, RFC1, and PCFT proteins (67). FRα and the PCFT proteins were found to be located on the maternal microvillus plasma membrane side while the RFC1 protein has been found at the fetal facing basal plasma membrane (67).

Table 1. Folate transporting genes studied in this thesis.

Gene name	Gene location	Protein name	Expressed in
FOLR1	Chr11q13	FRα	Epithelial cells of the lung, kidney, placenta
*SLC19A1** (also known as *RFC1*)	Chr21q22	RFC1	All tissues
*SLC46A1** (also known as *PCFT*)	Chr17q11	PCFT	Small intestine, placenta, kidney, liver

* In this thesis the *SLC19A1* and *SLC46A1*genes will be denoted as *RFC1* and *PCFT*.

Proton coupled folate transporter

The PCFT protein is encoded by the solute carrier family 46 A1 (*SLC46A1*) gene (Table 1) and consists of five exons (65, 66). The PCFT protein was identified by studying mutational analyses in subjects with hereditary folate malabsorption (65, 66). High levels of PCFT expression were found in the small intestine, liver and kidney (65, 66). The *PCFT* gene expression is regulated by nuclear respiratory factor 1 and vitamin D receptor (65, 66). Low *PCFT* gene expression and higher DNA methylation of the promoter region has been found in human leukemia cells (68) and anti-folate resistant HeLa cell line (69). One other study showed no difference in DNA methylation of the rat *Pcft* gene when comparing healthy jejunum and ileum that have different *Pcft* gene expression (70).

Reduced folate carrier

Reduced folate carrier belongs to the gene family of solute carriers 19 (*SLC19*) composed of three members out of which the *SLC19A1* gene codes for the RFC1 protein, see Table 1 (65, 66). This gene has six promoter regions (65, 66). The RFC1 protein is expressed in almost all tissues with highest levels in the placenta (65, 66). The gene expression is regulated by transcriptional factors Sp, USF, AP1, and C/EBP (65, 66) and DNA methylation. Lower DNA methylated fraction of the *RFC1* promoter region has been associated with expression in a breast cancer cell line (71, 72) but not in acute lymphoblastic leukemia (73), or osteogenic sarcoma (74). In ovarian cancer, higher *RFC1* promoter methylation was associated with lower expression (75). To date, there are no reports of DNA methylated fraction and expression of the *RFC1* gene in CRC specimens.

Folate receptor 1

The human folate receptor 1 (*FOLR1*) codes for the FRα protein (65, 76) and contains four promoter gene regions (65, 76). The FRα is expressed in leukocytes, epithelial cells of tissues such as the choroid plexus, lung, kidney, retina, and placenta (65, 76). The FRα has been found to be overexpressed in malignant tissues such as cervical and lung cancers (65, 76). The protein expression has also been associated with clinical prognosis and tumor stage (65, 76).

Folate deficiency

Folate is one of the key molecules in the nucleotide synthesis and a deficiency of this nutrient will affect tissues with a high cell division such as blood cells, or epithelial cells in the intestinal tract. Folate deficiency causes megaloblastic anemia which is an enlargement of the erythroblasts caused by defects in cellular proliferation and maturation (77). Suboptimal folate levels have been linked to neurological impairment, birth abnormalities, and cancer.

Neural tube defects

The most common birth defects are neural tube defects (NTD) and congenital heart defects. NTD is an opening in the spinal cord or brain that does not close during the development of the fetus (78). The causes of NTDs are linked to a combination of environmental factors such as smoking, diet and several genetic variations in the enzymes of folate metabolism (78). Higher intake of folate by women in periconceptional period reduces the risk of birth defects such as neural tube defects (79) and congenital heart defects (80). Folate fortification has led to a decreased incidence of NTDs. Because NTDs are not abolished in countries with folic acid fortified food, it suggests that additional mechanisms are involved in the development of NTDs.

Diseases due to loss-of-function mutations

Mutations in the human *FOLR1* and *PCFT* genes were associated with folate deficiency in central nervous system and developmental disorders (81, 82). Mice lacking the folate transporter *Folr1* die in utero and mice with a defective *Folr1* display developmental malformations, but can be rescued if supplemented with high folic acid (83). Mice that lack functional *Rfc1* gene die despite the supplementation with folic acid (84). These

studies suggest that the expression of folate transporting genes is important during embryogenesis but also later in the development.

Folate and cancer

The mechanisms of cancer development and the role of folate are complex and not clear as folate may both inhibit tumor growth and promote mutations leading to the development of the tumor. Anti-folate drugs are used in cancer treatment and lead to a slower tumor growth by inhibiting the function of folate dependent enzymes in the one carbon metabolism (85). On the other hand, low folate intake has been associated with genomic hypomethylation (86) and chromosome breaks (87), which are frequent events in cancer progression. Mice supplemented with folate showed a decrease in the number of colonic neoplasms in a time- and dose-dependent manner (88). One recent epidemiological study did not find any statistically significant effects on cancer incidence due to folate supplementation (89). More studies are needed to elucidate by which mechanism and what dose of folate may promote tumor growth.

Aims of the thesis

The two main aims of this thesis were, firstly, to analyze whether or not the genes responsible for the folate transport could be regulated by DNA methylation in placenta and cancer and secondly to comprehensively evaluate the DNA methylation changes in cancer. To meet these aims we used two different methodological approaches measuring locus specific and genome-wide DNA methylation.

The specific aims were to

I. Develop Pyrosequencing assays to measure DNA methylation of the folate transporting genes *FOLR1*, *PCFT* and *RFC1* and to identify T-DMRs in these genes (Paper I)

II. Analyze if the folate transporting genes are aberrantly methylated in placental specimens from fetuses with NTDs and in blood leukocytes from subjects with high total homocysteine (Paper I)

III. Study the DNA methylated fraction of the folate transporting genes and their protein expression in colorectal cancer specimens (Paper II)

IV. Perform a high-density genome-wide analysis of the DNA methylation signature in colorectal and cervical cancers (Papers III & IV)

Materials and methods

Study groups (Paper I-IV)

Placentas and leukocytes

In Paper I we performed a pilot study using samples with no clinical data denoted as "training set of samples" in the text. The training set consisted of 56 whole blood samples and four placental tissues (Dept. of Laboratory Medicine, Örebro University Hospital, Sweden). The blood samples were selected based on the total homocysteine (tHcy): a low tHcy in range of 5–10 µmol/L (n =25) and high tHcy in the range of 20–113 µmol/L (n = 25). We then analyzed a clinical cohort of placental specimens from deliveries of healthy fetuses (n = 48) and fetuses with neural tube defects (n = 75), from Shanxi Province, China.

Colorectal tissues

In Paper II we analyzed 28 CRC tissues and 33 healthy colonic mucosal tissues. The tissues from this cohort were formalin-fixed paraffin embedded (FFPE) and archived at the Dept of Laboratory medicine, Örebro University hospital, Sweden between the years 2008-2012. In Paper III, we used fresh frozen CRC tissues (n = 12) and adjacent colonic mucosal tissues (n = 12) recruited between 2009-2013 at the Thomayer Hospital (Prague, Czech Republic).

Cervical tissues

In Paper IV, we studied healthy cervical scrapes (n = 20), cervical scrapes with high-grade squamous lesions (CIN3, n = 18) and squamous cell cancer specimens (n = 6). All samples were collected at the Sister of Mercy Hospital, Zagreb, Croatia, between the years 2004-2011. The clinical characteristics were obtained from cytological smears or histopathology and all of the specimens were typed for HPV (90).

Ethical consideration

All clinical cohorts were collected with appropriate informed consent with ethical approval from the counties where the specimens were collected. The Regional Ethics Review Board, Uppsala, approved the Swedish part of the project performed in Paper I-IV (Dnr 2011/087, and Dnr 2012/242).

DNA extraction

We extracted genomic DNA for the methylation analyses performed in Papers I-IV. The genomic DNA from placental specimens, whole blood, and cervical specimens, were extracted using the GentraPure Gene, QIAmp EZ1 DNA blood 200 µl kit, or QIAmp DNA mini kit (Qiagen Inc.). DNA from fresh frozen and FFPE colorectal specimens was isolated with the QIAmp DNA mini kit or AllPrep DNA/RNA Isolation Kit (Qiagen Inc.). All tissue specimens were homogenized prior to DNA extraction. The FFPE specimens were de-paraffinized according to a standard routine protocol with several xylene and ethanol washes DNA extraction. All procedures were according to manufacturers protocol. The DNA concentrations were measured using NanoDrop ND-1000 UV-Vis Spectophotometer (NanoDrop technology).

RNA extraction

In Paper I we analysed mRNA expression of the folate transporting genes *FOLR1, PCFT,* and *RFC1* in placental tissues (n=4) and blood leukocytes (n=6) using the method real time polymerase chain reaction (RT-PCR). The total RNA was extracted as follows. The placental tissues were homogenized in TRIzol. Chloroform was added to each sample and then vortexed; after subsequent incubation and centrifugation the water phase containing the RNA was transferred to a clean vial. Isopropanol was added to the samples which were then incubated to precipitate the RNA. The samples were centrifuged to recover the RNA precipitate, which was washed with ethanol prior to dissolving in RNase free water. The samples were DNAse I treated to remove potential genomic DNA leftover.

The extractions from whole blood samples were made with the QIAmp RNA Blood Mini Kit (Qiagen Inc.). All RNA products were quantified by NanoDrop ND-1000 UV-Vis Spectophotometer (NanoDrop technology).

DNA methylation analyses

Bisulphite treatment of DNA

Most DNA methylation analyses require sodium bisulphite treatment of the DNA (91). The bisulphite treatment deaminates the unmethylated cytosine to uracile while the methylated cytosine remains unchanged (92). This process makes it possible to identify methylated cytosine positions and the unmethylated ones by comparing the bisulphite modified DNA sequence to a reference (Figure 6).

The studies performed in this thesis used the EZ DNA methylation Gold Kit for bisulphite treatment of the DNA, according to manufacturer's instruction (Zymo Research, Orion Diagnostica, Sweden). Briefly, 500 ng of DNA was denatured and bisulphite treated for 2.5 hours. The DNA was loaded on a spin-column and subsequent washing and desulphonation procedures were performed prior eluting the converted DNA in a buffer solution. The DNA was quantified by NanoDrop using the settings for RNA due to the single strand formation of the DNA that occurs upon bisulphite treatment.

The crucial steps in PCR based methylation analyses, regardless of the choice of methodology (Pyrosequencing assay technology, methylation specific PCR or high resolution melting), is a complete conversion of unmethylated cytosines to uracils and a specific PCR amplification targeting only the region of interest (93). To evaluate the quality of the methylation assay controls monitoring the bisulphite conversion efficiency of each DNA sample should be included.

Pyrosequencing methylation assay technology (Papers I, II & IV)

This sequencing by synthesis method is based on a PCR amplification of a defined locus of the bisulphite treated DNA (Figure 6), and the amplicon should be no more than 300 bases (94). In the PCR, the target region is amplified and the product represents both methylated and unmethylated cytosines (Figure 6). In this step the uracil nucleotides are substituted with thymines. The template is mixed with a sequencing primer and a synthesis reaction is then performed upon all the templates (Figure 6). The methylated cytosines in a CpG context are detected as C while the unmethylated ones are detected as T and the ratio of un-methylated cytosines and methylatied cytosines can calculated.

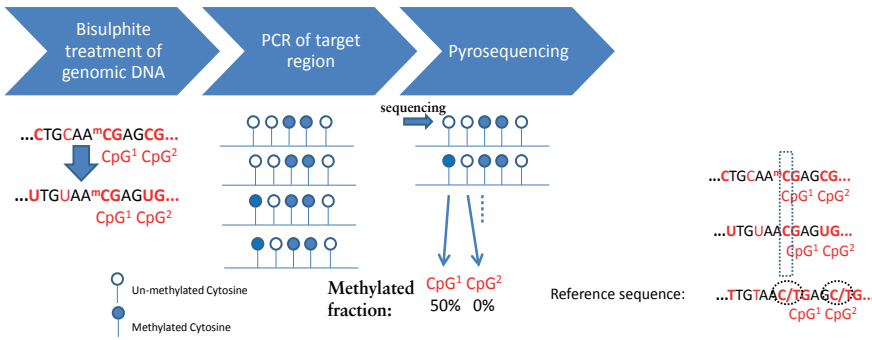

Figure 6. *The workflow of the Pyrosequencing method. The genomic DNA is bisulphite treated and a region of interest is amplified. The PCR product is sequenced and the methylated fraction of each CpG site is calculated.*

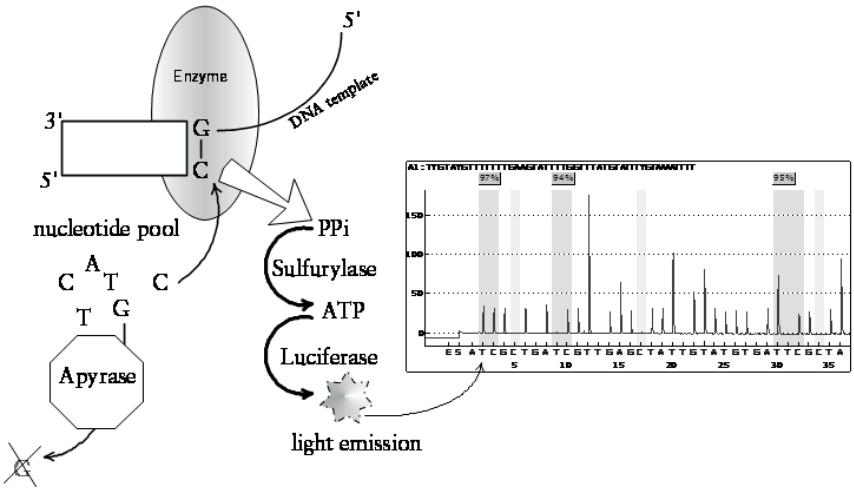

Figure 7. *The enzymatic reactions that occur in Pyrosequencing. The DNA template is produced in the PCR. When a nucleotide is incorporated to the new strand a light emission occurs that can be detected. The number of incorporated nucleotides is proportional to the peak height. The enzyme Apyrase degrades the unused nucleotides between the nucleotide additions.*

The methodological principle of pyrosequencing is the detection of light that is generated when a nucleotide base pairs with the complementary DNA strand (94). When a nucleotide binds, a pyrophosphate (PPi) is released which is used by the enzyme adenosine-tri-phosphate (ATP) sulfurylase to produce ATP. With the energy from ATP, the luciferase enzyme oxidises D-luciferin to oxyluciferin which emits a photon that can be detected with a camera (Figure 7). The nucleotide addition is predefined and there are inbuilt controls that allow the user to monitor the sequence specificity and the bisulphite treatment efficiency in every sample. The level of methylation is calculated automatically by the PyroMark software and presented in the pyrogram as percentage methylation.

Design of primers

The PCR and sequencing primers for the DNA methylation assays in the *FOLR1*, *PCFT*, and the *RFC1* genes (Papers I & II) were designed using the PyroMark assay design software 2.0 (Qiagen). One of the PCR primers was biotin labeled in order to isolate a single strand of the target product that serves as template in the sequencing reaction. The primers were chosen based on the location of the CpG island. We used the online

software CpG island searcher (http://cpgislands.usc.edu/) to locate CpG sites of interest both in the CGI but also outside the CGI. Eventually primers that had a high quality score, calculated by the Pyro Mark assay design software were selected. Primer details can be found in Paper I.

Illumina Infinium 450K DNA methylation array (Papers III & IV)

Array methods are based on short DNA probes of a specific target gene bound to a chip. The samples to be analyzed are added to this bead and upon binding a signal is detected. In the following sections the Illumina Infinium 450K DNA methylation array used in Papers III & IV will be described.

This array includes 485,764 CpG sites dispersed all over the genome, covering 99% of the annotated genes (95, 96). The detection of differentially methylated cytosine is based on a sample binding to the probe bead with the CpG site of interest. When a nucleotide extension of the methylated (C) or unmethylated (T) allele occurs a signal is emitted. There are several beads per locus, and a ratio in terms of β-value is calculated as an estimate of percentage methylation.

Assay procedure

The DNA was subjected to whole genome amplification and enzymatic digestion with reagents provided with the Illumina Infinium 450K kit (Illumina, Sweden). The fragmented DNA was hybridized on the bead chip and washing procedures were performed to remove all DNA that was not hybridized. A single nucleotide extension occurs with differentially labeled nucleotides (biotin labeled ddCTP and ddGTP; 2,4-dinitrophenol ddATP and ddTTP) and signal is detected. The β-value is generated by the signals obtained from the methylated (M) and unmethylated (U) allele and the β-value can range from 0 to 1.

Pre-processing of raw data

The β-values for CpG sites that had a detection *p* value above 0.01 were filtered away and not used in the subsequent data analyses. The detection *p* value is a measure of the probe quality, if it is distinguished from the background intensities. The array has several built in controls that can be used to monitor experimental factors such as bisulphite conversion of the DNA, staining efficiency, hybridization, and extension.

Data analysis

The results were analyzed either deductively (hypothesis based, Paper III) or inductively, exploring the top most striking differences (Paper IV).

The analysis of the >480 000 CpG sites was performed site-wise, using the mean β-value to calculate the differences ($\Delta\beta = \beta_{group\ A} - \beta_{group\ B}$) between groups, and regionally, using the median β-values to calculate the differences. Differentially methylated CpG sites or regions were considered relevant if the Δβ-value was ≥ 0.2 between groups and the *p* value measuring the statistical significance was < 0.05. The CpG loci in a specific group of samples could be classified as either hypermethylated (positive Δβ-value) or hypomethylated (negative Δβ-value). The regional analysis was based on the CpG islands, CpG shore, CpG shelves, TSS1500, TSS200, 5'UTR and 1st exon, gene body and 3'UTR.

Differentially methylated genes were analyzed bioinformatically using the Database for Annotation, Vizualization, and Intergrated Discovery (DAVID) (97) to analyze biological features associated with the hypo- or hypermethylated genes.

Gene expression

Real-time PCR

The samples used in Paper I were newly collected (blood, n=6) or fresh frozen (placentas, n=4) and we therefore used the method RT-PCR for the analysis of the *FOLR1*, *PCFT,* and *RFC1* gene expression. RT-PCR is a very sensitive method for detecting low amounts of a specific mRNA present in a sample (98). The quantification can be relative to a standard curve or to a co-amplified control sample. Briefly, the RNA was converted to cDNA prior to the RT-PCR which was performed on an ABI7500 Fast Real-Time PCR instrument (Applied Biosystems). The genes were quantified relative to the reference gene 18S. The probe sequences for each assay are presented in Paper I, Table 8.

Publicly available data sets

In Papers III & IV we chose to externally validate the methylation array results by using publicly available mRNA data sets. The gene expression data profiles are today frequently uploaded to publically available repositories (99). This has made it possible for other researchers to explore the deposited experimental results with other scientific questions and to test hypotheses of genes being expressed (100). There are several repositories

that maintain the data, such as Stanford Microarray Database (SMD), European Bioinformatics Institute's ArrayExpress, the Gene Expression Omnibus (GEO) kept by the National Center for Biotechnology Information (NCBI), or The Cancer Genome Atlas (TCGA), from the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) (101).

The studies in this thesis used data sets deposited to the GEO database and were analyzed by the GEO2R online application to identify differential gene expression (100). The data sets used were cited in the respective study.

Immunohistochemistry

In Paper II we had access to FFPE specimens and therefore it was appropriate to use immunohistochemistry (IHC) for the analysis of the *FOLR1*, *PCFT*, and *RFC1* expression. IHC measures protein expression by staining the target protein with a primary antibody and then detected using a secondary anti-rabbit antibody. The FFPE blocks (n = 56) from CRC specimens and adjacent colonic mucosal tissue, were sectioned in 4μm slices using a Leica microtome (Buffalo Grove, USA). The sections were mounted on super-frost slides and pretreated in a Dako Link at High pH (9.3). The stainings with the primary polyclonal rabbit antibodies anti- FRα (ab67422), anti-PCFT (ab25134), and anti-RFC1 (ab62302) from Abcam (Cambridge, UK) were performed according to the manufacturer's instructions (Dakocytomation, Denmark).

The slides were scored according to the number of stained cells as well as the intensity of the staining. Each sample was then given a total score which was then dichotomized to low/medium expression (total score 0-6) or high expression (total score 7-9).

Statistical analyses

In Papers I & II, the means were compared using Students t-test, ANOVA, binary logistic regression or Chi square test where appropriate. All tests were performed using the statistical software SPSS.

In Papers III & IV, the β-values were arcsine square root transformed and the empirical Bayes moderated t-statistic was used to generate the *p* values for the differences between the groups. The Benjamini-Hochberg method was used to adjust the *p* values for multiple testing.

Results and Discussion

DNA methylation of the folate transport genes (Paper I - II)

We have performed DNA methylation analysis of the folate transporting genes *FOLR1, PCFT,* and *RFC1* using a locus specific method, the Pyrosequencing assay technology (Paper I-II). With this method we have analyzed blood leukocytes, placental (cohort from China) and colonic mucosal specimens (cohort from Örebro). With the genome-wide approach to study DNA methylation we were able to obtain the DNA methylation signature of the folate transporting genes in two additional cohorts: colonic mucosal specimens (cohort from Prague) and cervical specimens (cohort from Zagreb).

Pyrosequencing methylation assays design

The methylation assays of the *FOLR1*, *PCFT* and *RFC1* genes were designed to cover CpG islands and -shore regions. They ultimately corresponded to 5' UTR and intragenic regions, the nucleotide numbers of the analyzed CpG sites are presented in Paper I in Tables 1-3. Totally, 24 assays covering 121 CpG sites were developed. The assays were tested for potential PCR bias by evaluating samples of known methylation percentage. Due to the bisulfite conversion of the DNA, a biased amplification of the unmethylated allele may occur (102). Our results showed slope values between 0.93 and 0.98 indicating a linear amplification of methylated and unmethylated allele. The intra-assay precision was calculated for selected assays (*FOLR1*, CpG sites 3-5; *PCFT*, CpG sites 45-48; *RFC1*, CpG sites 15-17), and the coefficient of variation was ≤ 4.4%.

Tissue-specific DNA methylation and mRNA expression

Identification of T-DMRs

In Paper I we performed an initial study using a training set of samples composed of placental specimens (n=4) and blood leukocytes (n=50) to identify putative T-DMRs in the *FOLR1*, *PCFT,* and *RFC1* genes. This approach aimed at identifying which gene regions are important to analyze in the clinical cohorts, as we did not want to confine our analysis to the CpG islands as most studies until now have been focused on this region. Genome-wide approaches have shown that variations in DNA meth-

ylation between diseased and healthy specimens (of the same tissue type) often co-localize with variations between tissues (T-DMRs) (24, 39, 103).

We obtained the following results in the initial study. The CpG sites 1-4 and 12-14 (flanking the predicted CGI) in the *FOLR1* gene were more methylated ($p < 0.05$) in blood leukocytes compared to the placental specimens; CpG sites 38 – 52 (CpG island and shore regions) in the *PCFT* gene were more methylated ($p < 0.05$) in blood leukocytes compared to the placental specimens; In the *RFC1* gene CpG sites 15-17 and 18-30 (CGI shore and CGI) were less methylated ($p < 0.05$) in leukocytes compared to the placental specimens.

Based on the above results we selected the following CpG sites to analyze in the clinical cohort of placental specimens from healthy fetuses or with NTD (Paper I), and the CRC specimens and healthy colonic mucosal specimens (Paper II): *FOLR1* CpG sites 1-2 and 12-14; *PCFT* CpG sites 1-5 and 45-48; *RFC1* CpG sites 8-14, 18-23, and 50-55. The sites 8-14 in the *RFC1* gene were also included because they showed differences in the methylated fraction when comparing blood leukocytes from subjects with high and low tHcy (Paper I, Table 5). Plotting methylated fractions in the *FOLR1, PCFT,* and *RFC1* genes for healthy placental specimens, blood leukocytes, and healthy colonic mucosal specimens, strengthen our findings that CpG sites 1-2 and 12-14 in the *FOLR1* gene, CpG sites 1-5 and 45-50 in the *PCFT* gene and CpG sites 8-14, and 18-23 in the *RFC1* gene may be functionally important T-DMRs (Figure 8).

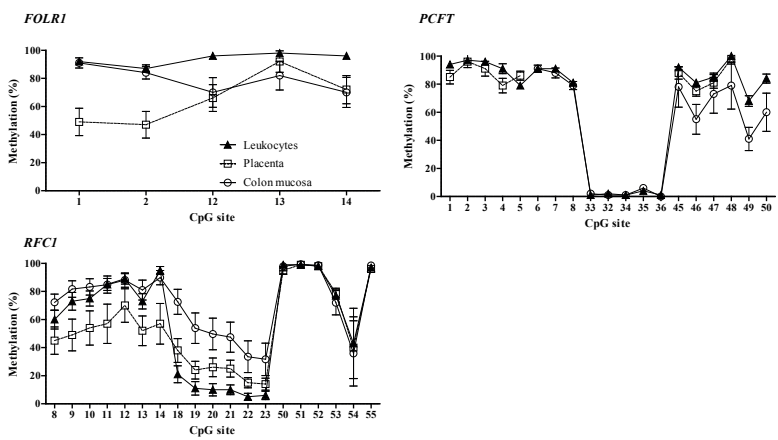

Figure 8. *Tissue-specific differential methylation of the folate transporting genes in leukocytes from healthy subjects (n = 25), placentas from healthy fetuses (n = 46), and healthy mucosal tissues (n = 15-25).*

Gene expression in relation to methylation

To further study the biological role of DNA methylation of the analyzed regions in the *FOLR1, PCFT,* and *RFC1* genes in the blood leukocytes, placental specimen and colonic mucosal tissues we studied their mRNA expression using the RT-PCR method and public data bases.

We analyzed gene expression with RT-PCR in blood leukocytes (n=6) and placental specimens (n=4). Higher *FOLR1* mRNA expression has been found in the four placental tissues compared to the leukocytes and considerably lower *PCFT* and *RFC1* mRNA expression in the placental tissues compared to leukocytes (Paper I, Table 4).

The inverse relationship between higher mRNA expression and lower methylated fraction (CpG sites 1-2) of the *FOLR1* gene in placental specimens compared to leukocytes, and lower mRNA expression and higher methylated fraction (CpG sites 18-23) of the *RFC1* gene in placental tissues compared to leukocytes underpins our hypothesis of a regulatory role of DNA methylation in these T-DMRs (Paper I, Table 8 and Figures 1 and 3). Our gene expression results are in accordance with the annotations found in the BioGPS data base (104).

The role of CpG sites 18-23 in the *RFC1* as T-DMR is further supported when comparing mRNA expression (data from the BioGPS data base). In the colonic mucosal specimens, placental specimens and blood leukocytes the expression was as found more in colon tissue and less in placental tissue and blood leukocytes. There is a reversed magnitude of DNA methylated fraction at CpG sites 18-23: colon tissue > placental tissue > blood leukocytes, Figure 8. A larger study is warranted to analyze the correlation between DNA methylation and expression of these genes.

RFC1 gene methylation in subjects with high or low tHcy

High plasma tHcy concentrations can be used as a proxy for low folate status. We were interested to find out if subjects with high plasma tHcy levels (used as proxy for poor nutrition, low folate status) have an aberrant DNA methylation pattern of the folate transporting genes when compared to subjects with low tHcy (proxy for sufficient nutritional status, high folate status). Our hypothesis was that gene regulation could be affected by DNA methylation in response to folate status in the cells. We observed differences in the DNA methylated fraction in the *RFC1* gene, but not in the *FOLR1* or *PCFT* genes. Subjects with high tHcy had a lower methylated fraction compared to subjects with low tHcy at 17 CpG sites (Paper I, Table 5). These statistically significant ($p < 0.05$) differences

ranged between 2-10%. A higher methylated fraction could respond to a lower RFC1 protein expression in subjects with low tHcy as a mechanism to prevent folate efflux (105). However, further studies with larger cohorts including folate status, and mRNA expression of *RFC1* gene are needed to settle this issue.

Methylation in placentas from subjects with neural tube defects

Folic acid supplementation pregnant women has been shown to reduce the risk of NTDs (106). Studies on mice models showed that folate transporting genes are highly important for the maintenance of folate homeostasis which is critical during embryonic development (83, 84). We wanted to see if there is an aberrant DNA methylation pattern of the human folate transporting genes *FOLR1*, *PCFT*, and *RFC1* in placental specimens from births with NTDs compared to the healthy ones. We did not find any difference in DNA methylation of these genes (Paper I, Figure 4). Our results suggest that DNA methylation of these genes does not have a role in the development of NTDs. We cannot rule out that these genes may be aberrantly regulated by other mechanisms that reduce their function and in that way contribute to NTD development, or that aberrant methylation of these genes is present in other cell types such as epithelial cells during neural tube closure. However, the collection of human embryonic cells is neither ethical nor practical to perform.

We observed large variations of the methylation in the two groups of placental specimens (Paper I, Figure 5). Placental tissue is composed of many cell types, and this can actually prevent identification of differences in methylation between the groups. One way of reducing this variation would be to isolate specific cell types and then measure DNA methylation.

DNA methylation and the role of *RFC1* 80G>A genotype

The 80G>A polymorphism (rs1051266) is located in exon 2 and causes a substitution of a histidine for an arginine in the protein. Individuals can have a wild-type genotype "GG" meaning that both alleles have a G nucleotide at this specific location; they can be heterozygous "GA" and carry one allele with A and one G nucleotide or they can be homozygous "AA" carrying two A alleles. Correlations of this polymorphism with plasma tHcy levels, serum folate levels and neural tube defects are not uniform (107-110). Some show association between AA genotype and higher folate levels while other no association. There is no conclusive finding of the risk for NTDs and the AA genotype. Therefore, we were interested to see if

there is a difference in methylated fractions in the *RFC1* gene between the genotypes (GG, GA, and AA) in subjects with high tHcy and NTDs.

This polymorphism (rs1051266, G>A) is placed after a cytosine (C) in the nucleotide sequence, and therefore causes loss of a CpG site in subjects with a GA or AA genotype. Consequently, we found the methylated fraction of this specific CpG site in subjects with a GA genotype was ~40%, and AA genotype ~ 3% (Paper I, Table 7: CpG site 54).

When we compared the methylated fraction of the *RFC1* gene between subjects of high or low tHcy and stratified by genotype, we found that the genotype is a predictor of the methylated fraction of a large number of CpG sites, see Figure 9 and in Paper I Table 7 the p^2 column. There is a statistically significant difference at 22 CpG sites between the high and low tHcy groups.

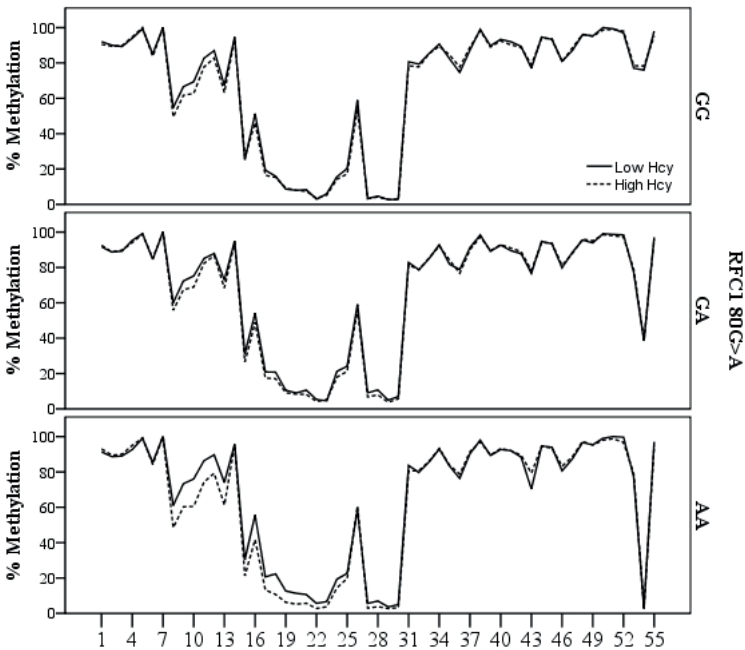

Figure 9. *The methylated fraction of the RFC1 gene in subjects with high or low tHcy stratified by 80G>A genotype.*

In the placental specimens from fetuses with NTDs we observed a lower methylated fraction in fetuses carrying the AA allele compared to the placental specimens from subjects with NTDs carrying the GA or GG alleles (Paper I, Figure 5). These differences were not found in placental specimens from healthy births.

Combining the observed lower methylation in subjects with high tHcy (proxy for poor nutrition) and subjects with NTD, specifically with AA genotype, suggests an interaction between nutritional factors, genotype and methylation of the *RFC1* gene. These data raise the question whether there is a feedback loop of high *vs* low folate status in the cell affecting the methylation of the *RFC1* gene in subjects with AA genotype. Future studies including intervention of folate intake and DNA methylated fraction of the *RFC1* gene in subjects with AA genotype may provide further insights into whether or not the *RFC1* gene methylation is affected by nutrition.

CRC and healthy mucosal tissues

The emerging role of folate transporters in various cancers types as either a therapeutic target or prognostic marker (111) has inspired us to investigate further the regulation mechanisms by DNA methylation in cancer specimens. The aim of the study in Paper II was to compare the DNA methylation pattern and protein expression of the folate transporting genes in CRC specimens and healthy colonic mucosal specimens. We chose to analyze the T-DMRs that were identified in Paper I. Genome-wide approaches showed that variations in DNA methylation between diseased and healthy cells often co-localize with tissue-specific methylated regions (T-DMRs) (24, 39, 103). In total, we analyzed five assays in the *FOLR1* gene, three assays in the *PCFT* gene and four assays in the *RFC1* gene (Paper II, Table 2). Our hypothesis was that the folate transporting genes would be hypomethylated and over-expressed in CRC specimens compared to healthy colonic mucosal specimens as a response to the higher nutritional needs that cancer cells have to maintain a high cellular division.

Differential methylation in CRC

We compared the methylated fractions of CRC specimens and healthy colonic mucosal specimens to understand the role of differential methylation of folate transporting genes in CRC. The differential methylation in CRC was defined in terms of hypomethylation (less methylated fraction in CRC specimens) or hypermethylation (more methylated fraction in CRC specimens).

The *FOLR1* gene CpG sites 1 and 2 located in the vicinity of the translation start site were found to be hypomethylated in the CRC specimens, while the CpG site 14 located downstream of the gene was hypermethylated in the CRC specimen ($p < 0.05$, Paper II, Figure 1).

In the *PCFT* gene, the CRC specimens were found to be hypermethylated at the CpG sites 9 – 12 and 14 ($p < 0.05$, Paper II, Figure 1). These CpG sites are located in the gene-body, in the 3' shore of the CGI-1. In vitro studies on methylation of the *PCFT* gene showed associations between methylation status and gene expression in leukemia cell lines, antifolate resistant HeLa cell line, but not in Caco2 cell lines (68, 69). Our results showed that there is no methylation in the CGI located in vicinity of the promoter/translation start site region neither in the normal nor cancer tissues (Paper II, Figure 1 CpG sites 4-8). Our results suggest that in CRC the *PCFT* gene is not affected by DNA methylation. The study by Furumiya et al (70) analyzed methylation of the corresponding rat *Pcft* gene in the healthy jejunum and ileum and found no difference in methylation between these regions that have a different gene expression of the *Pcft* gene. They suggested that DNA methylation was not involved in the regulation of the *Pcft* expression.

When comparing the methylated fraction of the *RFC1* gene, six CpG sites (CpG 3-5 and 11-13) were found to be hypermethylated in CRC specimens ($p < 0.05$, Paper II, Figure 1). These CpG sites are located in the vicinity of the gene promoter region. There are no previous studies analyzing the methylated fraction of the *RFC1* gene in CRC specimens. However, several studies have reported an association between hypermethylation and reduced expression in cell lines (72). In ovarian cancer, which is a solid tumor type, hypermethylation was associated with a lower expression (75) supporting our findings from the CRC specimens. The methylation of the folate transporting genes in relation to protein expression is presented in the following section.

Protein expression measured by IHC

The FRα and the RFC1 proteins were expressed in a high fraction of the cells in both CRC specimens and the healthy specimens. The mean total staining score (composed of both the cell population and intensity score) for the FRα and the RFC1 proteins was found to be significantly lower in the CRC specimens compared to the healthy colonic mucosal specimens (Paper II, Table 3), suggesting a lower expression of these proteins in the cancer specimens. The RFC1 protein expression has not been previously

addressed in CRC tissues, but has been reported in the healthy colon (112). We found that higher methylated fraction of the CpG sites 3-8 (located in the promoter region) in the *RFC1* gene correlated with a lower protein expression (Paper II Table 4). In healthy enterocyte cells of the small intestine, the RFC1 protein is located in the apical side of the cell membrane but is not functional at the low pH (65), which means that it is not mediating the uptake of folate from the lumen. In other cell types such as renal tubules the RFC1 protein is located at the basolateral side of the cell (65), functioning to transport folate from blood to the tissues. To understand the role of the RFC1 protein in CRC, further studies addressing its precise location and function in the cancer cells is needed. If the protein is located on the apical side of the cell, then the interpretation of the down-regulated protein in CRC could mean that the cancer tissues transports less folate into the cell compared to the healthy colonic mucosa. But, if the location of the protein is on the basolateral side of the cell then the down-regulated RFC1 protein expression mean that the cancer cells are "saving" their intracellular folate. Cancer cells often have a disrupted cell polarity and to elucidate the direction of the folate transport could be difficult. Perhaps analysis of premalignant cancer stages could be used to locate the protein changes. We did not see any association between *FOLR1* gene methylation and protein expression.

The PCFT protein was expressed in only 15% of all the specimens and the staining intensity was weak (Paper II, Table 2). The PCFT expression is confined normally to the small intestine (82), and is not likely to be involved of folate uptake in the CRCs.

Methylation and expression of folate transport genes in relation to tumor characteristics

To determine whether methylation or protein expression of folate transporting genes can be used as diagnostic or prognostic markers in CRC, we analyzed the association between DNA methylation, protein expression and tumor characteristics (differentiation, TNM stage, and localization). We found that there was a slightly lower methylation in the *FOLR1* gene of CpG sites 4, 5, and 9-11 ($p < 0.05$) in the primary tumor stage (pT) 4 compared to pT2 and pT3 (Paper II, Figure 2A). These results indicate that the methylated fraction of the *FOLR1* gene has a potential to differentiate between tumor stages. However these findings need to be validated in a larger cohort to draw firm conclusion. The tumors located in the distal colon and rectum had a lower methylated fractions at CpG sites 1, 2,

8, and 15 ($p < 0.05$) in the *RFC1* gene, compared to tumors located in the proximal colon (Paper II, Figure 2B). These results suggest that tumors which appear in different locations of the intestine show epigenetic variation.

In contrast to the methylation data, we found no an association between protein expression by IHC and various tumor characteristics such as differentiation, location or TNM (Paper II, Table 3), which could be due to a small number of samples. The RFC1 and FRα protein expression have been suggested as prognostic markers in other cancers. In ovarian and endometrial cancers, an increased FRα expression was associated with tumor progression and poor prognosis (113, 114). Higher expression of the RFC1 protein in lung cancer cells has been found to be associated with the extent of the tumor (115, 116).

DNA methylation of the folate transport genes measured by the array

We were interested in investigating the differences in the DNA methylated fraction of the folate transporting genes in CRC specimens and cervical cancer specimens measured by the Illumina Infinium 450 methylation array. We were interested to see if the array data would show similar DNA methylation pattern of the folate transporting genes in CRC as obtained by the pyrosequencing assays. We were also interested to see whether or not there are any differences in the methylated fraction between cervical cancer specimens and healthy cervical scrapes. The array measures 13 CpG sites in the *FOLR1* gene, 19 CpG sites in the *PCFT* gene and 24 in the *RFC1* gene. Most of these CpG sites are not covered by the Pyrosequencing assays developed in Paper I but many are in the same region (data not shown).

Methylation in the CRC tissues

We performed a genome-wide DNA methylation analysis of 8 CRC specimens and 8 healthy colonic mucosal specimens. The array detected statistically significant ($p < 0.05$) differences at CpG site D in the *FOLR1* gene, CpG sites L-O in the *PCFT* gene, and CpG sites K-M and O in the *RFC1* genes, Figure 10. In comparison with CRC specimens analyzed with Pyrosequencing, both methods showed a similar DNA methylation pattern in the folate transporting genes: heavy methylation of all gene regions in the *FOLR1* gene, and varied gene methylation of the *PCFT* and *RFC1* genes (Figure 10, and Paper II Figure 1).

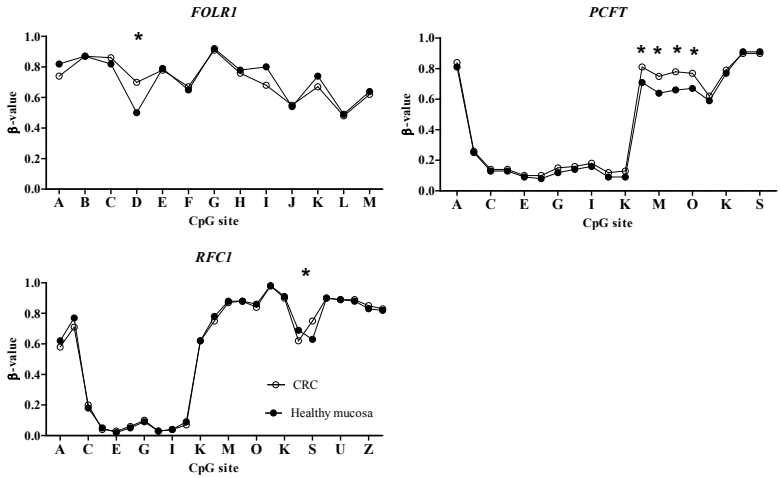

Figure 10. *The methylated fraction of the folate transport genes in colorectal cancer (CRC) and healthy adjacent mucosal tissues. Data were compiled from the array study in paper III. CpG sites with statistically significant differences are marked with (*).*

Methylation in the cervical cancer tissues

The DNA methylation pattern of the folate transporting genes has not been previously studied in cervical cancer specimens. We analyzed healthy cervical scrapes (n=20), CIN3 lesions (n=18) and cervical cancer specimens (n=6), Figure 11. In the *FOLR1* gene, six CpG sites were statistically significantly hypermethylated in cancer specimens compared to healthy cervical scrapes. Pillai et al (117) showed that a higher frequency of women with low grade squamous lesions expressed the FRα protein compared to women with high grade squamous lesions (CIN3) and invasive cervical cancer. Further studies with a larger cervical cancer cohort, low and high grade squamous lesions would give insight whether or not there is a functional role between DNA methylation and the expression of the *FOLR1* gene. We found five hypermethylated CpG sites ($p < 0.05$) in the *PCFT* gene in cancer specimens compared to healthy cervical scrapes. In contrast, in the *RFC1* gene four CpG sites were hypomethylated ($p < 0.05$) in cancer specimens compared to healthy cervical scrapes. The most intriguing finding is the step-wise increment in methylation from the healthy specimens to CIN3 and cancer specimens in the *FOLR1* and *PCFT* genes.

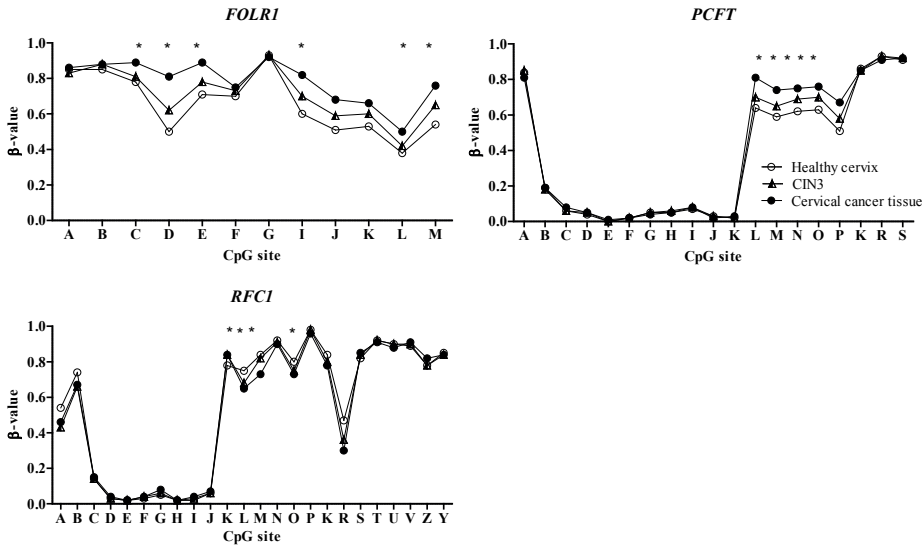

Figure 11. *The methylated fraction of folate transport genes cervical cancer specimens, CIN3 and normal cervical specimens. Data were compiled from the array study in paper IV. CpG sites with statistically significant differences are marked with (*).*

High-density DNA methylation array study in CRC (Paper III)

Initial data analysis

Four pairs of samples were excluded from the analysis as they were regarded as outliers. In total, 459,540 CpG sites were included in the analysis after pre-processing of the raw data. We observed a small fraction of CpG sites differentially methylated in CRC specimens when applying the criteria of $\Delta\beta \geq |0.2|$ and an adjusted p value of < 0.05, Figure 12 and Table 2. The differences in the methylated fraction were mostly in the range of $\Delta\beta \sim 0.2$ (Figure 12). These results suggest a high biological heterogeneity among our CRC specimens. We therefore chose to analyze this data set deductively, with pre-defined hypotheses of specific pathways to analyze. We focused on a comprehensive analysis of the DNA mismatch repair system (MMR), Wnt/β-catenin pathway and genes frequently mutated in CRC.

Table 2. *Hypo- and hypermethylated CpG sites in CRC specimens identified with a crude p value and adjusted (Adj.) p value for multiple testing.*

Identification criterion	Hypermethylated ($\Delta\beta \geq 0.2$), N	Hypomethylated ($\Delta\beta \leq -0.2$), N
$p < 0.05$	5233	6410
Adj. p value	48	284

N, the number of observations.

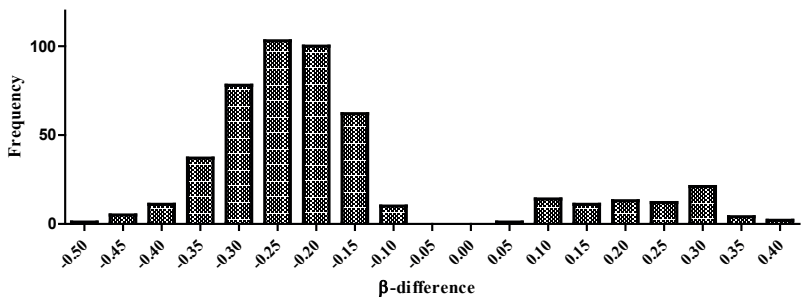

Figure 12. *All β differences (Δβ) in DNA methylation between CRC and healthy colonic mucosal specimens, adjusted p < 0.05.*

The DNA repair system

The DNA repair system includes genes in the nucleotide excision repair (NER), base excision repair (BER), direct reversal of DNA damage (RDD), homologous recombination (HR), non-homologous end-joining (NHEJ) and mismatch repair (MMR) (118).

Our data indicates that DNA methylation changes in genes of the DNA repair system are not a high-prevalence feature. Eighty-five genes were included in the analysis and six genes of the BER, NER, and NHEJ pathways (*NEIL1, NEIL3, NHEJ1, GTFH5, DCLRE1C*, and *CCNH*) were found to be differentially methylated in CRC specimens according to the criteria $\Delta\beta \geq 0.2$ and a p value < 0.05 (Paper III, Table 3). The differential methylation was in the gene body region and the role of these changes needs further investigation.

The DNA mismatch repair gene *MLH1* has been extensively studied in CRC specimens, especially due to its relationship to the CIMP pathway in CRC. The Infinium 450K array included 47 CpG sites in the gene promoter region. When comparing the CRC specimens and healthy adjacent colonic mucosa we found that the $\Delta\beta$ differences were < 0.1, none of them passed the threshold of $\Delta\beta \geq 0.2$. The meta-analysis by Li et al (119) included 19 studies (5584 subjects) with *MLH1* gene promoter methylation analysis and suggested a 20% methylation frequency in CRC. They did not however report the extent of methylation in CRC and healthy colonic mucosa.

Wnt/β-catenin signaling pathway

We analyzed the DNA methylation pattern of key genes in the canonical Wnt pathway (120). The Wnt signaling pathway is involved in cell differentiation, growth, and apoptosis (121). Continuous activation of β-catenin and the downstream Wnt target genes can lead to a disrupted balance of "normal" gene expression (121). In our study eleven genes (*CTNNB1, DKK2, DKK3, FZD5, WNT2, WNT3A, WNT6, LRP5, TLE3, TCF7,* and *TCF7L1*) out of 43 analyzed were found to be differentially methylated in CRC specimens (Paper III, Table 4).

We found hypomethylation of the *CTNNB1* gene in CRC compared to healthy mucosal tissues, while in urological cancer (122) it was hypermethylated. The role of hypomethylation in CRC remains unclear and needs further investigations.

We found several hypermethylated CpG sites in the *DKK2* and *DKK3* genes located in the 5' region. Others have reported frequent methylation

in the *DKK2* gene in CRC (123) and ovarian cancer (124). The *DKK3* gene has been found to be methylated in several cancers such as CRC (125, 126), bladder cancer (127), breast cancer (128), cervical cancer (129), and lung cancer (130).

The *WNT*-genes have been reported to be aberrantly methylated in various cancers: the *WNT2* gene has been found to be hypermethylated in sporadic CRC (131), the *WNT3* gene in cholangiocarcinoma (132), and the *WNT6* gene has been suggested as a potential tumor biomarker in brain cancer (133).

Genes frequently mutated in CRC

The aim of this approach was to examine if genes frequently mutated in CRC (48) are also affected by aberrant DNA methylation. We analyzed 30 genes out of which eight (*CASP8, CTNNB1, GPC6, EDNRB, KIAA1804, MYO1B, SMAD2,* and *TTN*) were found to be aberrantly methylated.

CASP8 gene has a function in the intra-cellular signaling of mitochondria-dependent apoptosis (134). We found hypomethylation and over expression of this gene in CRC specimens (Paper III, Table 7). Our data is in concordance with a recently published genome-wide study that showed hypomethylation of the *CASP8* gene in CRC, breast cancer, and hepatocellular carcinoma (135).

GPC6 gene in our CRC specimens is of special interest because it is coding for a cell surface protein that interacts with many proteins and among them the Wnt proteins (136). We noted hypermethylation of this gene in CRC specimens. There are no previous studies analyzing the role of DNA methylation of the *GPC6* gene in cancers. It has been associated with the progression of retinoblastoma due to its involvement in the JNK and p38 MAPK signaling pathways (137, 138). Further studies validating its role in CRC are needed.

Validation of the array data

The validation approach with an external data set can help to identify genes of interest for further analysis. This is especially useful when working with a large gene list of potential biomarkers. We therefore validated our DNA methylation results by using the publicly available mRNA expression data set GSE23878 (139). We found differential methylation in 14 genes (*DCLRE1C, CTNBB1, DKK2, DKK3, EDNRB, GCP6, SMAD2, TTN, WNT2, WNT3A, TCF7, TCF7L1, FLI1,* and *HOX5A*) located in the 5' gene region. Methylation status of the genes *CASP8,*

DCLRE1C, *GPC6*, and *EDNRB1* was in agreement with the mRNA expression (Paper III, Table 7). We found hypermethylation in the *EDNRB1* and *GPC6* genes and down-regulated mRNA expression (fold change ~ -2) in CRC specimens compared to healthy mucosal tissues. The mRNA expression of the hypomethylated *CASP8* and *DCLRE1C* genes were up-regulated (fold change ~ 1.5) in CRC specimens compared to healthy mucosal tissues. The *SMAD2* gene had a partially hyper- and hypomethylated 5' gene region and association with the mRNA expression needs to be further studied. The methylation status of the *FLI1*, *TCF7*, and *TTN* genes did not correlate with the mRNA expression. There were no statistically significant differences in *CTNNB1*, *DKK2*, *DKK3*, and *WNT2* mRNA expression in CRC and healthy mucosal tissues.

The *EDNRB1*, *GPC6*, *CASP8*, *DCLRE1C*, *SMAD2* genes need to be further validated in a larger cohort to address the correlation between DNA methylation and mRNA expression in the same samples. Their prognostic potential as well as their role in CRC development need to be investigated.

High-density DNA methylation array study in cervical cancer (Paper IV)

The aim of this study was to identify differentially methylated genes in cervical squamous cell carcinoma specimens, cervical scrapes with high-grade squamous lesions (CIN3) and healthy cervical scrapes using a genome-wide approach.

DNA methylation profile of the clinical groups

The comparison of the DNA methylation was made between the cancer *vs* CIN3 specimens, cancer *vs* healthy specimens, and CIN3 *vs* healthy specimens. This data set has been analyzed inductively. We found a large fraction of the CpG sites equally methylated comparing cancer specimens and healthy specimens. However, most of the differentially methylated ($\Delta\beta > 0.2$) CpG sites were hypermethylated (Paper IV, Supplementary Table 4).

Both the site-wise analysis and regional analysis (see Methods, Data analysis) showed that most differences occurred between cervical cancer and healthy specimens (Paper IV, Suppl. Table 1, and Suppl. Table 4).

The location of the differentially methylated CpG sites were predominantly gene related (Paper IV, Figure 2). Also, irrespective of group comparison, most of the hypermethylated CpG sites were situated in the CpG island and the hypomethylated CpG sites in the CpG island shores (Paper IV, Figure 2). Previous studies on methylation in cervical cancer report both global DNA hypomethylation and hypermethylation of specific genes promoter regions (140, 141).

We then analyzed the biological features associated with the hypomethylated and hypermethylated genes in cervical cancer specimens using the Database for Annotation, Visualization and Discovery (DAVID). The ontology terms overrepresented for the hypomethylated genes were related to the immune system and cell development and differentiation for the hypermethylated genes (Paper IV, Suppl. Table 2 and Suppl. Table 3). Our observations are supported by a study analyzing a smaller number of CpG sites (Infinium Human Methylation 27 BeadChip) in colon, kidney, stomach, lung and breast cancers as well as the corresponding normal tissue (142). These findings suggest that hypomethylated genes of the immune system are a general cancer event. At the early stages of HPV- infection the innate and cell-mediated immunity is down-regulated (143, 144). Our findings in cervical cancer are consistent with an immune response against HPV, which is activated through hypomethylation of specific genes included in the immune response and inflammatory reactions.

Candidate biomarker genes

The selection of candidate biomarkers was based on two approaches, the site-wise analysis (Paper IV, Figure 1) and on the regional analysis of the 5' and upstream regions (Paper IV, Tables 2 and 3) between cancer and healthy tissues. A list of 24 genes emerged one was hypomethylated (*S1PR4*) and twenty three genes were hypermethylated (*RGS7, LHX8, STGALNAC5, TBX20, KCNA3, ZSCAN18, ACAN, AJAP1, BARHL2, BOLL, C1orf114, FBXL7, GALR1, GYPC, KIF19, MIR663, PTGDR, SORCS1, TRIM58, TTYH1, VSTM2B, ZIK1,* and *ZNF582*), see Paper IV, Table 4. Several of these candidate genes are of interest for further functional analysis in cervical cancer, especially those not previously reported methylated in cancer: *ACAN, Clorf114, FBXL7, GYPC, KCNA3, KIF19, LHX8, MIR663, RGS7, S1PR4, SORCS1, TBX20, TRIM58, TTYH1,* and *VSTM2B*. The *ZNF582* gene was previously shown to be hypermethylated in cervical cancer suggesting silencing of the *ZNF582* gene in cervical neoplasms (145), and it could be a potential candidate gene for molecular cervical cancer screening. The product of the *GALR1* gene inhibits a key regulatory enzyme adenylyl cyclase and was suggested to be hypermethylated in a subset of head and neck cancer caused by oncogenic HPV types, notably HPV 16 which is one of the most common types of HPV (146).

Validation of the array data

In this study we applied two validation approaches of the array data. We performed an external validation and a technical validation by analyzing the same patient samples and exact CpG location with the Pyrosequencing assay technology.

We performed an external validation of our results by using three publicly available gene expression data sets (GSE6791, GSE9750, and GSE7803). They were selected based on HPV status of the samples. We wanted the cancers to be HPV positive and the healthy tissues to be HPV negative in order to resemble our study population. We chose to use the Affymetrix array platform because of in-house expertise. Most of the genes in the list (Paper IV, Table 4) were present in the datasets. We found good association between DNA methylation and mRNA expression. The hypermethylated state of the genes was in concordance with a down-regulated gene expression. However, the potential biomarker genes (*ACAN, Clorf114, FBXL7, GYPC, KCNA3, KIF19, LHX8, MIR663, RGS7, S1PR4, SORCS1, TBX20, TRIM58, TTYH1, VSTM2B, AJAP1,*

BARHL2, BOLL, GALR1, PTGDR, ST6GALNAC5, ZIK1, and *ZSCAN18*) need to be further validated in a larger cohort of cervical cancers, cervical low-grade and high-grade squamous lesions to address their prognostic potential.

We selected four genes (*TBX20, RGS7, KCNA3,* and *S1PR4*) for the validation with the Pyrosequencing assay technology. Six cervical cancers and six healthy tissues were analyzed. We obtained similar results as measured by the array, the *TBX20, RGS7,* and *KCNA3* were hypermethylated in cancer and the *S1PR4* was hypomethylated (Paper IV, Supplementary Figure 4). Others have shown that the measurements obtained by the array are reproducible when also using Pyrosequencing (147, 148).

Limitations of the studies (Papers I-IV)

Studies in this thesis were performed on placental, colorectal, and cervical tissues. DNA methylated fraction can vary between different cell types and be a source of variation. Higher standard deviation of the DNA methylated fraction in our results could be due to either the presence of a mixture of different cells or a cell population with a high epigenetic variation.

In Paper II, we studied the expression of the folate transporting genes with immunohistochemistry. It is a good method to use for the localization of the protein expression in the tissue but this method, like all immunological methods is dependent of the specificity of the antibodies used and is only at best semiquantitative. Immunohistochemistry analysis is also observer-dependent so the evaluation could be biased.

In Papers III & IV, we found statistically significant differences in the methylated fractions between cancer and healthy tissues. However, our cancer cohorts were small and therefore the identified biomarkers genes need to be further validated. Our selection criteria may have been too restrictive, for instance the requirement that hypermethylation should be accompanied by down-regulated mRNA levels. Recent data suggest that the correlation may for some genes be the reverse, or that there actually is no correlation with expression. Therefore we may have built in a type 2 error in the data evaluation.

Conclusions and future perspectives

We identified CpG regions of the folate transporting genes *FOLR1* and *RFC1* that could be functionally important (Papers I & II). There was a genotype (80G>A) dependent *RFC1* gene methylation in fetuses with NTDs and in blood leukocytes from subjects with high homocysteine. These findings need to be further investigated in relation to other birth defects and in subjects with folate supplementation. Intervention studies with folate combined with DNA methylation and expression analysis would give more insight of the relation between nutrition, genotype and *RFC1* gene methylation.

In CRC specimens, the *RFC1* gene was hypermethylated and the protein was down-regulated (Paper II). To answer the question whether or not the reduction of the RFC1 protein leads to the development or susceptibility to CRC needs to be studied further in a larger cohort including premalignant tissues. Also, additional studies of the potential clinical value of the *RFC1* and *FOLR1* gene DNA methylation should be addressed in a larger cohort with different CRC stages. We found the *FOLR1* gene aberrantly methylated in cervical cancer specimens and the functional role of this epigenetic change should be further evaluated.

Using a high-density genome-wide approach we identified five potential biomarker genes in CRC and twenty-four candidate genes in cervical cancer (Papers III & IV). To evaluate the clinical value of these potential biomarkers further studies analyzing premalignant tissues as well as different cancer stages should be performed. The putative role of these genes could be further evaluated by using *in vitro* studies in cancer cell lines.

In conclusion, the work of this thesis has filled a relevant knowledge gap regarding the role of differential methylation of the folate transport genes in NTD and colorectal cancer. This thesis work also provided insights into the functional role of DNA methylation in cancer specific pathways and identified novel potential biomarker genes.

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