Analysis of DNA methylation alterations in cell-free DNA fraction during colorectal cancer development

Ph.D. thesis

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1. INTRODUCTION

Cancers are among the most common cause of death worldwide. Colorectal cancer (CRC) is characterized by increasing incidence and mortality: it is the second most common type of cancer in Europe after breast cancer with 447,000 newly discovered cases per year, and it is also on the second place in terms of mortality following lung cancer. In Hungary, the number of newly registered patients is around 9,000; and CRC causes about 6,000 deaths a year.

Colorectal cancer is induced by carcinogenic or hereditary genetic and epigenetic alterations in the healthy colonic mucosa. DNA methylation is one of the most important epigenetic modifications. During the methylation process, cytosine has a methyl group on the 5th carbon atom in cytosine-guanine dinucleotide (CpG) sites located in the promoter regions of the genes. Increased methylation level in the promoters causes the decrease or complete inhibition of transcription. Methyl group physically inhibits the binding of transcription factors, and methyl-CpG binding proteins can also link to methyl groups, that recruit additional factors to modify the chromatin structure, and thereby induce gene inactivation. DNA methylation pattern changes during aging called age-related (A-type) methylation. In case of cancer, global genomic hypomethylation and tumor-specific local hypermethylation can be observed called cancer-related (C-type) methylation that occurs in CIMP+ phenotypic tumors.

Nucleic acids can enter to the bloodstream from different type of tissues including tumors; hence the different genetic and epigenetic modifications such as DNA methylation can be analyzed in the cell-free DNA (cfDNA) fraction in plasma samples, which can be a simple and promising minimally invasive method to diagnose cancers. Early detection of CRC can greatly increase patient survival, thus the development of screening techniques is crucial. Colonoscopy is the most accepted and effective gold standard method for cancer screening. However, this technique is costly, uncomfortable for patients and the rate of complications is relatively high, so the patient compliance is low. Therefore, the non-invasive (e.g. gFOBT, FIT test) or minimally invasive screening techniques become more popular and available. The latter group contains blood-based screening methods that target the identification of different biomarkers in the free circulating nucleic acid – including cfDNA – fraction.

There are several theories about the origin of cfDNA. Basically, two main pathways may occur during the release of cfDNA to the bloodstream: after cell death with apoptosis or necrosis, or with direct secretion by living healthy and tumorous cells. However, the most probable hypothesis is that these processes happen together in the body. The concentration of cfDNA is higher in patients with malignant tumors than in healthy individuals or in the presence of a benign tumor. The increase of cfDNA amount cannot be used as an individual cancer biomarker, as higher level of cfDNA can be observed in the case of strong physical activity and also during the first trimester of pregnancy. Therefore, it is necessary to investigate tumor-specific alterations in the cell-free DNA fraction, such as somatic mutations, abnormal microsatellite patterns or epigenetic modifications, including DNA methylation changes. According to literature data, there are genes that show elevated methylation level in colorectal cancer, and this change can be also detected in plasma samples. One of the well-known CRC-specific epigenetic markers is the septin 9 gene (*SEPT9*). The method for analyzing the altered methylation status of *SEPT9* gene has been developed a commercially available screening test for CRC called Epi proColon 2.0 by Epigenomics AG. The test can discover CRC cases with 68-72% sensitivity and with 89-93% specificity; however, it has a higher detection rate primarily in advanced stages, in the case of adenoma and early CRC stages the sensitivity is lower. Consequently, it is essential to identify new and more reliable biomarkers based on DNS methylation alterations in the cell-free DNA fraction.

2. AIMS

The aims of my Ph.D. work were summarized in the following points:

1. Investigation of the rate of cfDNA's release in SHO mice/HT-29 colorectal adenocarcinoma cell line xenograft model.

2. Determination of cfDNA's stability by the analysis of methylated and non-methylated DNA fragments' degradation in the bloodstream of healthy and tumorous C57BL/6 mice.

3. Analysis of the methylation pattern of *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1* genes in matched tissue and plasma samples of healthy, adenoma and colorectal cancer patients.

4. *In silico* validation of the selected four genes' DNA methylation pattern on independent tissue samples.

5. Examination of the effect of DNA methylation alterations on the protein expression level during colorectal adenoma – carcinoma sequence.

6. Analysis of the influence of different blood collection tubes and cfDNA isolation methods on the cfDNA amount and the four target genes' promoter methylation pattern.

7. Comparison of the sensitivity of the commercially available Epi proColon 2.0 test based on the SEPT9 gene's methylation level with our biomarker panel.

3. MATERIALS AND METHODS

During our experiments, we used animal models and investigated human colon tissue and plasma samples. The release and degradation of cell-free DNA were studied on 8-8 mice. Moreover, 193 healthy/normal adjacent tissue (NAT), 191 adenoma and 254 CRC colon tissue or plasma samples were examined with different methods.

3.1. Analysis of cell-free DNA fraction using animal models

3.1.1. Examination of the release of cfDNA

The first step in our work was the analysis of the cfDNA's origin and release rate. The 8-weeks long experiment was performed using SHO mouse/HT-29 human colorectal adenocarcinoma cell line xenograft model. Blood samples were collected from the mice once a week then plasma fraction was separated, and cfDNA was isolated. We quantified the human tumor-derived cfDNA amount with real-time PCR based on the copy number of mouse and human genes which were selected with bioinformatic methods and preliminary experiments. In addition to samples, a dilution series with 7 samples were amplified, and the mouse/human ratio was defined correlated to the calibration curve for the determination of our methods' sensitivity. Based on our results we could quantify the cfDNA fraction from human tumor cells in the mouse bloodstream, thus measuring the rate of release.

3.1.2. Investigation of the degradation of cfDNA

To assess the degradation rate of cfDNA, 4 healthy and 4 C38 colorectal adenocarcinoma tumor cells vaccinated C57BL/6 mice were injected with *in vitro* methylated and non-methylated DNA sequences. DNA was isolated from human HT-29 colorectal adenocarcinoma cell line for the treatment, and a 3000bp sized sequence of the human Glyceraldehyde 3-phosphate dehydrogenase (GAPD) gene was amplified by PCR. The purified PCR amplicons were injected into 2 healthy and 2 tumorous mice in methylated form, while the other groups were treated with non-methylated DNA fragments, respectively. Blood samples were collected from the mice 5 times, immediately before the injection, then 1, 3, 6 and 24-hours after the treatment. After plasma separation, cfDNA was isolated, and the decay of the 3000bp sized amplicons was measured with 19 specific, consistently located and overlapping PCR primers. Following real-time PCR, specified Ct-values were used for the determination of

degradation rate, and it was compared between the methylated and non-methylated fragments in healthy and tumorous mice.

3.2. DNA methylation analysis of *SFRP1, SFRP2, SDC2* and *PRIMA1* genes in human samples

After animal experiments, analysis of DNA methylation pattern and the cell-free DNA fraction were achieved using human samples. Four genes (*SFRP1*, *SFRP2*, *SDC2* and *PRIMA1*) were selected based on previous experiments of our research group and literature data, and the DNA methylation levels of these markers were examined using a total of 166 patients' tissue and plasma samples.

3.2.1. Analysis of the methylation level of the four selected markers in fresh-frozen tissue samples

Pyrosequencing results of our research group were used to study the DNA methylation pattern of *SFRP1*, *SFRP2* and *PRIMA1* genes. An additional marker, *SDC2* was selected based on literature data. In order to verify the promoter methylation of *SDC2*, pyrosequencing was also performed using GS Junior System (Roche Applied Science, Germany) utilizing the 454 technology on 15 NAT, 15 adenoma and 15 CRC fresh-frozen tissue samples. DNA isolation from macrodissected samples and bisulfite conversion were done with EZ DNA Methylation Direct Kit (Zymo Research, USA). After bisulfite-specific PCR, samples were pyrosequenced, and the methylation status of 44 CpG positions located in the promoters of the 4 selected genes was determined. Subsequently, the same regions were analyzed by other methods on independent tissue and plasma samples.

3.2.2. *In silico* verification of the methylation pattern of the four markers on independent tissue samples

Methyl capture sequencing data of 6 NAT, 15 AD and 9 CRC biopsy samples published by our research group were reanalyzed *in silico*. Differentially methylated regions (DMRs) overlapping with the sequences analyzed by pyrosequencing and subsequently with MethyLight PCR of the four methylation markers (*SFRP1*, *SFRP2*, *SDC2* and *PRIMA1*) were evaluated. The methylation status of DMRs (β-values) was defined, and Δβ-values were calculated as the differences of the β-values of samples groups.

Furthermore, the methylation status of the four candidates' whole promoter regions was analyzed using methylation array data of colonic tissue samples downloaded from The Cancer Genome Atlas (TCGA) database and from NCBI Gene Expression Omnibus Database (GEO accession number: GSE48684). The methylation level (β-values) of 99 CpG sites' in the promoter regions was studied, and Δβ values were also calculated.

3.2.3. Effect of altered promoter methylation level on protein expression

In order to examine the effect of promoter hypermethylation of the four genes on the protein expression, immunohistochemical analyses were performed on 11 healthy, 11 AD and 10 CRC formalin-fixed paraffin-embedded samples. Following deparaffinization and rehydration, microwave-based antigen retrieval was performed in Tris-EDTA buffer (pH 9.0). After immunostaining, slides were digitalized by Pannoramic 250 Flash II scanner (3DHISTECH Ltd.), then digital slides were semi-quantitatively analyzed with Pannoramic Viewer (3DHISTECH Ltd.) based on Quick-score (Q) method.

3.2.4. DNA methylation analysis of *SFRP1, SFRP2, SDC2* and *PRIMA1* genes in human tissue and plasma samples by MethyLight PCR

For MethyLight reactions, plasma samples were collected from 37 controls, 37 AD, and 47 CRC patients, and matched biopsy samples were obtained from 32 (11 healthy, 11 AD and 10 CRC) patients. DNA from colonic biopsies was extracted using High Pure PCR Template Preparation Kit (Roche Applied Science), in case of plasma samples High Pure Viral Nucleic Acid Large Volume Kit (Roche Applied Science) was used for DNA extraction. After bisulfite conversion of tissue and plasma DNA samples, multiplex preamplification step was carried out with bisulfite-specific primers in order to amplify the selected regions independently from methylation status. MethyLight PCR was applied to detect the methylation-dependent sequence differences after bisulfite conversion using methylation-specific primers. The methylation levels of the samples were determined based on the standard curves generated from the Ct-values of methylated and non-methylated standards preamplified in parallel with the analyzed samples. Valid methylation percentage range was defined by the Ct-values between 0% and 100% methylated standard samples.

3.2.5. The effect of different blood collection tubes and cfDNA isolation methods on the cfDNA level and the methylation status of the four selected genes

We aimed to test different blood collection tubes and cfDNA isolation methods to determine whether these factors influence the cfDNA amount and the promoter methylation level of the four analyzed hypermethylated biomarkers. A total of 139 blood samples were collected, five-five blood samples were obtained from patients with colorectal adenoma and cancer in Cell-Free DNA BCT® (Streck, Germany) collection tubes, all the other blood samples were drawn in K3EDTA Vacuette tubes. Streck tubes have been developed to increase the stability of cfDNA from the blood collection time to plasma separation, even in case of an extended time period. After plasma separation, three manual isolation methods (High Pure Viral Nucleic Acid Large Volume Kit (Roche Applied Science); Epi proColon 2.0 Kit (Epigenomics AG); Quick-cfDNA™ Serum & Plasma Kit (Zymo Research)) and automated sample preparation systems (InviGenius and InviGenius PLUS (STRATEC Biomedical AG)) were also examined. Following bisulfite conversion, methylation levels were determined with the above-mentioned MethyLight PCR method.

3.2.6. Comparison of the sensitivity of Epi proColon 2.0 test and our biomarker panel

Blood samples were collected in K3EDTA tubes from 10 healthy individuals, 10 adenoma, and 20 CRC patients. Plasma separation was performed with three different methods using High Pure Viral Nucleic Acid Large Volume Kit, Epi proColon 2.0 and the InviGenius system. The methylation status of SEPT9 was determined using the Epi proColon Sensitive PCR Kit (Epigenomics AG). Exact methylation level was measured based on a calibration curve generated from the Ct-values of bisulfite-converted methylated control DNA samples. After PCR, the sensitivity and specificity values were quantified. Finally, the values were compared with the methylation results of *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1* genes in healthy, adenoma and CRC sample groups.

4. RESULTS

4.1. Analysis of cell-free DNA using animal models

4.1.1. Examination of the release of cfDNA

The rate of cell-free DNA release from human tumor tissue was examined in mouse/human xenograft model, and the sensitivity of the method was determined by a dilution series with 7 samples. Based on our results, the applied method is sensitive enough to detect 0.1% human DNA content in mouse plasma samples. Tumor-originated DNA from plasma samples was quantified based on the calibration curve. Following the HT-29 injection to the mice, the amount of human DNA until the 2nd week was below the limit of detection, while at the end of the third week it reached 0.1% ratio. In the next days, a continuous growth was experienced, which reached 18.26% for the 56th day, meaning that approximately 20% of cfDNA in the bloodstream was derived from human tumor cells.

4.1.2. Investigation of the degradation of cfDNA

There was a difference between the degradation rate of non-methylated and methylated fragments. It was found that in samples of healthy mice the human non-methylated DNA fractions were detected at high concentrations 1 hour after treatment. In 3-hour samples, a high intense degradation was observed, which increased in 6-hour samples, and in 24-hour samples, the fragments were below the detection limit. In contrast, in vitro methylated sequences were found to be more stable, as the DNA fragments were observed in high concentration in 3- and 6-hour samples. Moreover, certain fragments of the 3000bp amplicon were still detectable after 24 hours. Comparing blood samples from tumorous and healthy mice, we observed that the non-methylated fragments in cancerous animals were found in 6-hour samples, and the methylated sequences were measurable after 24 hours in a larger amount than in healthy animals.

4.2. DNA methylation analysis of *SFRP1, SFRP2, SDC2* and *PRIMA1* genes in human samples

4.2.1. Evaluation of pyrosequencing data

44 CpG sites located in the promoter region of the four analyzed genes (*SFRP1*, *SFRP2*, *SDC2* and *PRIMA1*) were selected, and the methylation status of the positions was

determined after pyrosequencing. In case of *SFRP1*, 9 out of 10, for *SDC2* 17 out of 18, and in *SFRP2* and *PRIMA1* all CpG sites showed significantly higher methylation levels in CRC compared to healthy controls ($p<0.05$). Most of the CpG positions showed increased methylation level already in adenoma samples.

4.2.2. *In silico* analysis of DNA methylation status of the four selected markers

DNA methylation status of the promoter regions of the four markers was verified in silico using methyl capture sequencing data from our research group. The selected regions of *SFRP1* and *SDC2* promoters showed intensive and highly significant hypermethylation both in adenoma (*SFRP1* $\Delta\beta=0.60$; *SDC2* $\Delta\beta=0.65$ and 0.50) and CRC (*SFRP1* $\Delta\beta=0.49$; *SDC2* $\Delta\beta=0.59$ and 0.37) tissues compared to normal adjacent tissue (NAT) samples ($p<0.01$). DNA methylation of the *PRIMA1* promoter increased intensely in adenomas ($\Delta\beta=0.29$ and 0.43) ($p<0.01$) compared to NAT specimens. Remarkably higher *PRIMA1* promoter methylation levels ($\Delta\beta=0.07$ and 0.18) could be detected in CRC tissue compared to controls. Moderate hypermethylation of the examined *SFRP2* region was found both in adenoma and CRC samples, but it was significant only in the adenoma vs. NAT comparison ($\Delta\beta=0.29$ and 0.07) ($p<0.05$). DNA methylation of the whole promoter regions of the four genes was also studied using methylation array data downloaded from TCGA and GEO databases. According to both databases, the majority of the 99 analyzed CpG sites showed elevated methylation level in adenoma and CRC samples in comparison with controls.

4.2.3. Effect of promoter methylation of SFRP1, SFRP2, SDC2 and PRIMA1 on protein expression

In case of SFRP1 decreased protein expression (+2 and +1) was observed in both epithelial and stromal cells of adenoma (ΣQ-score: 237.22±51.96) and CRC samples (ΣQ-score: 199.16± 54.71); however, significant differences occurred only between CRC and normal samples ($p<0.001$). The cytoplasmic cell type expression was strong (+3) in the healthy surface in case of SFRP2, in contrast, lower ($p<0.001$) protein expression (+1 and 0) were seen in adenomas and CRCs (ΣQ-scores: 253.34±43.01 and 228.75±40.86, respectively). *SDC2* showed moderate (+2) cytoplasmic and nuclear protein expression in epithelial and stromal components of normal colonic samples (ΣQ-score; 281.42±44.13). Significantly decreased ($p<0.001$) syndecan 2 expression levels (+2 and +1) were detected in both adenoma (ΣQ-score:

202.00±30.84) and CRC tissues (ΣQ-score: 198.34±53.14). Strong cytoplasmic PRIMA1 protein expression was found in normal colonic samples (ΣQ-score: 416±32.86), and significantly lower ($p<0.05$) protein expression was observed in adenoma (ΣQ-score: 305± 33.91) and in tumor samples (ΣQ-score: 281.66±64.93).

4.2.4. DNA methylation analysis of *SFRP1, SFRP2, SDC2* and *PRIMA1* genes in human tissue and plasma samples

The average methylation rate of *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1* in adenoma samples was 41.39 ± 29.58%; 6.7 ± 6.61%; 35.48 ± 38.69% and 10.76 ± 25.46%, respectively. Methylation rates in CRC biopsies showed increased level, while in healthy controls, we observed decreased methylation percentages for all four markers ($p<0.05$). In paired plasma samples the same tendency was noticed. The analysis of 121 plasma samples showed that the methylation frequency was significantly higher for all four markers compared to healthy controls ($p <0.0002$ in all cases). DNA methylation of *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1* was observed in 85.1% (40/47), 72.3% (34/47), 89.4% (42/47) and 80.9% (38/47) in the plasma fraction of patients with CRC, and 89.2% (33/37), 83.8% (31/37), 81.1% (30/37) and 70.3% (26/37) of adenoma patients, respectively. DNA methylation status of the genes showed a continuous increase along the normal-adenoma-carcinoma sequence. Average percentage DNA methylation was <1 % for all four markers in healthy control samples. In adenomas, the highest methylation rate was found in *PRIMA1* promoter (4.73 ± 6.41%), while in CRC plasma samples aberrant hypermethylation of *SFRP1*, *SFRP2*, *SDC2* and *PRIMA1* was observed with methylation percentage values of 21.77 ± 33.32%; 6.82 ± 17.1%; 12.06 ± 24.37% and 13.66 ± 25.14%. Receiver operating characteristic (ROC) curves were applied to determine the sensitivity and specificity of the four markers in plasma samples. We observed highly sensitive and specific differentiation of CRC patients from healthy controls (91.5% sensitivity, 97.3% specificity). Adenoma samples could be differentiated from controls with 89.2% sensitivity and 86.5% specificity.

4.2.5. Analysis of the effect of different blood collection tubes and cfDNA isolation methods on the DNA methylation pattern

After the use of different blood collection tubes (Streck and K3EDTA) we did not found significant differences in terms of cell-free DNA content between the two collection modes in either adenomas or CRCs ($p = 0.86$). However, the methylation level of the

four genes was higher by using standard K3EDTA tubes in CRC samples. The difference was significant for *SFRP1* and *SDC2* genes ($p < 0.05$). Moreover, we determined that isolated cell-free DNA level is influenced by the different DNA extraction methods. In case of InviGenius system, we observed higher cfDNA content, while after using InviGenius PLUS, lower amounts were detected in comparison with manual isolations. Elevated methylation level was detected after manual DNA extraction in all four markers in adenoma and CRC samples compared to automated methods.

4.2.6. Comparison of the sensitivity of Epi proColon 2.0 test and our biomarker panel

Methylated *SEPT9* (*mSEPT9*) positivity was calculated based on the Ct-values after RT-PCR. In healthy controls 2-2 from 10 samples showed methylated *SEPT9* using Epi proColon 2.0 and InviGenius isolation methods. None of the adenoma showed *mSEPT9* positivity after automated isolation, but by using Epi proColon kit manually, 6 from 10 adenomas were positive for m*SEPT9*. Moreover, 95% of CRC plasma samples were detected based on *SEPT9* methylation pattern with the usage of Epi proColon kit, while the automated method could detect only the half of the cancerous samples. We compared the SEPT9 test sensitivity with the selected four markers, and we have ascertained that in adenoma samples, higher sensitivity (above 80%) was found in case of all four genes (*SFRP1, SFRP2, SDC2* and *PRIMA1*) compared to *SEPT9*. Similar sensitivity results were obtained in case of CRC samples to Epi proColon test. Regarding specificity, *SFRP1*, *SFRP2* and *SDC2* genes showed higher values than m*SEPT9* gene.

5. CONCLUSIONS

First steps of my Ph.D. work were the analyses of the quantitative and qualitative characteristics of cell-free DNA (cfDNA) with animal models. According to my observation, DNA molecules were released from the human HT-29 tumor cells into SHO mouse' bloodstream, representing 20% of the total DNA fraction. Therefore, in accordance with literature data, it has been found that both healthy and cancerous cells contribute to the increased level of cfDNA during tumor growth. Studies about the stability of cfDNA in healthy and C38 tumor cells vaccinated C57BL/6 mice showed that *in vitro* methylated human DNA fragments are characterized with slower degradation since these amplicons could be detected by PCR in blood 24 hours after the injection, contrary to non-methylated fragments. DNA sequences were more stable in cancerous animals compared to healthy mice partially due to decreased DNase enzyme activity in tumorous animals. Based on the results of animal experiments, studies on human samples were also performed analyzing cell-free DNA fraction and DNA methylation pattern. According to the observations on human plasma samples – based on the methylation status of the four selected markers (*SFRP1*, *SFRP2*, *SDC2*, and *PRIMA1)* – adenoma and CRC samples can be distinguished from healthy individuals with high sensitivity and specificity. These observations about methylation changes were verified also in human tissue samples. Due to the two-step MethyLight PCR method with multiplex preamplification, I could identify genes with increased methylation level in plasma samples containing low amount of cfDNA, and the methylation rates have also become measurable. Several samples preparation protocols were tested, and according to the results, different cfDNA extraction techniques influence the amount of isolated cfDNA and the methylation status. Finally, I found that the methylation panel compiled from the selected four genes can indicate the premalignant stages with high sensitivity in comparison to other minimally invasive colorectal cancer screening tests. In conclusion, a potential colorectal adenoma and cancer prescreening method was established based on methylation changes that can indicate the disease with high sensitivity using peripheral blood samples. My results provide an opportunity to develop an alternative, minimally invasive diagnostic procedure with potentially higher compliance among patients.

6. PUBLICATIONS

6.1. Publications related to the Ph.D. dissertation

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