Analysis of genes with altered expression along colorectal tumor formation and and their regulatory processes

PhD thesis

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1 INTRODUCTION

Although screening tests are getting greater emphasis over time, colon cancer (CRC) cases is usually diagnosed only at an advanced stage of the tumor, when chances of survival are greatly diminished. In the developed Western world, including our country CRC is characterized by extremely high incidence and mortality. Despite the fact that to date - as a result several studies – more and more knowledge is accumulated, molecular alterations during the development of colorectal tumors are only partially known. It is well known that during the development of colon cancer various gene expression differences can be detected. Numerous studies aimed to identify clinically useful markers on the basis of whole genome expression studies, that later can impove on the classification of tumors as a part of routine diagnostics. Our working group identified a set of 11 transcripts for the discrimination of healthy colon tissue and colon cancer cases.

During the development of a variety of cancers including CRC - in addition to genetic changes - the altered function of epigenetic mechanisms can also regulate gene expression. The most studied epigenetic phenomenon is DNA methylation, whose aberrant levels can lead to gene silencing. The posttranscriptional regulation by miRNAs can also reduce gene expression levels.

Several genes were identified during the development of colon tumors that are subjected to DNA methylation silencing. Certain hypermethylated genes can be detected already in aberrant crypt foci (ACF) such as *RASSF1A, SFRP1, SFRP2* and *MINT31 MINT1* loci and several hypermethylated genes are identified along the adenoma-carcinoma sequence. Beside tissue samples, with non-invasive sampling DNA methylation markers can also be detected in blood plasma and stool that are characteristic to CRC development. The best known plasma DNA methylation marker of CRC is Septin 9 (SEPT9), its increasing hypermethylation is detectable in the blood plasma during adenoma-carcinoma sequence. Although levels of circulating SEPT9 DNA methylation can be detected in plasma samples with high sensitivity and specificity, to date relatively little information is available about the DNA hypermethylation of the colon tissue and how its effect on the degree of expression on mRNA and protein levels.

For gene expression studies fresh frozen tissue samples are the most ideal type of specimens, from which extracted nucleic acids are characterized by high integrity and purity.

The diagnosis of pathological alterations of colon biopsy or surgically removed tissue specimens obtained by routine endoscopy is made on the basis of sections from FFPE blocks.

Formalin fixation perfectly preserves tissue structural architecture, however, it has technical disadvantages regarding molecular structure. Thus, the isolated RNA from FFPE samples often have relatively low integrity, so FFPE tissues are considered to be challenging samples type in gene expression studies. In order to increase the usability of FFPE samples, manual and automated nucleic acid isolation methodologies are constantly being improved.

Cells constituting a specific tissue type have different transcriptional profile. *In situ* hybridization can be a useful technique for detecting these differences, providing information not only about the level of expression, but also about the histological and cellular localization.

In order to obtain more information about the cells in tissue samples, it is necessary to separate the different cell types, which can be achieved by laser microdissection (LCM) of histological sections.

2 AIMS

The aims of my PhD study were summarized in the following points:

1. Analysis of the applicability of automated RNA isolation from fresh frozen and formalin-fixed, paraffin-embedded (FFPE) surgically removed colon tissue.

2, Analysis of a CRC-specific transcript set published recently by our research group on FFPE tissue samples identified on the basis of whole genome expression experiments, discriminating diagnostic groups.

3, Analysis of the applicability of automated DNA isolation from fresh frozen surgically removed colon, FFPE tissue and biopsy samples

4, Identification of genes with altered gene expression along colorectal cancer formation and analysis of their potential regulatory mechanisms (DNA methylation, miRNA)

5, DNA methylation analysis of *SEPT9* plasma DNA methylation marker, also used in clinical practice with characteristic hypermethylation along colon adenoma-carcinoma sequence on epithelial and stromal cells isolated from colon tissue samples.

3 MATERIALS AND METHODS

In our study a total of 268 colorectal tissue samples were analyzed. The analyzed sample group contained 114 CRC, 34 adenomas, 107 adjacent normal (NAT) and 18 healthy colon tissue samples.

3.1 Analysis of colorectal cancer-specific mRNA markers fresh frozen biopsy and FFPE samples isolated with automated method

3.1.1 Analysis of the applicability of automated RNA isolation

3.1.1.1 Automated RNA isolation

Automated and manual RNA isolation from 10 CRC and the corresponding 10 NAT fresh frozen, furthermore 10 CRC and the corresponding 10 NAT FFPE specimens from the same biological tissue samples were performed. Automated isolation was performed with MagNA Pure 96 Cellular Large Volume Kit (Roche, Penzberg, Germany) using the MagNA Pure 96 automated nucleic acid isolation system and RNeasy Mini Kit or RNeasy FFPE kits (Qiagen, Hilden, Germany) were used for manual isolation.

RNA concentration was measured with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). The quality of the RNA samples was measured with RNA 6000 Pico LabChip kit on the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, USA) microcapillary electrophoresis system.

3.1.2 Analysis of colorectal cancer-specific mRNA markers on FFPE samples

3.1.2.1 Automated RNA isolation

MagNA Pure 96 automated nucleic acid isolation was performed from 15 CRC and 15 healthy colon biopsy, and 15 CRC and the corresponding 15-NAT FFPE with MagNA Pure 96 Cellular Large Volume Kit (Roche, Penzberg, Germany). The quality control was performed as described in Section 3.1.1.1.

3.1.2.2 Gene expression analysis

Reverse transcription was performed with 150 ng of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Gene expression of the 11 CRC-specific transcripts previously identified on the basis of microarray analyses (CA7, COL12A1, CXCL1, CXCL2, CHI3L1, GREM1, IL1B, IL1RN, IL-8, MMP-3, SLC5A7) and 18S ribosomal RNA internal control was performed with real-time PCR using Real Time using Ready® assays. RT-PCR reactions were performed with LightCycler® 480 instrument (Roche).

3.1.2.3 In situ hybridization

mRNA *in situ* hybridization was performed for two selected transcripts from the 11 markers, carbonic anhydrase VII (CA7) with decreasing, and chemokine (CCC motif) ligand 1 (CXCL1) with expression along CRC formation. During the test, two so-called non-overlapping Locked Nucleic Acid (LNA) oligonucleotide probes were designed for both mRNA including a negative and a positive (miR-126) test. The labeled sections were digitalized with Pannoramic 250 scanner (3DHISTECH Ltd., Budapest, Hungary).

3.2 Identification of DNA methylation markers of colon tissue

3.2.1 Analysis of automated DNA isolation applicability

3.2.1.1 Automated DNA isolation

Automated and manual DNA isolation from 10 CRC and the corresponding 10 NAT fresh frozen tissue sample, 10 CRC and 10 healthy colon biopsy, and 10 CRC and the corresponding 10 NAT FFPE samples was performed. Automated isolation was carried out with MagNA Pure DNA and Viral NA Small Volume Kit manual DNA extraction on the MagNA Pure 96 system, manual isolation was performed with QIAamp DNA Mini Kit and QIAamp DNA FFPE Kit (Qiagen). The quantity (OD 260) and purity values (OD 260/230, OD 260/280) of the isolated DNA was measured with NanoDrop 1000 spectrophotometer.

3.2.1.2 DNA methylation analysis

DNA samples were bisulfite converted with EZ DNA Methylation Kit (Zymo Research, Irvine, USA) with 1 μg input DNA amount. DNA methylation level of three different genes (MAL, SFRP1, SFRP2) was determined with bisulfite-specific polymerase chain reaction (BS-PCR) followed by methylation-specific high-resolution melting analysis (MS-HRM) using 10 ng bisulfite converted DNA input. The reaction was performed by PCR LightCycler 480 instrument, the HRM curve analysis was carried out using the LightCycler GeneScanning software with methylation standard samples with known percentage of DNA methylation.

3.2.2 Identification of DNA methylation markers in colon tissue samples

3.2.2.1 Laser capture microdissection

Colonic epithelial and stromal cells (10^3 cells / sample) cells were separated from fresh-frozen tissue using a PALM Microbeam laser microdissector. Macrodissected samples were collected from toluidine blue-stained fresh frozen sections.

3.2.2.2 DNA methylation analysis

The bisulfite conversion from the collected cells was performed without prior DNA extraction using EZ DNA Methylation Direct Kit (Zymo Research) according to the manufacturer's instructions. BS-PCR reactions were performed with 1ng bisulfite converted DNA input with LightCycler 480 instrument in case of 18 genes (*ALDH1A3, BCL2, CDX1, COL1A2, CYP27B1, ENTPD5, FADS1, MAL, PRIMA1, PTGDR, PTGS2, SFRP1, SOCS3, SULT1A1, THBS2, TIMP1 SFRP1, SULF1*). In this study, we used PyroMark Q24 and GS Junior sequencers.

3.2.2.3 Immunohistochemistry

SFRP1 protein levels in CRC (n = 10), adenoma (n = 10) and CRC FFPE samples (n = 10), was detected with anti-SFRP1 polyclonal antibody (ab4193, Abcam, UK, 1: 800 dilution).

3.2.2.4 miRNA analysis

CRC (n=3), adenomas (n=3) és NAT (n=3) független FFPE mintákból a High Pure miRNA kit (Roche) miRNS izolálást végeztük, majd közel 800 miRNS kifejeződését a Human Panel I + II (Exiqon) RT-PCR módszeren alapuló array segítségével végeztük, majd kiválasztott miRNS-ek expresszióját hasonlítottuk össze a csoportok között. In silico miRNS predikciót a miRWALK programmal végeztünk.

miRNA isolation was performed from independent CRC (n = 3), adenoma (n = 3) and NAT (n = 3) FFPE samples using High Pure miRNA kit (Roche), and expression of 800 miRNAs were analysed with Human Panel I+II (Exiqon) RT-PCR method, and then expression levels of selected miRNAs were compared between the groups. *In silico* miRNA prediction was performed with miRWALK program.

3.3 *SEPT9* DNA methylation in laser microdissected epithelial and stromal cells

3.3.1 Laser microdissection

Colonic epithelial and stromal cells were collected from 3 CRC and from the 3 corresponding NAT fresh frozen tissue samples and from 3 healthy colon biopsies using LCM. Normal adjacent areas from 1 cm (NAT1) or 10 cm (NAT2) distance from the tumor were also examined.

3.3.2 DNS methylation analysis

Bisulfite conversion from the collected laser microdissected cells was performed with EPI proColon 2.0 kit with a modified protocol. A two-step multiplex amplification were performed using 2 ng bisulfite converted DNA per sample in order to amplify 13 regions.

Sanger sequencing was performed on the amplicons on ABI 3730 XL instrument (Applied Biosystems, Waltham, USA). The methylation percentage of CG dinucleotides was calculated on the basis of the ratio of C nucleotides compared to total C and T nucleotides. CG methylation in a given amplicon was averaged and then these results were compared between the sample groups.

3.3.3 Immunohistochemistry

Septin 9 protein expression in NAT (n = 3), adenoma (n=3) and CRC (n=3) FFPE samples was detected with anti-Septin 9 primary antibody (Abnova PAB4799, Germany, 1:50 dilution).

4 RESULTS

4.1 Analysis of colorectal cancer-specific mRNA markers fresh frozen biopsy and FFPE samples isolated with automated method

4.1.1 Analysis of the applicability of automated RNA isolation

Fresh frozen and FFPE samples from the same biological samples showed similarly high yield of RNA both after automated (mean ± standard deviation RNA quantity of fresh frozen normal: 8.3 ± 4.16 μg RNA, fresh frozen CRC: 15.6 ± 10.28 μg RNA; FFPE normal: 2.7 ± 1.7 μg RNA; FFPE CRC: 6.2 ± 2.4 μg RNA) and after manual isolation (mean ± standard deviation RNA quantity of fresh frozen normal 7.88 ± 4.32 μg RNA; fresh frozen CRC: 16.28 ± 12.38 μg RNA; FFPE normal: 1.87 ± 1.55 μg RNA; FFPE CRC: 2.8 ± 1.51 μg RNA) methods.

Purity values (OD 260/280 and and OD260/230) of the isolated RNA samples were found to be different after the two isolation methods. The automatic isolation of fresh frozen samples values were similar to the manual isolation results, but in the case of FFPE samples these values were lower, while OD 260/280 ratio of RNA samples after manual isolation were high in both sample types. RNA intactness RIN (1-10) values were higher in case of fresh frozen samples compared to FFPE samples both with automated and with manual isolations.

4.1.2 Analysis of colorectal cancer-specific mRNA markers on FFPE samples

Fresh frozen and FFPE samples from the same biological samples showed similar RNA yield both after automated isolation (mean ± SD ; biopsies : normal = 5.98 ± 1.72 μg RNA; tumor = 5.77 ± 2.27 μg RNA; FFPE: normal = 20.04 ± 3.70 μg RNA; tumor = 7.10 ± 3.30 μg RNA). The OD 260/OD 260 and OD280/230 ratios in biopsies were significantly ($p < 0.001$) higher than in the FFPE samples. RIN values of RNA samples isolated from frozen biopsies were significantly ($p < 0.001$) higher than RNA samples' from FFPE tissue.

The unsupervised hierarchical cluster analysis of the previously identified 11 CRC-specific markers revealed one group of CRC samples and one misclassified normal samples, while in the other group almost all normal samples (14/15) and misclassified CRC (8/15) cases. Discrimination of FFPE samples almost perfectly separated the CRC and healthy cases. With discrimination analysis biopsies could be separated with maximum efficiency (100%, 100%). The FFPE samples from 6 normal and tumor sample 8 were excluded automatically from the analysis, the remaining 7 tumor samples and 9 normals were classified correctly (100%, 100%).

With multiple logistic regression based on the gene expression results of 11 markers healthy and tumor biopsies were separated with 93.3 % sensitivity and 86.7% specificity. In contrast,

FFPE tissues could be distinguished with a higher specificity (96.7%), but lower (70.0%) sensitivity. *In situ* mRNA hybridization of carbonic anhydrase 7 and chemokine ligand 1 transcripts showed similar gene expression alterations as seen in RT-PCR reactions.

4.2 Identification of DNA methylation markers from colon tissue samples

4.2.1 Analysis of automated DNA isolation applicability

DNA yield of samples extracted from fresh frozen automated and manual methods were found to be similar. Comparing the manual method to the automated protocol the average of yield showed no significant difference ($p < 0.01$) (mean ± SD of DNA quantity ; automated: 10.48 ± 6.16 µg DNA, manual: 14.61 ± 14.05 µg DNA). DNA yield of biopsies (mean ± SD of DNA quantity ; automated: 3.86 ± 1.42 µg DNA / 3-5 mg tissue; manual: 8.50 ± 3.34 µg DNA/3-5 mg tissue) and FFPE samples (the average amount of DNA ± SD ; automated: 4.61 ± 2.36 µg DNA/section, manual: 11.51 ± 6.89 µg DNA/section) were significantly ($p<0.01$) higher after the manual method.

The OD 260/280 ratios in fresh frozen samples (mean OD260/280 ± SD ; automated: 1.87 ± 0.07; manual: 1.88 ± 0.11) and biopsies (average OD260/280 ± SD; automated: 1.94 ± 0.04; manual: 1.93 ± 0.04) were found to be similar. OD260/280 ratios of certain automatically isolated normal FFPE samples were lower than fresh frozen and biopsy samples. OD260/280 ratio of the manually isolated samples were significantly ($p < 0.01$) higher for FFPE samples (mean OD260 / 280 ± SD ; automated: 1.83 ± 0.06; manual: 2.00 ± 0.04). OD260/230 ratio of fresh frozen samples were relatively high (mean OD260 / 230 ± SD ; automated: 2.01 ± 0.46; manual: 1.85 ± 0.35), these values were higher in the automatically isolated samples. Similar results were obtained in the case of biopsy specimens (median OD 260/230 ± SD; automated: 2.71 ± 0.56; manual: 2.23 ± 0.13). After manual isolation of FFPE samples high OD26 / 230 ratios were measured (average OD 260/230 ± SD; automated: 1.81 ± 0.35; manual: 1.95 ± 0.27). The biopsy and fresh frozen samples of DNA methylation results were similar after the different isolation methods (biopsy: $R^2_{MAL} = 0.93$; $R^2_{SFRP1} = 0.61$ $R^2_{SFRP2} = 1.00$, fresh frozen: $R^2_{MAL} = 0.72$; $R^2_{SFRP1} = 0.69$, $R^2_{SFRP2} = 0.76$). In contrast, FFPE samples showed lower correlation (FFPE . $R^2_{MAL} = 0.09$, 0.89 R^2_{SFRP1}; $R^2_{SFRP2} = 0.89$). Among the three genes MAL primer pairs differed mostly, while strong correlation was found in case of *SFRP1* and *SFRP2* genes.

4.2.2 Identification of DNA methylation markers in colon tissue samples

On the basis of a previously published gene expression study certain genes were identified with gradually altering gene expression ($p \leq 0.05$) along the colorectal adenoma-carcinoma sequence. The following genes showed significant ($p<0.05$) gene expression alterations in the

adenoma vs. normal and also in the adenoma vs. tumor comparisons: *BCL2*, *CDX1*, *CYP27B1*, *ENTPD5*, *MAL*, *PRIMA1*, *PTGDR*, *PTGS2*, *SFRP1*, *SOCS3*, *SULT1A1*, and *TIMP1*. Furthermore, ALDH1A3, COL1A2, FADS1, SFRP2, SULF1 and THBS2 genes showed significant differences ($p < 0.01$) in tumor vs. normal comparison.

Lézer mikrodisszektált hámsejtekben kimutatható szignifikáns expressziós változást ($p<0,05$) a *SOCS3* és a *PRIMA1* gének mutattak az adenoma vs. ép összehasonlításban. A *BCL2*, a *CYP27B1*, a *COL1A2*, a *FADS1* és a *SULT1A1* gének kifejeződése csak a tumor-normál összehasonlításokban bizonyult szignifikánsnak ($p<0,05$), míg a *CDX1*, a *ENTPD5*, a *PTGDR* és a *TIMP1* gének kifejeződése mindkét összehasonlításban változónak adódott.

Laser microdissected epithelial cells demonstrated significant expression changes ($p < 0.05$) in *SOCS3* and *PRIMA1* genes in the adenoma vs. normal comparison. The *BCL2*, *CYP27B1*, *COL1A2*, *FADS1* and *SULT1A1* gene expression alterations were significant ($p < 0.05$) only in the tumor vs. normal comparison, while *CDX1*, *ENTPD5*, *PTGDR* and *TIMP1* gene expression altered in both comparisons.

Demethylation treatment of HT-29 cells with 5-aza-2'-deoxycytidine resulted in moderate gene expression differences in case of 4 examined genes (*TIMP1*, *FADS1*, *CYP27B1*, *SULT1A1*), whereas the expression of *PTGS2* gene increased and expression of *SOCS3* genes slightly decreased.

Our results show that from the 18 transcripts with altered expression 6 analyzed region belonging to 4 genes showed significant DNA methylation difference. Among them, *COL1A2*, *SOCS3* and *SFRP2* were hypermethylated while *THBS2* showed hypomethylation in adenoma and cancer samples compared to NAT cases.

DNA methylation data were examined with unsupervised hierarchical cluster analysis. The first major group (*SFRP2, COL1A2, THBS2, SOCS3, CYP27B1, SULT1A1, PRIMA1, MAL*) showed relatively high levels of DNA methylation in adenoma and cancer samples. Genes classified in the second group showed no significant difference in DNA methylation, while the third cluster contained *THBS2* gene with relatively high DNA methylation levels. The laser microdissected results were similar to the biopsy and macrodissected results. Relatively high level of DNA methylation was found in adenoma and cancer samples in case of *PRIMA1, SFRP1, SFRP2, MAL, SOCS3, CYP27B1, SULT1A1* and *COL1A2* genes both in macrodissected and also in epithelial cells.

Target prediction of miRNAs showing altered expression was performed with miRWALK database. We were able to identify miRNAs targeting certain transcripts analyzed in our study in silico with miRWALK validated module. For instance expression of miR-21 potentially

targeting *BCL2, MAL, PTGS2, SFRP1, SOCS3* transcipts was significantly increased along colon tumor formation. The miR-21 * (targeting *BCL2, MAL, PTGS2, SFRP1, SOCS3*), the let-7i * (targets: *BCL2, CYP27B, SOCS3*) and miR-181c (targeting *ALDH1A3, BCL2, MAL*) expression also increased in tumor samples.

Based on our results, SFRP1 protein level decreased in colorectal adenoma-carcinoma sequence.

4.3 *SEPT9* DNA methylation in laser microdissected epithelial and stromal cells

Based on our results CRC-specific DNA methylation level alteration was seen only in the third CpG island (CGI3), while other regions showed no significant differences. The fifth major amplicon localized centrally in CGI3 showed remarkable DNA methylation differences between the normal and adenoma, as well as between normal and tumor tissues. This difference was more pronounced in epithelial cells. DNA methylation level difference between normal and tumor epithelial cells reached 80% ($p<0.0001$), that was detected in the same degree in the normal vs adenoma comparison. In case of two among the three tumors the NAT1 samples within less than one centimeter distance from tumor areas, DNA methylation levels showed significant differences compared to the control samples ($p<0.0001$), while this difference was not detected ($p>0.05$) in the distal NAT (NAT2) areas.

In contrast, approximately 50% hypermethylation could be detected in stromal cells isolated from tumor tissue compared to normal stromal cells, while this value was 30% in adenomas. In contrast to the epithelial cells, stromal cells showed no difference in level of DNA methylation in NAT areas. Significantly ($p <0.001$) higher Septin 9 protein levels could be detected in the healthy samples and NAT1 epithelium and stromal cells compared to adenoma or tumor samples, where decreased Septin 9 protein expression was found.

5 CONCLUSIONS

On the basis of the results of my PhD I could conclude that the automated nucleic acid isolation methods are ideal alternatives for laboratories processing large samples numbers. Gene expression markers can be useful for the objective classification of colon cancer cases, on the basis of them the discrimination of healthy and tumorous samples can be achieved. My results show that with this approach the automatically processed FFPE samples can be grouped with high sensitivity and specificity.

The automated DNA extraction can be also used efficiently, and the processed samples can be an adequate basis for further molecular studies

Based on gene expression alterations it is possible to identify markers potentially regulated by DNA methylation.

With this approach I could identify a group of genes whose DNA hypermethylation was characteristic especially in the epithelial cells of colon adenoma and cancer tissue samples.

Based on the results of *SEPT9* in colorectal adenomas and tumors located DNA hypermethylation in the promoter region of a the 3 larger CpG islands are is restricted to only one of them, which overlaps with the region analyzed by Epi proColon 2.0 plasma pre-screening test.

In summary, it can be concluded that the identified genes regulated by DNA methylation may play important role along the colorectal adenoma-carcinoma sequence. These results might contribute to the better understanding of the molecular background of tumor formation and the identified markers can enhance the early detection of CRC in the future.

6 THE MOST IMPORTANT NEW STATEMENTS

The main findings of the thesis are:

- With automated RNA isolation using MagNA Pure 96 instrument it is possible to extract RNA samples with sufficient quantity and quality from biopsy and also from FFPE samples.
- The analyzed 11 mRNA marker set could discriminate healthy and CRC FFPE tissue samples with 96.7% sensitivity and 70% specificity, thus I established that the CRC-specific transcript set can be suitably used on FFPE tissue material, as well.
- According to my results DNA samples isolated with automated method had sufficient yield and purity ratios to be applicable for further experiments determining DNA methylation levels.
- I have identified genes with decreasing gene expression that may be under control of DNA hypermethylation or miRNA during colorectal adenoma-carcinoma sequence
- Among the identified genes showing decreased gene expression in parallel with increased DNA methylation levels decreased protein level could be confirmed for SFRP1.
- In case of genes without significant DNA hypermethylation in the adenoma-carcinoma sequence, other epigenetic control mechanisms, including miRNA regulation can be hypothesized.
- I observed similar DNA methylation results with automatically and manually isolated biopsy, fresh frozen and FFPE surgical samples.
- DNA methylation level of *SEPT9* was studied on laser microdissected epithelial and stromal cells isolated from normal, NAT, adenomas, and tumor samples.
- I found that epithelial cells of colorectal tumor and adenoma samples show high *SEPT9* DNA hypermethylation.
- -Based on the results of *SEPT9* DNA hypermethylation located in the promoter region of a the 3 larger CpG islands are is restricted to only one of them, which coincides with the region analyzed by Epi proColon 2.0 pre-screening test.

7 PUBLICATIONS

7.1 Publications related to the PhD dissertation

7.1.1 Publications related to the PhD dissertation published in international journals

Kalmár A., Péterfia B., Hollósi P., Galamb O., Spisák S., Wichmann B., Bodor A., Tóth K., Patai V. Á., Valcz G., Nagy Zs. B., Kubák V., Tulassay Zs., Kovalszky I., Molnár B. - DNA hypermethylation and decreased mRNA expression of MAL, PRIMA1, PTGDR and SFRP1 colorectal adenoma and cancer. (2015) BMC Cancer, (2015) 15:736. doi:10.1186/s12885-015-1687-x **IF: 3,362**

Kalmár A., Wichmann B., Galamb O., Spisák S., Tóth K., Leiszter K., Schnack N. B., Barták B. K., Tulassay Zs., Molnár B. - Gene-expression analysis of a colorectal cancer-specific discriminatory transcript set on formalin-fixed, paraffin-embedded (FFPE) tissue samples. (2015) Diagnostic Pathology, 10: (1) paper 126. 12 p. doi:10.1186/s13000-015-0363-4 **IF: 2,597**

Kalmár A., Péterfia B., Wichmann B., Patai V. Á., Barták Barbara K., Nagy Zsófia B., Fűri I., Tulassay Zs., Béla Molnár – Comparison of automated and manual DNA isolation methods for DNA methylation analysis of biopsy, fresh frozen and formalin-fixed, paraffin-embedded colorectal cancer samples. (2015) Journal of Laboratory Automation, 1-10. doi:10.1177/2211068214565903 **IF: 1,879**

Kalmár A.; Péterfia B., Hollósi P., Wichmann B., Bodor A., Patai V. Á., Krenács T., Tulassay Zs., Molnár B. - Bisulfite-based DNA methylation analysis from recent and archived formalin-fixed, paraffin embedded colorectal tissue samples. (2015) Pathology and Oncology Research, May 2015: Paper doi:10.1007/s12253-015-9945-4. **IF: 1,855**

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Spisák S., **Kalmár A.***, Galamb O., Wichmann B., Sipos F., Péterfia B., Csabai I., Kovalszky I., Semsey S., Tulassay Z., Molnár B. - Genome-Wide Screening of Genes Regulated by DNA Methylation in Colon Cancer Development. (2012) PLoS ONE, 7(10): e46215. doi:10.1371/journal.pone.0046215 **IF: 3,730**

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Galamb O., Wichmann B., Sipos F., Spisák S., Krenács T., Tóth K., Leiszter K., **Kalmár A.**, Tulassay Z., Molnár B. - Dysplasia-carcinoma transition specific transcripts in colonic biopsy samples. (2012) PLoS ONE 7(11): e48547. doi:10.1371/journal.pone.0048547 **IF: 3,730**

Patai Á.V., Molnár B., **Kalmár A.**, Schöller A., Tóth K., Tulassay Z. - Role of DNA Methylation in Colorectal Carcinogenesis. (2012) Dig Dis 2012;30:310-315 (DOI: 10.1159/000337004) **IF: 2,725**

Tóth K., Sipos F., **Kalmár A.**, Patai A.V., Wichmann B., Stoehr R., Golcher H., Schellerer V., Tulassay Z., Molnár B. - Detection of methylated SEPT9 in plasma is a reliable screening method for both left- and right-sided colon cancers. (2012) PLoS One. 2012;7(9):e46000. doi: 10.1371/journal.pone.0046000. **IF: 3,730**

Patai Á.V., Valcz G., Hollósi P., **Kalmár A.**, Péterfia B., Patai Á., Wichmann B., Spisák S., Barták B. K., Leiszter K., Tóth K., Sipos F., Kovalszky I., Péter Z., Miheller P., Tulassay Z., Molnár B. - Comprehensive DNA methylation analysis reveals a common ten-gene methylation signature in colorectal adenomas and carcinomas. (2015) PLoS One. 2015;10:e0133836.**IF: 3,234**

7.1.2 Publications related to the PhD dissertation published in Hungarian journals

Spisak S., **Kalmar A**., Molnár B., Sipos F., Wichmann B., Galamb O., Tulassay Zs. - Metilációs szabályozás alatt álló gének azonosítása lézerrel kimetszett vastagbéldaganat-sejtekben az adenoma-carcinoma sorrend vizsgálata során. (2010) Orvosi Hetilap 151:(20) pp. 805-814. **IF: 0**

7.2. Publications not related to the PhD dissertation

7.2.1 Publications not related to the PhD dissertation published in international journals

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