Molecular connections of aging and colorectal carcinogenesis

Ph.D. thesis

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1. INTRODUCTION

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. According to the data of Hungarian National Cancer Registry, recently almost ten thousand new colorectal cancer (CRC) patients were registered in our country per year. The incidence of sporadic CRC is low under the age of 40, and it occurs more frequently in older ages. Many macroscopic and microscopic changes occur in the colorectal epithelium during aging, which can be associated with the development of certain gastrointestinal disorders. Molecular changes of the aging colorectal epithelium can be in connection with the carcinogenesis. However, the alterations of proliferative and apoptotic activity, furthermore the changes in mRNA expression of proliferation- and apoptosis-regulating genes of colorectal epithelium during normal aging and carcinogenesis are less known.

Somatostatin (SST) is a regulatory-inhibitory peptide, and is mainly produced in the central and peripheral nervous system, in the endocrine pancreas and in the gut. SST has endocrine and paracrine/autocrine effects, and can bind to its cell surface G protein-coupled receptors (SSTR1-5) initiating signal transduction pathways. It can inhibit cell growth in normal and neoplastic tissues as well. In the scientific literature only few articles can be found about the alterations of colorectal SST production during normal aging and carcinogenesis. The effects of somatostatin analogue octreotide on cell proliferation and apoptosis are also less well known in human colorectal adenocarcinoma cell lines.

Increased methylation in the promoter region of genes can be a possible molecular link between aging and carcinogenesis. DNA promoter hypermethylation is an epigenetic gene silencing mechanism that regulates gene expression without changing the DNA sequence. There are few data in the literature on how the methylation rate changes in the promoter region of SST encoding gene, and demethylating 5-aza-2-deoxycitidine treatment effects on SST mRNA expression in colon adenocarcinoma cell lines.

2. OBJECTIVES

I had the following objectives during my Ph.D. work:

- to examine the intensity of cell proliferation and apoptosis in colorectal biopsy samples during normal aging and colorectal adenoma-carcinoma sequence, on tissue level;
- to analyse the mRNA expression of apoptosis- and proliferation-regulating genes in colorectal biopsy samples during aging and carcinogenesis;
- to investigate the somatostatin production of colorectal epithelium during normal aging and colorectal carcinogenesis, both on mRNA and protein levels;
- to examine the proliferation and apoptosis regulating effects of somatostatin analogue octreotide on Caco-2 cell line;
- to examine the methylation level in the promoter region of somatostatin encoding gene, furthermore to analyse the gene expression alteration following demethylation treatment on HT-29 cell line.

3. MATERIALS AND METHODS

3.1. Samples

I did my researches in the Cell Analysis Laboratory of the 2nd Department of Internal Medicine. After informed consent, colonic biopsy specimen were taken during routine colonoscopies in the Endoscopy Units of 2nd Department of Internal Medicine and 1st Department of Paediatrics, Semmelweis University. Six histologically intact juvenile samples, 41 normal adult samples and 34 colorectal cancer (CRC) samples were used for Affymetrix microarray analysis. The microarray results were confirmed using Taqman real-time RT-PCR on juvenile (n=6), adult (n=6) and tumorous samples (n=6). PCR validation was performed on original and independent sets of samples. Tissue sections made from diagnostic paraffin blocks (28 normal juvenile, 30 normal adult, 10 colorectal adenoma and 33 CRC) were used for immunohistochemical analysis. Methylation array analysis was performed on colorectal biopsy samples from healthy children (n=5), healthy adults (n=5) and CRC (n=9). The samples taken for RNA isolation were placed into RNAlater Stabilization Reagent and stored at –80°C until the RNA extraction. Immunohistochemical investigations were implemented on paraffin embedded tissue microarray (TMA) slides and biopsy specimen were taken during routine, diagnostic colonoscopies. The number of samples according to their histologic stages (female/male) used in different experiments can be seen in the table.

	Children (Ch)	**Adult (N)**	**Adenoma (Ad)**	**CRC**	**Totally**
Affymetrix microarray (F/M)	6 (2/4)	41 (26/15)	-	34 (19/15)	**81 (47/34)**
Taqman RT-PCR – Original samples (F/M)	6 (2/4)	6 (3/3)	-	6 (4/2)	**18 (9/9)**
Taqman RT-PCR – Independent samples (F/N)	6 (3/3)	6 (3/3)	-	6 (1/5)	**18 (7/11)**
Ki-67 / TUNEL immunohistochemistry	14 (7/7)	10 (4/6)	10 (4/6)	10 (3/7)	**44 (18/26)**
Somatostatin immunohistochemistry	14 (7/7)	20 (11/9)	-	23 (13/10)	**57 (31/26)**
Methylation array analysis (F/M)	5 (2/3)	5 (2/3)	-	9 (5/4)	**19 (9/10)**

3.2. Microarray analysis

Total RNA was isolated from colonic biopsy samples. Following the quantity and quality control of isolated RNA biotinylated cRNA probes were synthesized. After the fragmentation, ten micrograms of each fragmented cRNA sample was hybridized into HGU133 Plus2.0 array (Affymetrix). The microarrays were washed then stained with antibody amplification staining method, according to the manufacturer's instructions. The fluorescent signals were detected using GeneChip 3000 scanner. For the statistical evaluation of microarray data, the Affymetrix expression arrays were primarily pre-processed by GCRMA background correction method. SAM analysis was applied for determination of proliferation- and apoptosis-regulating genes with altering mRNA expression. The following SAM criteria were used: LogFC≥abs 1, p-value<0.05. The expression of each selected genes with different expression among the sample groups was further analysed by ANOVA and post-test Tukey HSD. The datasets are available in the Gene Expression Omnibus databank (http://www.ncbi.nlm.nih.gov/geo/), series accession numbers: GSE10714, GSE37364 and GSE37267.

3.3. RT-PCR validation

Knowing the microarray data, 10 selected genes (*CDKN2B, MKI67, CDC2/CDK1, CCNE1, ACVR1B, TNFSF10, DYRK2, SOCS3, IFI6* and *SERPINB9*) with altering mRNA expression between the examined sample groups were further investigated for verification using polymerase chain reaction (PCR). Twelve samples were tested (6 dependent samples and 6 independent samples) in each sample groups (children, normal adult, CRC). For statistical analysis ANOVA test and Tukey HSD post-test were applied. The following criteria were used: Fold change≤0.5 or Fold change≥2 and p-value<0.05.

3.4. Protein level analysis

For detection of proliferating cells Ki-67 immunohistochemistry was used. Apoptosis was examined with TdT-mediated dUTP Nick End Labeling (TUNEL) method. Nuclei of Ki-67 and TUNEL negative cells were stained with Hoechst.

The somatostatin producing cells were detected with rabbit, anti-human polyclonal antibody. Following the signal conversion, hematoxylin co-staining was carried out.

Stained biopsy samples and tissue microarray (TMA) slides were digitalized with a high resolution digital scanner. Digital slides were accessed through a computer monitor

and analysed using the Pannoramic Viewer software. The Marker Counter software module resulting in permanent annotations on the counted cells was used to count the epithelial cells. Knowing the exact number of proliferating cells (Ki-67 positive /red/), apoptotic cells (TUNEL positive /green/), normal cells (Hoechst or hematoxylin positive /blue/) and somatostatin producing cells (brown), we have determined the proliferative-apoptotic ratio (PAR: ratio of proliferative and apoptotic cells in crypts), the mitotic index (MI: the ratio of proliferative cells and total counted cells in crypts), the apoptotic index (AI: the ratio of apoptotic cells and total counted cells in crypts) and the ratio of somatostatin producing cells.

For statistical analysis of Ki-67, TUNEL and SST immunostaining results, ANOVA test and Tukey HSD post-test were applied. In both methods significance criteria was $p<0.05$.

3.5. Methylation array analysis

DNA extraction was performed on colonic biopsy samples. The methylation analysis was carried out using Methyl-Profiler DNA methylation PCR array method (Qiagen), investigating 96 genes, which can be characterised with altering methylation profile in gastrointestinal tumours. The results of methylation array analysis were evaluated by ANOVA and Tukey HSD post-test.

3.6. Cell culture experiments

Caco-2 human epithelial adenocarcinoma cells were grown to confluence in MEM medium, supplemented with Fetal Bovine Serum (FBS) and gentamycin. Caco-2 cells were settled to a 24-well treatment plate. After 24 hours medium was changed: MEM was added with gentamycin and with 0.1, 1.0, 2.5, 5.0 and 10.0 nmol/l of octreotide without FBS. After 24, 48 and 72 hour treatment the cells were harvested and stored at -20ºC. Flow cytometry analysis was used to determine the ratio of apoptotic cells in Sub-G1 phase and other cells in the active cell cycle (G1+S+G2+M). Mann-Whitney test was used to compare the proportion of Caco-2 cells in different cell cycle stages (Sub-G1, G1, S, G2 and M) of octreotide-treated groups and in the control group. $p<0.05$ was considered to be statistically significant.

The mRNA expression alteration of somatostatin encoding gene was analysed on HT-29 colon adenocarcinoma cell line after 5-aza-2-deoxycitidine (5-Aza-dC)

demethylation treatment. HT-29 cells were cultured for 24 hours in cell culture flask, then were treated with demethylating agent 5-aza-2-deoxycitidine for 72 hours. Total RNA was extracted from the treated HT-29 cells, then the quantity and quality of isolated nucleic acid were tested. Single round in vitro transcription was performed by using the One-Cycle Target Labeling and Control Kit for amplification and labeling of the transcripts. The samples were hybridized on HGU133 Plus2.0 arrays, and the microarrays were washed and stained using an antibody-based signal amplification method. Fluorescent signals were detected with GeneChip Scanner 3000. The datasets are available in the Gene Expression Omnibus databank (series accession number: GSE29060). Student t-test was performed for comparison of 5-Aza-dC-treated and control samples. The significance criteria was $p<0.05$.

4. RESULTS

4.1. The alterations of proliferative and apoptotic activity of colorectal epithelium during aging and carcinogenesis

Proliferative-apoptotic ratio (PAR) and mitotic index (MI) were significantly higher in children (PAR=3.51±2.49; MI=0.33±0.06) and CRC samples (PAR=9.83±7.72; MI=0.42±0.10) than in healthy adult samples (PAR=0.88±0.22; MI=0.15±0.06) ($p<0.05$). They showed continuous increase in the course of adenoma-carcinoma sequence (ACS). The highest PAR and MI were found in colorectal cancer samples. Apoptotic index (AI) was decreased in healthy children samples (0.13±0.06) and significantly lower in colorectal cancer samples (0.06±0.03) than in the histologically intact adult colonic samples (0.17±0.05) ($p<0.05$). AI showed continuous decrease in parallel with the colorectal ACS. The lowest AI was detected in colorectal cancer samples. Significant alteration in proliferative activity was not found between healthy adult colonic mucosa (PAR=0.88±0.22; MI=0.15±0.06) and adenoma samples (PAR=1.45±0.89; MI=0.13±0.05); while the AI was found to be significantly lower in adenoma samples (Normal AI= 0.17±0.05; Adenoma AI=0.10±0.04) ($p<0.05$).

4.2. mRNA expression analysis of proliferation and apoptosis regulating genes in colorectal biopsy samples during aging and carcinogenesis

mRNA expression of 117 proliferation-regulating genes were studied using HGU133 Plus2.0 microarrays. Gene expression of 4 genes altered in the course of aging alone in histologically intact colonic mucosa; mRNA expression of 13 genes were altered during colorectal carcinogenesis; and 8 genes (*BRCA1, CCNB1, CCNE1, CDC20, CDK1, CDKN2B, MKI67* and *TFDP1*) were differently expressed in both processes. Similarly, gene expression of 534 apoptosis-regulating genes was also analysed in this study. mRNA expression of 9 genes altered in the course of aging alone in histologically intact colonic mucosa; gene expression of 32 genes showed changes during colorectal carcinogenesis; and 11 genes (*ACVR1B, BRCA1, CHEK2, DYRK2, IFI6, SERPINB9, SFRP1, SOCS3, SST, TNFSF10* and *ZAK*) were differently expressed in both processes.

4.3. mRNA expression alterations of proliferation and apoptosis regulating genes between juvenile and CRC samples

Increased cell proliferation was detected in juvenile and tumorous samples as compared healthy adult samples on tissue level. However, the increased cell proliferation is controlled and well-balanced in children, while it is uncontrolled in CRC. Therefore proliferation- and apoptosis-regulating genes were further investigated to find genes with dissimilar mRNA expression that can explain the cell kinetic differences between juvenile and tumorous samples. Eight proliferation-controlling genes (*BCL2, CDKN2B, RAD9A, BRCA2, CCND1, CDK1, CDK6* and *RBL1*) and 26 apoptosis-regulating genes (*AIFM2, AIFM3, BTK, CIDEB, CIDEC, DAPK2, MAL, NLRP1 (LOC728392), SFRP1, SIVA1, SPN, SST, TR53I3, TNFRSF25, ANXA1, CBX4, CASP4, INHBA, MYC, PLAGL2, PMAIP1, POLB, PROK2, SOCS3, TNFRSF10B* and *ZAK*) showed significant alteration between children and cancer groups, according to the p-value.

4.4. Validation of microarray gene expression data using real-time polymerase chain reaction

mRNA expression of 10 selected proliferation- and apoptosis-regulating genes (*CDKN2B, MKI67, CDC2/CDK1, CCNE1, ACVR1B, TNFSF10, DYRK2, SOCS3, IFI6* and *SERPINB9*) were further investigated using Taqman RT-PCR method. The mRNA expression of these genes showed differences between sample groups in microarray study. PCR validation confirmed the tendency of gene expression alterations in all cases with respect to proliferation regulation. *CDKN2B, MKI67, CDC2/CDK1* and *CCNE1* showed borderline significant mRNA expression changes in Children vs. Normal and Normal vs. CRC comparisons, according to Fold change. Tukey post-test recruited gene expression alterations during aging and colorectal carcinogenesis in case of *CDC2/CDK1* ($p<0.05$). PCR validation confirmed the tendency of mRNA expression alterations of five apoptosis-regulating genes (*TNFSF10, DYRK2, SOCS3, IFI6* and *SERPINB9*) between Children vs. Adult Normal and Adult Normal vs. CRC. Four genes (*TNFSF10, DYRK2, SOCS3* and *IFI6*) showed statistically significant ($p<0.05$) mRNA expression differences in the above-mentioned comparisons.

4.5. Investigation of colorectal somatostatin production during aging and colorectal carcinogenesis, on mRNA and protein levels

Affymetrix microarray method was firstly used for mRNA expression analysis of somatostatin encoding gene. *SST* expression was investigated in colonirectal biopsy samples from children, adults and CRCs. Based on the results of microarray study, mRNA expression of *SST* did not alter during normal aging as compared healthy juvenile and adult samples, however, gene expression significantly decreased in colorectal cancer ($p<0.05$) compared to normal adult samples.

mRNA expression of *SST* was further investigated for verification using polymerase chain reaction (PCR). Real-time PCR validation on original, independent and combined sets of samples verified the significantly reduced SST expression in CRC ($p<0.05$), however, the *SST* production does not alter significantly during normal aging in healthy samples.

In the immunohistochemical study I have determined the ratio of somatostatin producing and non-producing cells in healthy young, adult and tumorous colonic epithelium. SST protein expression data were in correlation with SST mRNA expression data from both microarray and real-time PCR analysis. Immunohistochemical analysis confirmed the nearly absent somatostatin production in colorectal carcinoma samples as compared to young and adult healthy colonic mucosa on protein level ($p<0.05$).

4.6. Investigation of proliferation- and apoptosis-regulating effects of somatostatin analogue octreotide on Caco-2 cells

The effects of effects of somatostatin analogue octreotide on cell proliferation and apoptosis was investigated on Caco-2 colorectal adenocarcinoma cell line. Octreotide was added to Caco-2 cells in different concentrations (0.1 nmol/l, 1.0 nmol/l, 2.5 nmol/l, 5.0 nmol/l and 10.0 nmol/l). The proportion of apoptotic cells (Sub-G1 phase) and cells in other cell cycle phases (G1+S+G2+M) was determined using flow cytometry 24, 48 and 72 hours after octreotide treatment. In cases of somatostatin analogue treatment at higher concentrations than 0.1 nmol/l, the proportion of apoptotic Sub-G1 fractions was significantly higher ($p<0.05$) than it was in the control group, while the proportion of cells in other cell cycle phases (G1+S+G2+M) was significantly lower. The highest apoptotic fraction (Sub-G1) and the lowest G1+S+G2+M population were measured at 5.0 nmol/l octreotide concentration. The results of Caco-2 cell culture experiment showed that

somatostatin analogue octreotide significantly increased the proportion of apoptotic cells and significantly decreased the proportion of cells in G1, S, G2 and M phase, in a concentration-dependent manner.

4.7. Analysis of *SST* promoter methylation and its reversibility

Methylation status in the promoter region of *SST* encoding gene was examined using methylation array analysis in histologically intact juvenile, adult and CRC samples. Promoter DNA methylation of somatostatin gene shows continuous increase during normal aging and carcinogenesis. The lowest *SST* promoter methylation was found in juvenile colonic epithelium which can be characterized by controlled, increased cell proliferation. However, in colorectal cancer wherein increased cell growth is dysregulated, SST methylation was significantly higher ($p<0.05$). The highest methylation status was detected in CRC.

Reversibility of *SST* promoter methylation was investigated after 5-aza-2-deoxycitidine demethylation treatment in HT-29 colon adenocarcinoma cell culture. Moderate *SST* expression differences were found between 5-Aza-dC-treated and control HT-29 cells. Mild somatostatin mRNA elevation was detected in demethylating agent-treated cells compared to the controls.

5. CONCLUSIONS

In the first part of my Ph.D. work I have analysed the proliferative and apoptotic activity of colorectal epithelium during normal aging and colorectal carcinogenesis. I have investigated on mRNA and protein levels the above-mentioned cell kinetic parameters in colorectal biopsy samples from children, adults and colorectal cancers. We found elevated proliferative and decreased apoptotic activities both in histologically intact children colonic mucosa and CRC samples compared to healthy adult samples, where cancer showed the significantly largest cell kinetic alterations. Physiological growth and development may result in an increased and controlled proliferative activity in children's colorectal epithelium which is a well-balanced process compared to the uncontrolled cellular proliferation in CRC. Our results suggest that significant mRNA expression alterations of certain cell proliferation- and apoptosis-regulating genes (e.g. *CDK1, CDK6, CCND1, CDKN2B, SFRP1, SOCS3* and *SST*) in juvenile and tumorous samples can cause the observed cell kinetic differences.

Somatostatin is one of the most important naturally occurring anti-proliferative hormone. I compared the somatostatin expression in colorectal epithelium of healthy children and adults to that found in colorectal cancer samples both at mRNA and protein levels. Our results show that somatostatin production does not alter significantly during normal aging, but it is nearly absent in CRC. Significantly reduced epithelial somatostatin production may contribute to the accelerated and deregulated cell proliferation in CRC. This was supported by the inhibition of cancer cell growth along with the induction of apoptosis upon somatostatin analogue octreotide treatment. Reduced somatostatin levels were associated with promoter hypermethylation of *SST* gene as a potential explanation for the missing hormone in CRC. Further investigations are needed to test somatostatin analogues and demethylating compounds as potential therapeutic agents against sporadic colorectal cancer.

6. LIST OF PUBLICATIONS

6.1. Publications directly related to the Ph.D. thesis:

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

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