

In situ and peripheral biomarkers of development of colorectal cancer

PhD thesis

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INTRODUCTION

The incidence and prevalence of colorectal cancer (CRC) increase worldwide. Nowadays, CRC is the most frequently diagnosed cancer after prostate and lung cancer in men and after breast and lung cancer in women. There are several methods for CRC screening, which can be divided into two categories: 1) stool tests or non invasive tests (gFOBT – guaiac fecal occult blood test, FIT or iFOBT – immunochemical fecal occult blood test and stool DNA test) and 2) instrumental examination or invasive methods (flexible sigmoidoscopy, double contrast barium enema, CT colonography – virtual colonoscopy and colonoscopy). However, the determination of diagnosis is difficult in several cases due to lack of typical and specific symptoms such as stool habits change, bloody stool, abdominal pain or loss of weight. The aim of colorectal cancer screening is to detect the cancer in early stages, and to provide a possibility for recognizing the precancerous adenomas, thereby to prevent the development of late stage cancers.

In the last years, more and more attention has been focused to research the cell-free circulating DNA (cfDNA) molecules in peripheral blood. CfDNA was firstly detected in plasma samples of patients with autoimmune disease, and significantly higher cfDNA levels were observed in cancer plasma samples compared to healthy controls.

Accumulation of several genetic and epigenetic changes occurs during colorectal cancer development, which can lead to the transformation of healthy colonic epithelium to adenocarcinoma. One type of the epigenetic alterations is the changed DNA methylation, which plays a role in transcriptional regulation of numerous genes in physiological conditions however it is also essential in carcinogenesis. Certain genes can be inactivated by hypermethylation of their promoter regions, therefore important cell functions are modified. For example, promoter hypermethylation of tumor suppressor genes can cause reduction/lack of gene expression, thus this process can lead to cancer development.

Hypermethylation of Septin 9 gene (SEPT9) in blood and biopsy samples of patients with colorectal cancer was described in several studies. SEPT9 belongs to the superfamily of GTP-binding and filament-forming proteins. SEPT9 takes part in various cell functions such as cell division, actin dynamics, cell motility, cell proliferation, vesicle transport and exocytosis. The reason for this complexity may be that several SEPT9 slice variants encode different proteins.

AIMS

The key questions of my PhD work were

- the examination of SEPT9 methylation changes and to compare in peripheral blood and in tissue samples during the colorectal adenoma-carcinoma sequence progression;
- the analysis of the effect of SEPT9 hypermethylation on mRNA and protein expression during the colorectal adenoma-carcinoma sequence progression;
- the analysis of expression levels of SEPT9 splice variants in laser microdissected healthy and colorectal cancer samples;
- to analyse the correlation between cfDNA amount and SEPT9 methylation level;
- the determination of sensitivity and specificity of methylated SEPT9 in colorectal cancers with different localization (right and left sided), and
- to compare the effectiveness of mSEPT9 method and other, non invasive methods such as FOBT or CEA which is usually used for follow-up.

MATERIALS AND METHODS

Altogether 141 healthy, 59 adenoma and 161 colon cancer plasma and tissue samples were involved in my studies. Patients participating in the study were given complete information about the trial. Patients were excluded, who has malabsorption, acute medical conditions requiring intervention and other malignant diseases (besides colorectal cancer) at the time of sampling. None of the patients with cancer received chemotherapy, radiotherapy, or surgical invention prior to sampling. All patients included in this study were scheduled for routine colonoscopy. After endoscopic examination, histological diagnoses were established by pathologists.

Duplex RT-PCR

Twenty-four healthy, 26 adenoma with low-grade dysplasia (more than 1 cm diameter or histologically tubulovillous or villous) and 34 colon cancer (according to the AJCC system: 6 stage I, 11 stage II, 11 stage III, 5 stage IV and 1 with unknown stage) blood and tissue samples were used for methylated SEPT9 pattern determination. After homogenization, DNA was extracted by High Pure DNA Mini kit (Qiagen) and QIAmp DNA Mini kit (Qiagen) from

biopsy samples. 3.5 ml plasma/patient was applied for DNA isolation using Epi proColon Plasma Quick Kit (Epigenomics).

92 healthy and 92 cancer (25 stage I, 14 stage II, 35 stage III and 18 stage IV) plasma samples were analysed in order to determine the sensitivity and specificity of mSEPT9 detection.

Before the PCR examination, bisulfite treatment of extracted DNA samples was carried out, during which the unmethylated cytosines were converted into uracil through sodium bisulfite salt treatment and this modification can be detected by duplex PCR method. The applied quantitative RT-PCR reaction shows the methylated CpG sites in the promoter region of v2 variant of Septin 9 gene, and measures the β -actin (ACTB) housekeeping gene region of the bisulfite treated DNA. Cycle threshold (CT) values were taken into consideration during the evaluation of RT-PCR results. Each sample was tested in triplicate PCR reactions, SEPT9 hypermethylation was established when the CT values of at least in 2 from 3 PCR reactions were within the detection border (SEPT9: <50 CT, ACTB: ≤ 33.7 CT). Two types of methods were applied during the evaluation. In 1/3 method, a sample was declared positive if 1 from 3 PCR replicates were within the detection border. In 2/3 method, the examined sample was considered as SEPT9 positive one, if at least 2 from 3 PCR reactions were found to be positive.

The ratio of the ACTB housekeeping gene amount detected during the duplex PCR was used for the quantitative determination of cfDNA amount.

Microarray analysis

Total RNA was isolated by RNeasy Mini Kit (Qiagen) from 7 healthy, 13 adenoma (six with low-grade dysplasia, seven with high-grade dysplasia) and 15 CRC (5 stage I, 6 stage II and 4 stage IV) biopsy samples for mRNA expression profiling. Laser microdissected samples were originated from 6 colorectal cancer (moderately differentiated, II stage, left sided adenocarcinoma) surgical specimens, with polypoid lesions adjacent to tumor. Epithelial and stroma cells were collected during laser microdissection, then RNA were extracted from these cells using RNeasy Micro Kit (Qiagen).

Affymetrix whole genomic microarray analysis was done both in biopsy and laser microdissected cases. During the statistical evaluation of microarray results, preprocessing was firstly applied, which contains background correction, normalization and summarization. After that, SAM (Significance Analysis of Microarray) was used for the determination of the

differentially expressed genes between the sample groups. ANOVA and Tukey HSD methods were used for the detection of differences between the disease groups.

Real-time PCR

After reverse transcription of the RNA isolated from 6 colorectal cancer (moderately differentiated, II stage, left-sided adenocarcinoma) surgical samples, SYBR Green real-time PCR assays were performed using primer sequences for β -actin housekeeping gene and for different SEPT9 splice variants (v1, v2, v4, v4*, v5).

Examinations in protein level

For studying the effect of SEPT9 methylation on protein expression, 10 healthy, 14 adenoma (villous and tubulovillous) and 13 colorectal cancer (stage II and III) biopsy samples were involved in immunohistochemical analysis. During the evaluation, semi-quantitative scoring system was applied for determination of the SEPT9 immunohistochemical staining intensity.

Analysis of other markers (gFOBT, CEA)

During retrospective data collection, the detection level of stool blood was 0.6 mg Hg/gm of feces. The measurement range of CEA test was 0.2–1,000 ng/mL of CEA. A CEA serum level higher than 4.3 ng/mL was considered to be in the pathological range.

RESULTS

1. DNA methylation examination of SEPT9 gene in plasma and tissue samples

Highly significant difference ($p < 0.001$) was observed between healthy vs. adenoma and healthy vs. cancer cases in tissue samples. No significant difference ($p = 0.14$) was found between adenoma and cancer group, namely the level of methylated SEPT9 showed excessive similarity. Significantly higher SEPT9 methylated levels were measured in tumorous tissue ($p < 0.001$) and plasma ($p = 0.01$) samples. Surprisingly, a similar correlation was not seen in adenoma samples in the two different sampling groups. Correspondingly to the tumor tissue results, elevated SEPT9 methylation was detected in adenoma biopsies, while the methylation patterns of adenoma plasma samples were rather similar to healthy group. Higher PMR value was measured only in one (1/24; 4.2%) healthy sample in the tissue group. From the healthy

plasma samples, only 2 cases (2/24; 8.3%) showed higher PMR %. Based on the above-mentioned observations, in adenoma samples, methylated SEPT9 positivity was measured in 30.8% (8/26) of plasma and in 100% (26/26) of tissue samples.

The majority of the CRC plasma samples (30/34; 88.2%) showed SEPT9 methylation, while it was detectable in 97.1% (33/34) of tissue cancer samples. The highest (29.41%) mean tissue PMR value was measured in the adenoma group, and similar methylation level was observed in cancer group (21.52%), while it was only 0.52% in healthy cases. Contrarily, in plasma samples the mean PMR values were elevated in parallel with the disease prognosis (0.01%, 0.17% and 5.95%).

2. Effect of SEPT9 hypermethylation on mRNA expression

According to the expression level of SEPT9 207425_s_at transcript, healthy biopsy samples could be separated from adenoma and cancer groups. Expression of the above transcript was suitable for distinguishing late stage cancer cases from adenoma and from early stage cancer, as well ($p < 0.01$).

Contrarily, the expression level of 1559025_at transcript was found to be similar in the healthy and low-grade dysplastic adenoma samples, while its expression in high-grade dysplastic adenoma samples was observed to be closer to the expression level measured in cancers.

3. Effect of SEPT9 methylation on protein expression

The strongest, 100% (10/10) SEPT9 protein production was observed in healthy samples: diffuse SEPT9 protein expression was shown which was more intensive towards the luminal epithelium. In most adenoma samples, moderate or weak immunoreaction was found to be localized mainly to the apical cytoplasm of epithelial cells, and the rate of +2 immunoreactive cells was 42.8% (6/14). SEPT9 staining was heterogeneous in most CRC samples, with +2 score value in 38.4% (5/13) of samples.

4. Examination of SEPT9 mRNA expression in laser microdissected samples

When the stromal cells were analysed, similar expression of 1559025_at SEPT9 transcript was found in healthy and adenoma samples, while cancer cells showed different mRNA

expression level. In epithelial cells, the SEPT9 mRNA expression was found to be continuously decreased according to the adenoma-carcinoma sequence progression. Significant difference was observed between healthy vs. adenoma ($p < 0.05$) and between healthy vs. cancer ($p < 0.01$) cases.

During the examination of epithelial cells, the highest difference (17.4x) was shown in case of SEPT9_v2 from SEPT9_v1, _v2, _v4, _v4* and _v5 splice variants in healthy vs. tumor comparison.

5. Correlation between cfDNA amount and SEPT9 methylation level

Significant difference ($p < 0.01$) was found only between healthy and tumor samples during the quantitative determination of cell-free DNA. The mean value of cfDNA amount was 20.52 ng/ml in healthy, 37.65 ng/ml in adenoma and 70.32 ng/ml in cancer samples.

In comparison of methylated SEPT9 level and cfDNA, lower ($R^2 = 0.254$) correlation was observed in early stage tumors and stronger correlation was established ($R^2 = 0.483$) in late stage tumors.

6. Determination of sensitivity and specificity of methylated SEPT9 in colorectal cancer with different localization (right-and left-sided)

According to the 1/3 analysis method, SEPT9 positivity was found in 15.2% (14/92) of healthy samples, while it was 96.5% (88/92) in cancer cases. No SEPT9 methylation difference was detected in the aspect of localization: positivity was found in 96.4% (54/56) of left-sided cancers and 94.4% (34/36) of right-sided cancers. Using the AJCC cancer stage classification, all CRC cases from stage II showed SEPT9 positivity, while it was only 84% (21/25) in stage I samples. Thus, the sensitivity of SEPT9 marker was found to be 95.6% (95% confidence intervals 89.2%-98.8%) and the specificity was 94.8% (95% confidence intervals 75.8%-91.44%).

Using the 2/3 analysis method, SEPT9 positivity was observed in only one healthy sample (1%; 1/92). Less SEPT9 positive samples were found in the tumor group, as well (79.3%; 73/92). Smaller difference was detected according to the localization, too. SEPT9 was found to be methylated in 85.7% (48/56) of the left-sided CRC and 69.4% (25/36) of the right-sided CRC cases. The specificity was 99% (95% confidence interval 94.1%-100%) and the sensitivity was 79.3% (95% confidence interval 69.6-87.1%) using 2/3 analysis method.

Lower values (60%-77.8%) were found in the comparison of tumor stages due to the lower sensitivity.

7. Comparison of effectiveness of SEPT9 method and other, non invasive methods

Occult blood was detected in 29.4% (5/17) of healthy subjects and 68.2% (15/22) of CRC patients. As expected, higher proportion ($p=0.09$) of left-sided CRC (83.3%; 10/12) showed gFOBT positivity compared to right-sided CRC (50%; 5/10). Thus, the specificity and sensitivity of gFOBT for CRC was found to be 70.6% (95% confidence intervals 44% to 89.7%) and 68.2% (95% confidence intervals 45.1% to 86.1%), respectively.

In our study, 14.8% (4/27) of healthy subjects and 51.8% (14/27) of CRC specimens showed elevated CEA levels. There was no significant difference ($p=0.34$) between the proportion of right-sided CRC cases (5/12, 41.6%) and left-sided CRC cases (9/15, 60%) with elevated CEA serum levels. Although the CEA level measurement is not used for screening, we determined its sensitivity and specificity for tumors: the specificity and sensitivity were 85.2% (95% confidence intervals 66.3% to 95.8%) and 51.8% (95% confidence intervals 31.9% to 71.3%), respectively.

CONCLUSIONS

In my studies I have established that methylated SEPT9 is a specific and sensitive biomarker for colorectal cancer from both tissue and plasma samples. This method is suitable for colorectal cancer screening from tissue and plasma samples equally.

Methylated SEPT9 analysis is an adequate method for premalignant screening only from tissues. It is usable for cancer detection from early stages with the proper analysis method. Detection of mSEPT9 marker can be carried out from peripheral blood as well, hereby it may have a great importance in colorectal cancer screening due to the favourable compliance. Since the participation rate of patients is very low for colonoscopy which is the most effective method used as far. It is important to note, that the mSEPT9 test does not replace the colonoscopy, but it could reduce the number of unnecessarily performed invasive examinations. Nowadays, the growing number of colorectal cancer cases requires more colonoscopic examinations with more competent professionals and devices. Thus, screening from peripheral blood suggests colonoscopy to those patients who had positive test, hereby not only time and energy, but also costs may be saved. Detection of mSEPT9 as an alternative

screening method would be suggested to the patients, thus increasing the proportion participants in screenings.

THE MOST IMPORTANT NEW STATEMENTS

- The mSEPT9 as peripheral blood-based biomarker was already known, but the methylated SEPT9 patterns have been compared in tissue and plasma samples from the same patients at first in my PhD study. Low methylation level was observed in healthy sample group and high in tumor cases. Difference was found in adenoma group between the two different origin of samples, since I have observed high SEPT9 methylation in tissue samples and low in adenoma plasma cases.
- The mSEPT9 marker is not suitable for adenoma screening from peripheral blood, however it can trustworthily detect the presence of adenomas from biopsy samples.
- I have established at first, with the help of laser microdissection, that SEPT9 methylation has epithelial origin.
- I have verified the effect of SEPT9 DNA methylation on protein expression with immunohistochemical examination.
- During SEPT9 gene slice variant (SEPT9_v1, _v2, _v4, _v4* and _v5) analysis, I have demonstrated that the expression of SEPT9_v2 transcript differs mostly between healthy and cancer samples.
- I have also shown a correlation between the cell-free DNA and methylation of SEPT9 in cancer samples, especially in late stage colorectal cancers.
- Comparing the effectiveness of mSEPT9 marker and the used non invasive methods (such as FOBT or CEA which is usually used for follow-up), the mSEPT9 detection was proved to be suitable for colorectal screening as the most effective blood based method.

PUBLICATIONS

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