Characteristic molecular changes during colorectal carcinogenesis and progression

Sándor Spisák

PhD thesis

Semmelweis University

Clinical Medicine Doctoral School

Gastroenterology PhD program

Program leader: Zsolt Tulassay MD, DSc

Supervisor: Béla Molnár MD, PhD

Opponents: Kiss András MD, PhD

Ponyi Tamás PhD

Head of Examination Committee: László Orosz DSc

Members of Examination Committee: Ilona Kovalszky MD, DSc

Attila Patócs MD, PhD

Budapest

2011

Introduction

Malignant diseases including colorectal cancer (CRC) are leading causes of death both in our country and worldwide. Diagnosis, treatment and research of CRC have become a central issue in medicine. In our country, approximately 9000 new colorectal cancer cases are registered, and almost 6000 CRC-related deaths appear in every year. Scientific literature and the exponentially expanding electronic databases provide more and more information about the molecular aspects of tumorous diseases.

In the last decade, high-throughput molecular biological methods provide more and more information about the molecular processes of tumor development, but there are still many questions regarding the early diagnosis, therapy and follow-up of cancers. Despite the intensive investigation and the increase of the available knowledge about the background of carcinogenesis and tumor progression, the previous research has not achieved breakthrough results in diagnosis and treatment of CRC.

According to our knowledge the tumor development is resulted by accumulation of errors in genetic information. During the disease progression, abnormal tissue increase appears in the affected caused by cell division error, impeding the normal function of the organ. The process causes not only local alterations, as the modification of the regenerative system contributes to the tumor growth and invasion which lead to drastic outcome of the disease. Consequently, the tumor is a systemic problem, rather than a local disorder. The high incidence and not fully understood molecular and pathological background of CRC necessitate the systemic research of this field.

In my PhD thesis I have searched tissue-specific biomarkers of the development and progression of colorectal carcinoma. Performing gene expression profile analysis of CRC samples using whole genomic oligonucleotide and antibody microarray technology I aimed the identification of methylation-regulated genes and examination of chemopreventive possibilities.

AIMS

The aims of my PhD work were

 Collection of surgical samples and testing their suitability for oligonucleotide and antibody microarray analyses

 Determination of diagnostic and prognostic protein patterns by antibody microarray technology

 Comparison of oligonucleotide and antibody microarray technologies and examination of their applicability, limitations, advantages and disadvantages

 Identification of correlation between the mRNA and protein level in CRC

 Development and testing of validation systems at mRNA and protein level, using TMA and RT-PCR platforms

 Identification of signaling pathways involved in colorectal pathogenesis and carcinogenesis

 Examination of molecular background of adenoma – dysplasia – carcinoma sequence using LCM samples and identification of carcinogenesis-related changes in epithelial and stromal regions.

 Determination and functional analysis of methylation-regulated genes

 Examination of molecular effect of NS398 selective COX-2 inhibitor and 5'-aza-2'-deoxycitidine potential therapeutic agents.

MATERIALS AND METHODS

1. Samples

I have performed my examinations on surgical and biopsy samples, furthermore in parallel on fresh frozen and FFPE samples and sections. Antibody microarray analyses were done on 16 surgical samples from 10 patients with Dukes B and 6 with Dukes D stage CRC. Fresh frozen sections from 6 patients with moderately differentiated, Dukes B stage colon adenocarcinoma with polyp were applied for LCM analysis. Formalin-fixed, paraffin-embedded (FFPE) sections were prepared from healthy and diseased samples which were the basis of pathological diagnosis and TMA immunohistochemistry. The sample collection and the study

were authorized by Semmelweis University Regional and Institutional Committee of Science and Research Ethics (ethical permission No.: TUKEB: 2005/037). Microarray analysis of HT-29 cell culture was performed using two chemical agents (5-aza-2'-deoxycitidine and NS398). Solvents without agent were used as controls.

2. Clontech AB 500 array analysis

Crude protein extract was prepared and labeled by monoreactive Cy3 and Cy5 (Amersham (GE Helthcare) dyes according to the manufacturer's instructions. The labeled samples were hybridized onto Clontech AB500 arrays, then the slides were digitalized by Axon 4000B instrument (Axon Instruments, USA) at 532 and 635 nm. According to the manufacturer's suggestion, the following scanning parameters were applied: 33% laser force, 560 and 670 PMT and 20um/pixel resolution. Slides were evaluated by GenePix 4.1 software. After fitting the array grid (Lot: 5010317), fluorescence intensity of the spots were determined. Statistical evaluation was performed using R software. After background correction (RMA and normexp) microarrays were normalized using quantile method.

3. Tissue microarray (TMA) validation

The antibody array results were confirmed by immunohistochemistry on TMA slides containing independent samples. The validation was performed on 15 healthy mucosa and 36 CRC samples with different localizations and degrees of differentiation. TMA contained scores with 2 mm diameter in 6x 4 blocks. Five um thin serial sections were cut from the TMAs and the slides were incubated with anti- APC, Caveolin, CBP, cyclinA, ERK, HSP60, Cox2, EGFR, C-myc, Cald, Top1, DARPP32, MRE11A, AndrogenR and EPS8 antibodies. The slides were digitalized and evaluated by Mirax Desk Scan system (3DHistech Ltd, Budapest).

4. Immunohistochemistry

After deparaffination, heat induced antigen retrieval was performed for 20 minutes in citrate buffer. Standard indirect immunoperoxidase reaction was applied for antibody visualization using DAB. Immunostaining was performed by Abgene and Dako antibodies according to the manufacturer's instructions.

5. LCM- Laser Captured Microdissection

In cryostat, 6 um thick sections were cut and placed onto special membrane-coated LCM slides (1um PEN) (Membrane Slide 1.0 PEN, Carl Zeiss, Jena, Germany). The sections were stored at -80^{O}C until use, then after fixation in ethanol dilution series, were stained using Cresyl Violet acetate. Laser captured microdissection was performed using PALM Microbeam system (PALM, Bernried). In average, 5000-10000 cells were collected.

6. Affymetrix whole genomic microarray analysis of LCM samples

The quality of the isolated total RNA was checked using Agilent Bioanalyzer Pico 6000 Chip kit, then biotin-labelled cRNA probes were synthesized using Affymetrix two-round IVT (in vitro transcription) Labeling Kit (Affymetrix, Santa Clara, California, USA). Ten ug of cRNA was hybridized from each sample onto HGU133 Plus2.0 microarrays (Affymetrix) for 16 hours at 45ºC. The microarrays were washed using Fluidics Station 450 device, and stained with antibody amplification staining method according to the manufacturer's instructions (EukGE_Ws_2v5 washing protocol and 10µg/ml streptavidin-phycoerythrin (Molecular Probes)). The fluorescent signals were detected using GeneChip 3000 scanner at 570nm.

7. Statistical evaluation of Affymetrix U113 Plus 2.0 whole genomic microarray results

The qualiy control was performed according to the suggestions of Tumour Analysis Best Practices Working Group. The pre-processing was done using GCRMA method. The significantly differentially expressed genes were determined using linear model and Bayesian approach by a Bioconductor program package containing SAM and PAM methods in R environment.

RESULTS

The results of my PhD work belong two main topics, namely the BIOMARKER research and the FUNCTIONAL analysis of the molecular processes of colon tumors. Briefly, I have performed mRNA and protein-based CRC biomarker identification using microarray technology. After validation of the results, I have searched connection between the mRNA and protein level results. The tissue specific expression of the examined biomarkers was determined using laser microdissected samples. Using LCM samples, I have identified the

genes with decreasing expression during the colorectal adenoma-dysplasia-carcinoma sequence progression, from which the genes potentially regulated by DNA methylation were determined. Also with the examination of the adenoma-carcinoma sequence I have identified the genes with reversible expression under selective COX-2 inhibitor treatment.

1. Identification of genes with expression changes in colon tumors at protein and mRNA levels

a. Whole genomic mRNA expression profiling

- Microarray results of 6 Dukes D stage CRC and 6 normal samples were applied for determination of gene expression pattern. For feature selection, SAM (Significance Analysis of Microarrays) were performed. Top500 most differentially expressed genes in normal vs. tumor comparison were selected. In all 199 downregulated and 301 overexpressed genes were identified and grouped by cell functions.

b. Determination of protein-based markers in colon tumors using antibody microarray technology

- Between tumorous and normal mucosa samples, 67 genes with protein expression changes were identified. These gene products were involved in apoptosis (5), cell cycle reulation (7), transcription regulation (4), DNA replication (6) and molecular transport, cell adhesion (45) cell functions.
- In the Dukes B group, 22 genes (9 downregulated and 13 overexpressed) were detected which showed significant expression changes at protein level compared to macroscopically normal mucosa.
- At protein level, 25 differentially expressed genes were found from which 13 were underexpressed and 12 were overexpresssed in Dukes D stage samples compared to healthy tissue.
- For the identification of progression markers, during the discrimination of Dukes B and Dukes D stages, 58 genes were found to be significantly differentially expressed, 11 of them showed considerable expression alterations between the two diagnostic groups.
- The selected proteins (APC, Caveolin, CBP, cyclin A, ERK, HSP60, Cox2, EGFR, C-myc, Cald, Top1, DARPP32, MRE11A, Androgen R, EPS8) determined by antibody microarrays were validated using TMA immunohsitochemistry.

2. Discovery of correlations between mRNA and protein levels

- When I have searched the connections between the mRNA and protein levels, positive correlation could be found in case of 143 transcripts and proteins (R^2> 0,8) which are mainly transport proteins. While 95 genes with opposite mRNA and protein expression were identified (R^2> 0,9), which are principally involved in cell process regulation.

3. Identification of genes with expression alterations during adenoma-carcinoma sequence using laser microdissected colonic samples

- During my PhD work, I have performed 42 microarray analyses from 6 distinct tissue regions of 6 tumorous patient: epithelial and stromal cells from 6 normal mucosa, 6 polyps, 6 CRC, furthermore 3 normal and 3 lymphoid follicle regions were analyzed. From each regions, 5000-10000 cells were collected using laser microdissection.

a. Analysis of epithelial and stromal regions

- The top100 most significantly differentially expressed genes during colorectal carcinogenesis were determined both in epithelium and stroma which classified into 17 cell function groups.
- The function and the role in genetic pathways of the one fourth of these genes (22 epithelial and 23 stromal markers) have not known yet. The apoptosis (2,3), the proteolysis (3,3), the cell migration (2,2) and the ubiquitination (1,1) are typical cell processes both in epithelium and stroma. Expression changes of osteogenesis-related genes (0,7) could not be detected in the epithelium, while the gene expression alterations involved in exocytosis and stress response are characteristic in the epithelium unlike the endocytosis- and immune response-associated genes which were found to be differentially expressed in stroma. In contrast, more intensive expression changes of transport (9,6), transcription regulation (20,6), metabolism (10,3) and cell division (2,5) genes could be observed in the epithelial layer referring to increased cell division and metabolism enhancing the cell proliferation. Upregulation of genes involved in angiogenesis (1,5), signal transduction (9,15) and changes of oxidation-reduction process genes (0,2) are typical in stroma. Other cell functions such as morphogenesis (5,7), cell adhesion (7,9) and migration (2,2) approximately similarly changed in both tissue regions.

- According to the results of the cluster analysis and the molecular pattern of the 100-100 selected genes, normal and adenoma samples showed higher similarity than the adenoma and tumor stages both in case of epithelium and stroma.

b. Analysis of lymphoid follicles

- Although the exact role of Peyer-patches in the regeneration and the tumor progression processes of the colon is not clarified yet, literature data implies active cell migration and differentiation in this tissue region. Fifty-four down- and 21 upregulated genes were identified in lymphoid follicles during the adenoma transition.

4. Determination of methylation-regulated genes during the adenoma-carcinoma sequence progression using gene expression profiling

- I have identified 110 genes with intensive expression in normal colon epithelial cells, whose expression begin to decrease in adenomatous epithelium, and further downregulates or ceases in tumorous epithelial cells. In parallel, HT-29 colon adenocarcinoma cells were treated by 5-aza-2'-dezoxicitidine demethylation agent. In the treated cells, 71 genes were found to be at least two-fold upregulated during the demethylation ($p<0,01$).. The 17 common elements of the two gene lists were further examined using RT-PCR technique.
- Using SAM method, top100 downregulated genes were determined in normal-adenoma, adenoma-tumor and normal-tumor comparisons. The decreasing expression observed in normal-adenoma comparison is not clearly caused by methylation, as only 12 of the 100 selected genes were found to be upregulated after 5-aza-2'-dezoxicitidine treatment in the demethylation model. Regulation by DNA methylation could be mostly (55%) confirmed in case of genes from the normal-tumor comparison. Between adenoma and tumorous samples, overexpression of 32 from the 100 downregulated transcripts could be detected in the demethylation model, confirming the regulation of these genes by DNA methylation.

a. RT-PCR validation on tissue samples

- According to the normal-adenoma results, 15 genes were found to be underexpressed, at least 2-fold decrease could be detected in case of the majority of these genes.

Between normal and tumorous samples 11 genes were downregulated. In CRC samples, 6 genes were identified whose expression reduced further during the malignant transition compared to adenoma samples.

b. Validation of PTGDR protein expression changes

- One selected gene, prostaglandin D2 receptor (PTGDR) was further analyzed whose downregulation in biopsy samples was also confirmed and 3 CpG islands were identified in the 5' regulatory region of the gene. For validating the methylation-associated regulation of this gene, bisulfite sequencing was designed. Before this I have analyzed whether the expression of PTGDR protein also decreases during the adenoma-carcinoma transition or not. According to the results of the PTGDR immunohistochemistry analyses, the downregulation along the tumor progression could be observed at the protein level as well.

c. Bisulfite sequencing and HRM analysis in the promoter region of PTGDR gene

- Bisulfite sequencing showed that CpG islands of the PTGDR gene promoter are slightly methylated in normal mucosa, partially methylated in the tumorous epithelium, while heavily hypermethylated in HT-29 colon adenocarcinoma cell line. Thymine peaks shows high values in samples from normal mucosa (N2) referring to the bisulfite conversion of the non-methylated cytosines. Higher cytosine values were found in the tumorous sample (T2), especially in the 2nd, 8th, 9th, 14th, 15th and 23rd CpG islands. During the bisulfite sequencing of the HT-29 sample only non-converted cytosines could be detected which implies strong methylation. With the HRM analysis of PTGDR gene promoter, the normal and the tumorous samples could be discriminated.

5. Analysis of the molecular mechanism of COX-2 inhibition

- Using PAM method, I have searched genes whose expression consequently changes during the disease development, thus according to the expression pattern of these genes the normal and adenoma, furthermore the normal and carcinoma stages could be discriminated. Between healthy and adenoma 20 discriminatory genes were identified, while the normal and CRC samples could be separated using 38 classifier transcripts.

- The expression of 17 of 20 adenoma-normal genes changed oppositely in HT-29 colon adenocarcinoma cells under NS398 COX-2 inhibitor treatment. In case of 14 genes (such as overexpressed somatostatin, claudin 8, YY peptid and downregulated cadherin 3, KIAA1199) the expression changes were found to be significant.
- Twelve of the 38 CRC-markers (like carbonic anhydrase 7, interleukin 8, melanoma cell adhesion molecule) expressed reversely under NS398 treatment.

a. Analysis of COX-2 protein expression

- Dose-dependent inhibition of COX2 protein expression was observed under NS398 treatment. COX2-positive cell/total cell ratio was 80,5% in untreated control samples, while it decreased to 77,0% under 10μM, to 61,2% under 25μM NS398 treatment. The further elevation of the NS398 dose (100μM) caused significant decrease in positive cell ratio (53,1%). Strong granular and/or diffuse cytoplasmatic immunostaining was detected in COX2-positive cells. Western blot results showed considerable reduction in COX2 protein expression after 96h NS398 treatment at 50μM and 100μM concentrations. The results of protein level analyses implies that NS398 exerts their effect through the inhibition of COX-2 protein production.

b. Analysis of tissue-specific expression

- In the LCM samples, 65% of the adenoma-related gene expression changes originated from epithelial cells, while 53% of CRC-related markers were epithelium-derived.

CONCLUSIONS

The molecular biological results of my PhD research suggest that colon tumors can be considered as systemic rather than local alterations. The tumor during their development and progression influences the surrounding cells, organs and organ systems.

Using high density oligonucleotide microarray technology and hereinafter NGS (next generation sequencing)-based transcriptome sequencing, mRNA molecules can be identified which have a diagnostic value. These techniques offer opportunities not only for automated diagnostic utilization and molecular pathological classification enhancing the histological diagnosis, but for prediction of therapeutic response and for realization of the individualized therapeutic possibilities.

The antibody microarray technology as a potentially high-throughput method can be suitable for identification of protein biomarkers and diagnostic patterns. Identification of marker combinations and their utilization in the daily diagnostic routine procedure is an important issue, where the antibody microarrays can be a great leap forward.

The development of microarray technology allowed that properly evaluable whole genomic microarray analyses could be performed even from rare cell samples. This plays a remarkable role in the understanding of the background of the genetic diseases. The microarray technology combined with LCM makes the genome-wide expression profiling of different histological regions possible. Thus it becomes definable which molecular processes in which histological regions are typical. Expression patterns from homogeneous enriched cell populations can have an important role in diagnostics, as well. The present limit is several thousand cells. Although it is small enough to characterize the instantaneous state of a histological region, the real-breakthrough would be the realization of whole genomic expression analysis of a single cell. The current technical limits are the isolation and the amplification techniques which will be expectedly solved by application of next generation sequencing methods.

Gene expression analysis provides a possibility for exploration of epigenetic changes such as DNA methylation. The degree of DNA methylation can be determined by bisulfite sequencing and HRM analysis both in normal and tumorous tissues. In cell culture almost completely methylated regions can be determined, while in the tumor tissue not such strong, but 20-25% hyper methylation can be detected. The reasons of this can be the unknown tumor/normal cell ratios in the tissues and the difference of the methylation extent of the epithelium and the stroma. Further experiments are required for studying this phenomenon, as the laser microdissected samples – because of quantitative limits - cannot be applied for bisulfite sequencing yet.

Using biopsy samples, the genes with altered expression during the colorectal adenoma-dysplasia-carcinoma sequence progression can be identified.

Among them there are such genes whose expression can be reversed, namely their expression can be modified to the normal level. Identification of further, adenoma-carcinoma transition related genes is still an important diagnostic issue, furthermore development and testing of new agents is also a major objective in therapeutic respect.

THE MOST IMPORTANT NEW STATEMENTS

 gene expression patterns of surgical colonic samples and laser microdissected histological regions from fresh frozen sections can be analyzed, determined and compared using whole genomic oligonucleotide microarrays. The microarray analyses of surgical and laser microdissected samples fulfill the Affymetrix quality requirements, and are highly standard, and reproducible.

 diagnostic and prognostic protein patterns can be determined using antibody microarray technology whose discriminatory power can be confirmed also by TMA immunohistochemistry.

 according to the results of oligonucleotide and antibody microarray analyses, correlation can be detected between protein and mRNA levels in colon tumors which can be validated by other methods (RT-PCR, TMA).

, the results of the microarray analyses can be validated by other methods both at protein (TMA) and mRNA (RT-PCR) levels.

 due to the gene expression profiling results, altered biological pathways can be identified which are associated with the pathomechanism of colorectal diseases.

 Gene expression changes during colorectal adenoma-dysplasia-carcinoma sequence progression can be determined using laser microdissected samples. The altered expression of these genes can be an indicator of the colorectal carcinogenesis in epithelial and stromal regions.

 with gene expression profiling, the methylation-regulated genes can be identified (PTGDR) using demethylation cell culture model and clinical samples.

 The expression of the genes involved in the adenoma-carcinoma transition can be reversed by NS398 selective COX-2 inhibitor.

International publications in connection with the PhD dissertation

Galamb O, **Spisák S**, Sipos F, Tóth K, Solymosi N, Wichmann B, Krenács T, Valcz G, Tulassay Z, Molnár B. Reversal of gene expression changes in the colorectal normal-adenoma

pathway by NS398 selective COX2 inhibitor. Br J Cancer. 2010;102(4):765-73. **IF: 4,346** (2009)

Spisák S, Galamb B, Sipos F, Galamb O, Wichmann B, Solymosi N, Nemes B, Molnár J, Tulassay Z, Molnár B. Applicability of antibody and mRNA expression microarrays for idetifing diagnostic and progression markers of early and late stage colorectal cancer. Dis Markers, 2010;28(1):1-14. **IF: 2,026** (2009)

Spisák S, Guttman A. Biomedical applications of protein microarrays. Curr Med Chem. 2009;16(22):2806-15. **IF: 4,708**

Galamb O**,** Sipos F, **Spisák S**, Galamb B, Krenács T, Valcz G, Tulassay Z, Molnár B. Potential biomarkers of colorectal adenoma-dysplasia-carcinoma progression: mRNA expression profiling and in situ protein detection on TMAs reveal 15 sequentially upregulated and 2 downregulated genes. Cell Oncol. 2009;31(1):19-29. **IF: 4,169**

Galamb O, Sipos F, Solymosi N, **Spisák S**, Krenács T, Tóth K, Tulassay Z, Molnár B. Diagnostic mRNA expression patterns of inflamed, benign and malignant colorectal biopsy specimen and their correlation with peripheral blood results. Cancer Epidemiol Biomarkers Prev. 2008;17(10):2835-45. **IF: 4,770**

Spisak S, Tulassay Z, Molnar B, Guttman A. Protein microchips in biomedicine and biomarker discovery. Electrophoresis. 2007 ;28(23):4261-73. **IF: 3,609**

Sipos F, Muzes G, Galamb O, **Spisak S**, Krenács T, Toth K, Tulassay Z, Molnar B. The possible role of isolated lymphoid follicles in colonic mucosal repair. Pathol Oncol Res. 2010;16(1):11-8. **IF: 1,152** (2009)

Hungarian publications in connection with the PhD dissertation

Spisák S, Kalmár A, Galamb O, Sipos F, Wichmann B, Molnár B, Tulassay Z. Identification of methylation related genes from laser capture microdissected colon samples during investigation of adenoma-carcinoma sequence Orv Hetil. 2010;151(20):805-14.

Spisák S, Galamb B, Wichmann B, Sipos F, Galamb O, Solymosi N, Nemes B, Tulassay Z, Molnár B. Orv Hetil. Tissue microarray (TMA) validated progression markers in colorectal cancer using antibody microarrays. 2009;150(34):1607-13.

Spisák S, Molnár B, Galamb O, Sipos F, Tulassay Z. Fehérje chipek elméleti alapjai és alkalmazási lehetőségük az orvosi diagnosztikában és kutatásban. Orv Hetil. 2007; 148(32):1511-20.

International publications in other topic

Tóth K, Galamb O, **Spisák S**,Wichmann B, Sipos F, Valcz G, Leiszter K, Molnár B, Tulassay Z. The influence of methylated septin 9 gene on RNA and protein level in colorectal cancer. Pathol Oncol Res. 2010 (nyomtatásban). **IF: 1,152** (2009)

Szoke D, Molnar B, Solymosi N, Racz K, Gergics P, Blasko B, Vasarhelyi B, Vannay A, Mandy Y, Klausz G, Gyulai Z, Galamb O, **Spisak S**, Hutkai B, Somogyi A, Berta K, Szabo A, Tulassay T, Tulassay Z. Polymorphisms of the ApoE, HSD3B1, IL-1beta and p53 genes are associated with the development of early uremic complications in diabetic patients: Results of a DNA resequencing array study. Int J Mol Med. 2009;23(2):217-27. **IF: 1,980**

Galamb O, Győrffy B, Sipos F, **Spisák S**, Németh AM, Miheller P, Tulassay Z, Dinya E, Molnár B. Inflammation, adenoma and cancer: objective classification of colon biopsy specimens with gene expression signature. Dis Markers. 2008;25(1):1-16. **IF: 2,303**

Galamb O**,** Győrffy B, Sipos F, Dinya E, Krenács T, Berczi L, Szőke D, **Spisák S**, Solymosi N, Németh AM, Juhász M, Molnár B, Tulassay Z. Helicobacter pylori and antrum erosion specific gene expression patterns: the discriminative role of CXCL13 and VCAM1 transcripts. Helicobacter. 2008;13(2):112-26. **IF: 2,470**

Galamb O, Sipos F, Molnár B, Szőke D, **Spisák S**, Tulassay Z. Evaluation of malignant and benign gastric biopsy specimens by mRNA expression profile and multivariate statistical methods. Cytometry B Clin Cytom. 2007 Sep;72(5):299-309. **IF: 2,307**

Galamb O, Sipos F, Dinya E, **Spisák S**, Tulassay Z, Molnár B. mRNA expression, functional profiling and multivariate classification of colon biopsy specimen by cDNA overall glass microarray. World J Gastroenterol. 2006;12(43):6998-7006.

Hungarian publications in other topic

Valcz G, Sipos F, Krenács T, **Spisák S**, Tóth K, Molnár B, Tulassay Z. Az őssejtek jellemzése, mozgása és terápiás lehetőségei a humán vastagbélben. Magy. Belorv. Arch. 2009;4:272-278.

Leiszter K, Galamb O, Sipos F, **Spisák S**, Tóth K, Valcz G, Kalmár A, Műzes G, Molnár J, Molnár B, Tulassay Z. Az öregedés jelei az emésztőrendszerben. Magy Belorv Arch. 2010;63:19-24.

Tóth K, Galamb O, **Spisák S**, Wichmann B, Sipos F, Leiszter K, Molnár J, Molnár B, Tulassay Z. Free circulating DNA based colorectal cancer screening from peripheral blood: the possibility of the methylated septin 9 gene marker Orv Hetil. 2009;150(21):969-77.

Galamb O, Sipos F, Dinya E, **Spisák S**, Somorácz A, Molnár B, Tulassay Z. Vastagbél-biopszia funkcionális mRNS-expressziós vizsgálata és osztályozása általános cDNS microarray technikával. Orv Hetil. 2008;149(5):219-32.

Galamb O**,** Győrffy B, Sipos F, **Spisák S**, Németh AM, Miheller P, Dinya E, Molnár B, Tulassay Z. Vastagbél-adenoma, vastagbélrák és IBD-specifikus génexpressziós mintázatok meghatározása teljes genomszintű oligonukleotid microarray-rendszerrel. Orv Hetil. 2007;148(44):2067-79. **Markusovszky díj 2008**

Tóth K, Galamb O, Hevér-Pálfy T, Solymosi N, Miheller P, Müllner K, **Spisák S**, Sipos F, Molnár B, Tulassay Z. Vastagbél betegségben szenvedők perifériás vérének mRNS expressziós vizsgálata. Magy Belorv Arch. 2007; 60:531–539.

Szoke D, Sipos F, **Spisak S**, Molnar B, Tulassay Z. A p53 gén és fehérje 2005-ben: új eredmények, ígéretes lehetőségek. Orv Hetil, 2005;l46(30):1587-94.

ACKNOWLEDGEMENT

Hereby I would like to thank everybody who helped me in my PhD work:

- my program leader, Zsolt Tulassay MD. DSc. and my supervisor, Béla Molnár MD. PhD. for allowing and supporting my PhD work in Semmelweis University 2nd Department of Internal Medicine;
- Orsolya Udvardyné Galamb Ph.D. for professional support and friendship;
- Emese Mihály MD. PhD. and Péter Igaz MD. PhD. opposers for the review of my PhD dissertation;
- Tibor Krenács PhD. for the professional support and for his indispensable help in tissue microarray analyses;
- Lajos Berczi MD. PhD. for his useful advices and directions during the personal consultation;
- Norbert Solymosi DVM. PhD. for the professional implementation of bioinformatic analyses
- Marica Csorba for her help in the laboratory experiments;
- every employee colleagues of the Cell Analysis Laboratory for their help;
- and last, but not least I thank my Family for taking my side every time.