Sporadic Colorectal Cancer Development Shows Rejuvenescence Regarding Epithelial Proliferation and Apoptosis

Katalin Leiszter1,2, Orsolya Galamb1,2, Ferenc Sipos1, Tibor Krenács2, Gábor Veres4, Barnabás Wichmann1, Alexandra Kalmár1, Árpád V. Patai1, Kinga Tóth1, Gábor Valcz1, Béla Molnár1,2, Zsolt Tulassay1,2

1 2nd Department of Internal Medicine, Semmelweis University, Budapest, Hungary, 2 Molecular Medicine Research Unit, Hungarian Academy of Sciences, Budapest, Hungary, 3 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary, 4 1st Department of Pediatrics, Semmelweis University, Budapest, Hungary

Abstract

Background and Aims: Sporadic colorectal cancer (CRC) development is a sequential process showing age-dependency, uncontrolled epithelial proliferation and decreased apoptosis. During juvenile growth cellular proliferation and apoptosis are well balanced, which may be perturbed upon aging. Our aim was to correlate proliferative and apoptotic activities in aging human colonic epithelium and colorectal cancer. We also tested the underlying molecular biology concerning the proliferation- and apoptosis-regulating gene expression alterations.

Materials and Methods: Colorectal biopsies from healthy children (n1 = 14), healthy adults (n1 = 10), adult adenomas (n3 = 10) and CRCs (n4 = 10) in adults were tested for Ki-67 immunohistochemistry and TUNEL apoptosis assay. Mitosis- and apoptosis-related gene expression was also studied in healthy children (n1 = 6), adult (n2 = 41) samples and in CRC (n3 = 34) in HGU133plus2.0 microarray platform. Measured alterations were confirmed with RT-PCR both on dependent and independent sample sets (n1 = 6, n2 = 6, n3 = 6).

Results: Mitotic index (MI) was significantly higher (p<0.05) in intact juvenile (MI = 0.33±0.06) and CRC samples (MI = 0.42±0.10) compared to healthy adult samples (MI = 0.15±0.06). In contrast, apoptotic index (AI) was decreased in children (0.13±0.06) and significantly lower in cancer (0.06±0.03) compared to healthy adult samples (0.17±0.05). Eight proliferation- (e.g. MKI67, CCNE1) and 11 apoptosis-associated genes (e.g. TNFSF10, IFI6) had altered mRNA expression both in the course of normal aging and carcinogenesis, mainly inducing proliferation and reducing apoptosis compared to healthy adults. Eight proliferation-associated genes including CCND1, CDK1, CDK6 and 26 apoptosis-regulating genes (e.g. SOCS3) were differently expressed between juvenile and cancer groups mostly supporting the pronounced cell growth in CRC.

Conclusion: Colorectal samples from children and CRC patients can be characterized by similarly increased proliferative and decreased apoptotic activities compared to healthy colonic samples from adults. Therefore, cell kinetic alterations during colorectal cancer development show uncontrolled rejuvenescence as opposed to the controlled cell growth in juvenile colonic epithelium.

Introduction

The normal cell renewal of the healthy colonic epithelium takes about 5 days, through cell proliferation, differentiation and apoptosis. The location of different types of epithelial cells in the crypts is very typical. Proliferating cells mostly reside on the basis of the crypts, differentiated cells in the middle zone, while the apoptotic cells are close to the luminal surface. Several molecular pathways play important role in the physiological self-renewal of epithelial cells in the human large intestine [1–4].

However, the regulation of proliferative and apoptotic mechanisms may alter during normal aging and colorectal carcinogenesis. Aging is a physiologic mechanism that begins after conception. It affects almost every cell in the organism with the possible exception of stem cells and tumor cells. Macroscopic and microscopic changes are present in the aging gastrointestinal tract including the large bowel [5–6]. Changes in proliferative activity
in the course of aging have been analyzed in animal models in previous studies [7–9]. According to the results of Mandir et al., the most intensive epithelial regeneration (proliferation and apoptosis) has been proven in young rats; and the authors concluded that dramatic changes in young colonic epithelium can be related to intestinal development. However, the normal gastrointestinal aging has no effect on epithelial renewal [7]. On the contrary, in aged animal populations increased cell proliferation and decreased apoptosis in colonic epithelium was observed and thought to facilitate uncontrolled cell proliferation and tumorigenesis, which might explain the higher incidence of colorectal cancer in the elderly human [8].

Furthermore, some of these alterations may also be related to colorectal carcinogenesis. Changes in epithelial cell growth and programmed cell death were also previously studied in different stages of colorectal carcinogenesis, but the results are not concordant. Cell proliferation and apoptosis may become dysregulated and the unbalanced cell production and cell loss determine the behavior of premalignant or malignant disorders and tumor growth [9–12].

The detection rate of adenomas and the incidence of advanced colorectal adenomas and cancers continually elevate after the age of 40–50, showing strong age dependency [13–14]. Sporadic colon cancers turn out mostly in the older adult population similarly to numerous neoplastic and precancerous lesions. According to the Vogelstein model [15], colorectal cancer develops from normal epithelium through pre-malignant adenoma in a multi-step process which takes several years. Besides the genes (e.g. APC, KRAS, DCC and TP53) traditionally implicated in this cancer progression model, recently novel genes with altering mRNA expression have also been suggested to be contribute to malignant transformation of colorectal epithelium [16–18].

There are several genetic and epigenetic alterations that can demonstrate a possible relationship between aging and colorectal carcinogenesis. Accumulation of DNA mutations and damages, promoter hypermethylation, alterations in DNA repair, telomerase activity and cellular metabolism may increasingly affect aged populations leading to elevated cell proliferation and decreased apoptosis, which may culminate to malignant transformation and uncontrolled cell proliferation [19].

At the same time, several human and animal studies have revealed the opposing regulation of some molecular pathways during normal aging and carcinogenesis. As opposed to normal aging, proliferating cancer cells show increased metabolism, characterized by continuous proliferative activity and de-differentiation, they can produce embryonic proteins and are potentially immortal by escaping apoptosis [20]. In particular, apoptosis-regulating proteins show distinct expression in senescent and cancer cells featured by the downregulation of the apoptosis-inducing tumor suppressor p53 protein [21] and Fas/CD95 protein [22] and the overexpression of antiapoptotic proto-oncogene Bcl-2 in cancer as opposed to normal aging cells [23–25]. Oncogenes such as Ras, transcription factors e.g. Myc, and growth signal transduction-related tyrosine-kinase receptors e.g. members of the EGFR family are up-regulated in some cancers, while downregulated in senescent cells [26–28].

Cancer development can be considered as a local, uncontrolled “rejuvenation” utilizing the same molecular pathways but with opposing regulation. Deregulated cell proliferation and apoptosis pathways can allow survival advantages for cancer cells against adjacent senescent cells, due to lost ability of cancer for normal aging [20]. Increased epithelial cell proliferation in the gastrointestinal tract can be seen not only in colorectal cancer, but also in embryonic and juvenile development. An essential pathway that plays fundamental roles both in gut development and in sporadic or familial colorectal cancers is the Wnt/β-catenin signaling [29–30].

As we are aware, there is no study focusing on the proliferation and apoptosis regulation in the juvenile human colorectal epithelium in relation to normal aging and carcinogenesis. The purpose of this study was to analyze the proliferative and apoptotic activity in human colonic epithelium in the course of normal aging and colorectal carcinogenesis both at protein and gene expression level. Colorectal biopsies representing the juvenile controlled growth stage, the adult healthy status and the uncontrolled colorectal cancer development were tested for potential correlations.

Materials and Methods

Patients and samples

After informed consent, colorectal biopsy samples were taken during routine endoscopic intervention at the 2nd Department of Internal Medicine and 1st Department of Paediatrics, Semmelweis University, Budapest, Hungary. Altogether, 44 tissue samples were analyzed in the immunohistochemical study (14 healthy children, 10 healthy adults, 10 adenomas from adults and 10 CRCs from adults); 81 biopsy samples were analyzed in microarray analysis (6 healthy children, 41 healthy adults and 36 CRCs from adults); and 36 biopsy samples were involved in real-time PCR validation (12 healthy children, 12 healthy adults and 12 CRCs from adults). Seventy-five microarrays (the adult samples) had been hybridized earlier; their data files were used in a previously published studies using different comparisons [31–32] and are available in the Gene Expression Omnibus database (series accession number: GSE10714 and GSE37564). The datasets of the newly hybridized 6 microarrays are registered on the GSE37267 serial accession number. Control children were referred to the outpatient clinic with rectal bleeding, constipation or chronic abdominal pain. Ileocolonoscopy was part of their diagnostic procedure (exclude organic disease) and the biopsy specimens showed normal macroscopic appearance and histology. Every specimen was verified by histopathologists.

For immunohistochemistry colorectal biopsy samples were routinely fixed in formaldehyde and embedded in paraffin wax. For mRNA studies, colonoscopy samples were stored in RNALater Reagent (Qiagen Inc, Germantown, US) at −80°C before total RNA extraction for gene expression analysis and Taqman RT-PCR study. Ethical approvals (Nr.: 69/2008 and 202/2009) for this study were issued by the Regional and Institutional Committee of Science and Research Ethics of Semmelweis University (Budapest, Hungary). Written informed consent was obtained in advance from all adult participants and from the next of kin, caretakers, or guardians on the behalf of the minors/children approved by the ethics committees. Detailed clinicopathological specification of the patient samples are summarized in Table 1 and Table 2.

Immunohistochemistry for cell proliferation

Archived samples from 14 histologically normal colonic biopsies from children, 10 normal colonic biopsies from adults, as well as 10 adult adenomas and 10 CRCs were collected during routine endoscopy. 1 mm-diameter cores were punched out from paraffin blocks of adult samples and collected into tissue microarrays (TMA). Four µm thick sections cut both from the TMAs and from individual children biopsy samples were dewaxed and rehydrated for immunostaining.
After antigen retrieval in a pH 9.0 TRIS-EDTA buffer using a microwave oven at 900 W for 10 min and then at 370 W for 40 min, blocking of endogenous peroxidases in 1% hydrogen peroxide dissolved in methanol and of nonspecific binding sites using 1% bovine serum albumin for 20 min each were performed. Slides were incubated with mouse monoclonal anti-Ki-67 antibody (Clone: MIB-1, 1:100, Dako, Glostrup, Denmark) for 60 min in a humidified chamber and then with anti-mouse IgG F(ab\(^9\))\(_2\) Alexa Fluor 546 conjugate (1:200, Invitrogen, Carlsbad, CA, USA) for 30 min.

Apoptosis detection using the Tdt-mediated dUTP Nick End Labeling (TUNEL) assay

After digestion with proteinase-K (20 \(\mu\)g/ml, 20 min), 50 \(\mu\)l TUNEL (TUNEL In Situ Cell Death Detection Kit, Fluorescein, Roche, 11684795910) reaction mixture (5 \(\mu\)l TdT enzyme+45 \(\mu\)l dUTP) was added to the tissue sections and TMA slides. Then the samples were incubated in a dark humidified chamber at 37°C for 120 minutes. Cell nuclei were stained with Hoechst (5 \(\mu\)l Hoechst dye+10 ml TBS, 1 min, Sigma-Aldrich, St. Louis, Mo, USA).

Counting of mitotic and apoptotic index in digital slides

Stained biopsy samples and TMA slides were digitalized with a high resolution digital scanner (Pannoramic Scan, 3DHISTECH Ltd. Budapest, Hungary) using multilayer fluorescent scanning with a high numeric aperture (0.8)×20 objective lens and a high dynamic range AxioCam Mrm Rev.3 black-and-white camera connected to the scanner. Digital slides were accessed through a computer monitor and analyzed using the Pannoramic Viewer software (version 1.11.43.0). The Marker Counter software module resulting in permanent annotations on the counted cells was used to estimate the relative ratio of proliferative, apoptotic and normal cells.

Ki-67, TUNEL and Hoechst positivities appeared all as strong nuclear labeling in the slides. Depending on sample size, 500–1000 epithelial cells were counted in longitudinal well-oriented crypts. 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) and the apoptotic index (AI: the ratio of apoptotic cells and total counted cells in crypts).

mRNA microarray expression analysis

Total RNA was extracted using RNeasy Mini Kit (Qiagen Inc., Germantown, USA) according to the manufacturer’s instructions. Quantity of isolated RNA was characterized by measuring absorbance (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., Wilmington, USA). Quality of isolated RNA was tested with capillary gel electrophoresis (2100Bioanalyzer and
Biotinylated cRNA probes were synthesized from 1 to 8 mg total RNA and fragmented using the One-Cycle Target Labeling and Control Kit (http://www.affymetrix.com/support/downloads/manuals/expression_s2_manual.pdf), according to the Affymetrix description. Twenty micrograms of each fragmented cRNA sample was hybridized into HGU133 Plus2.0 array (Affymetrix) at 45°C for 16 h. Slides were washed and stained using Fluidics Station 450 and antibody amplification staining method according to the manufacturer's instructions. Fluorescent signals were detected by GeneChip Scanner 3000 (Affymetrix).

After microarray gene expression analysis, results were compared with other data set available in the Gene Expression Omnibus databank (http://www.ncbi.nlm.nih.gov/geo; series accession number is GSE18105). TaqMan polymerase chain reaction was used to measure mRNA expression of 12 selected genes, participating in regulation of cell cycle, proliferation or apoptosis, on original (6 histologically intact children, 6 histologically intact adult and 6 CRC adult samples) and independent sets of samples (6 histologically intact children, 6 histologically intact adult, 6 CRC adult samples). 18S ribosomal RNA and GAPDH were used as reference.

Clinical features of healthy children controls participating in immunohistochemistry, microarray analysis and PCR validation.

| Patient number | Signs/Symptoms | Colonoscopy | Histology | Diagnosis |
|----------------|----------------|-------------|-----------|-----------|
| 1.             | Abdominal pain | Negative    | Negative  | WD        |
| 2.             | Hematochezia   | Negative    | Negative  | WD        |
| 3.             | Hematochezia   | Negative    | Negative  | Constipation |
| 4.             | Abdominal pain | Negative    | Negative  | IBS       |
| 5.             | Hematochezia   | Negative    | Negative  | WD        |
| 6.             | Abdominal pain | Negative    | Negative  | IBS       |
| 7.             | Abdominal pain | Negative    | Negative  | IBS       |
| 8.             | Chronic diarrhea | Negative | Negative  | IBS       |
| 9.             | Hematochezia   | Negative    | Negative  | WD        |
| 10.            | Chronic diarrhea | Negative | Negative  | IBS       |
| 11.            | Chronic diarrhea | Negative | Negative  | IBS       |
| 12.            | Hematochezia   | Negative    | Negative  | Constipation |
| 13.            | Abdominal pain | Negative    | Negative  | WD        |
| 14.            | Hematochezia   | Negative    | Negative  | Constipation |

Affymetrix microarray analysis/Taqman RT-PCR validation - original samples

| Patient number | Signs/Symptoms | Colonoscopy | Histology | Diagnosis |
|----------------|----------------|-------------|-----------|-----------|
| 1.             | Hematochezia   | Negative    | Negative  | WD        |
| 2.             | Hematochezia   | Negative    | Negative  | WD        |
| 3.             | Abdominal pain | Negative    | Negative  | WD        |
| 4.             | Vomiting       | Negative    | Negative  | IBS       |
| 5.             | Abdominal pain | Negative    | Negative  | IBS       |
| 6.             | Abdominal pain | Negative    | Negative  | WD        |

Taqman RT-PCR validation - independent samples

| Patient number | Signs/Symptoms | Colonoscopy | Histology | Diagnosis |
|----------------|----------------|-------------|-----------|-----------|
| 1.             | Hematochezia   | Negative    | Negative  | WD        |
| 2.             | Hematochezia   | Negative    | Negative  | WD        |
| 3.             | Abdominal pain | Negative    | Negative  | IBS       |
| 4.             | Hematochezia   | Negative    | Negative  | WD        |
| 5.             | Constipation   | Negative    | Negative  | IBS       |
| 6.             | Hematochezia   | Negative    | Negative  | IBS       |

WD = Without disease, IBS = Irritable bowel syndrome.
doi:10.1371/journal.pone.0074140.t002

Rejuvenescence-Like Cell Growth in Sporadic CRC
miRNA expression profiling was performed on tissue samples from juvenile, normal adult, and CRC. The results showed that CRC samples exhibited a distinct miRNA expression profile compared to juvenile and normal adult samples. The specific miRNAs that were upregulated or downregulated were identified, providing insights into the molecular mechanisms underlying CRC development.

Statistical evaluation

The statistical analysis was performed using the R software package. The p-values were calculated using the Student’s t-test for comparisons between groups. The results indicated that there were significant differences in miRNA expression between CRC and control groups.

Results

Proliferation and apoptosis in juvenile, normal adult, adenoma and colorectal carcinoma samples

Proliferating epithelial cells were predominantly localized at the base of crypts while apoptotic cells were close to the luminal surface in normal colonic mucosa (Figure 1). The proliferative-apoptotic ratio (PAR: ratio of proliferative and apoptotic epithelial cells in crypts) and 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). These results confirmed the findings of a previous study that indicated a decrease in apoptosis and an increase in proliferation with advancing age in normal colonic mucosa.

Expression of proliferation- and apoptosis-regulating genes in juvenile, normal adult and colorectal carcinoma samples

The expression of proliferation-regulating genes (ACVR1B, TNFSF10, DYRK2, IFI6, SERPINB9, SFRP1, SOCS3, SST, TNFSF10 and ZAK) was differentially expressed in CRC samples compared to juvenile or normal adult samples. The highest PAR and MI were observed in CRC samples (PAR = 3.51 ± 2.49; MI = 0.33 ± 0.06), while the lowest PAR and MI were detected in juvenile samples (PAR = 0.88 ± 0.22; MI = 0.15 ± 0.06).

Gene expression alterations between juvenile and CRC samples

Proliferation- and apoptosis-regulating genes were further investigated to find genes with dissimilar mRNA expression that could explain the differences between the controlled and uncontrolled cellular proliferation. Twelve probes belonging to 8 proliferation-regulating genes (BCL2, CDKN2B, RAD9A, BRCA1, CCNB1, CCNE1, CDC20, CDK1, CDKN2B, MKI67 and RBL1) were found to be significantly differentially expressed in CRC samples compared to juvenile or normal adult samples (p < 0.05). The expression of these genes was confirmed using TaqMan RT-PCR.

Validation of gene expression using TaqMan RT-PCR

mRNA expression of 10 genes including CDKN2B, MKI67, CDC2/CDK1, CCNE1, ACVR1B, TNFSF10, DYRK2, SOCS3, IFI6 and SERPINB9 were validated with TaqMan RT-PCR. The expression of these genes was confirmed using TaqMan RT-PCR.

According to the results of microarray analysis of proliferation-regulating genes (CDKN2B, MKI67, CDC2/CDK1 and CCNE1), significant mRNA expression alterations were detected by the value of Fold changes (FC ≤ 0.5 or FC ≥ 2) and the ANOVA test (p < 0.05) in Children vs. Normal and Normal vs. CRC comparisons; and these results were also confirmed with Tukey-test in case of CDC2/CDK1 and CCNE1. 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 previously mentioned 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).

Numbers of apoptosis-regulating genes (ACVR1B, TNFSF10, DYRK2, SOCS3, IFI6 and SERPINB9) were also analyzed with RT-PCR. Gene expression of ACVR1B, TNFSF10 and DYRK2
was significantly lower in children and CRC samples compared to normal adult colonic mucosa (FC ≤ 0.5 or FC ≥ 2; p ≤ 0.05); and these results were validated by RT-PCR as well. According to the results of Affymetrix study, mRNA expression of anti-apoptotic genes, such as SOCS3, IFI6 and SERPINB9, showed significantly higher expression in children and CRC samples as compared that to histologically intact adult colonic samples. PCR validation confirmed the tendency of gene expression alterations between Children vs. Adult Normal and Adult Normal vs. CRC. ANOVA and Tukey-test analysis of RT-PCR results have verified these alterations in case of SOCS3 and IFI6 (p < 0.05). Expression changes of the selected genes are summarized in Table 4.

**Figure 1. Detection of proliferative (red nuclei) and apoptotic (green nuclei) cells during aging and colorectal adenoma-carcinoma sequence (ACS) with fluorescent staining.** Blue spots represent the nuclei of inactive cells. Images were taken with digital microscope: normal child tissue (Ch), normal adult tissue (N), adenoma (Ad) and carcinoma (CRC) in adult. Mitotic activity decreases during aging and increases during the ACS in contrast to apoptotic activity. doi:10.1371/journal.pone.0074140.g001

**Discussion**

Aging is associated with increased incidence of sporadic colorectal malignancies, which is one of the leading causes of mortality in Western countries [33]. Colorectal cancer is related to uncontrolled cellular proliferation and dysregulated apoptosis. Juvenile growth, on the other hand, is characterized by controlled growth, cellular proliferation and apoptosis [34].

In this study the proliferative and apoptotic activity in intact human colorectal epithelium from children and adults compared to that in adenoma and colorectal cancer was investigated. Expression of the related genes was also tested in mRNA microarrays. We found an increased proliferative and a decreased...
apoptotic activity in children and cancer samples compared to normal adult epithelium.

These results suggest an opposing molecular regulation of proliferation and apoptosis during normal aging and colorectal carcinogenesis. Enhanced cellular proliferation of tumor cells without “aging” can contribute to their survival advantage over adjacent senescent cells.

An increased cellular proliferation detected in children colorectal mucosa can be related to the physiologic growth of the large bowel however, the cell renewal slows down during normal aging in the histologically intact adult colonic crypt. Cell proliferation and apoptosis regulation between controlled growth in childhood and uncontrolled growth in CRC have not been correlated before in the colonic mucosa. Here we found several proliferation promoting genes including cyclin B1 /CCNB1/, cyclin E1 /CCNE1/ and cyclin-dependent kinase (CDK1) to be upregulated both in young and cancer samples compared to normal adult mucosa (Figure 3). These results correlate well with our finding significantly higher proliferating cell fractions in children and cancer tissue sections compared to normal adult samples. CDKs, indeed, have crucial role in the regulation of cell-cycle and growth in eukaryotic cells. CDK complexes are a highly conserved family of Ser/Thr protein kinases, consisting of a catalytic CDK subunit and an activating cyclin subunit. Different CDKs can control different parts of the cell-cycle; these complexes can be activated by phosphorylation, binding activating cyclins or inhibiting subunits [35–36]. CCNB1, CCNE1 and CDK1 expressions, which can correlate to an increased proliferative activity, were higher in children and neoplastic colonic mucosa compared to normal adult mucosa. Furthermore, cyclin D1 (CCND1), CDK1 and CDK6 mRNA levels were significantly higher in CRC compared to normal children samples, which may be related to the uncontrolled cellular proliferation in cancer (Figure 4). Cyclin D1 has several regulatory effects in normal cellular differentiation,
growth and metabolism; however, the overexpression of CCND1 is one of the most commonly observed alterations in human cancers, as it has cell-cycle regulatory effects in G1 phase [37]. According to Zhang et al. results, the abnormal up-regulation of cyclin D1 can be an early event in intestinal carcinogenesis [38]. Sahl et al. have investigated the expression of different cyclin-dependent kinases in human colon cancer. They observed that the activation of CDK1, CDK2 and CDK6, which can phosphorylate the retinoblastoma protein, resulting in the release from the inhibition of forward progression of the G1 phase, is in connection with human colorectal carcinogenesis [39].

There are several regulatory molecules that can modulate and block the function of CKDs, called cyclin-dependent kinase inhibitors. In our present study, we investigated the mRNA expression alterations of CDKN2B in the processes of aging and colorectal carcinogenesis. CDKN2B is also known as multiple tumor suppressor 2 (MTS2), cyclin-dependent kinases 4 and 6 binding protein or p15-INK4b (p15). CDKN2B is located adjacent to tumor suppressor CDKN2A in the chromosome 9, which is frequently mutated and deleted in a wide variety of neoplasms. This gene encodes a cyclin-dependent kinase inhibitor that can produce complexes with CDK4 and CDK6, inhibiting the cellular growth or cell-cycle. In microarray analysis a moderate mRNA expression of CDKN2B was found in well-controlled, hyper-proliferative colonic biopsy samples from children as compared to histologically intact adult colonic mucosa, and a remarkable gene expression reduction was observed in CRC samples (Figure 3). According to previous studies, CDKN2B may act as a tumor suppressor gene and a potential effector of TGFb-induced cell-cycle arrest [40]. Herman et al. certified that gene

Table 3. Cell proliferation- and apoptosis-regulating genes analyzed in the study.

| Gene symbol | Affymetrix ID | Taqman ID | Gene name | Gene function |
|-------------|---------------|-----------|-----------|---------------|
| CDKN2B      | 236313_at     | Hs00365249_m1* | Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) | Negative regulation of (epithelial) cell proliferation. |
| MKI67       | 212022_s_at   | Hs01032443_m1* | Antigen identified by monoclonal antibody Ki-67 | Cell proliferation/cell cycle regulation. |
| CDC2/CDK1   | 210539_s_at   | Hs00364293_m1 | Cell division cycle 2, G1 to S and G2 to M | Cell cycle and cell division regulation. Anti-apoptosis. |
| CCNE1       | 213523_at     | Hs01026536_m1* | Cyclin E1 | Cell cycle and cell division regulation. Organ development and regeneration. |
| ACVR1B      | 208223_s_at   | Hs00923304_m1 | Activin A receptor, type IB | Induction of apoptosis. |
| TNFSF10     | 214329_s_at   | Hs0234356_m1* | Tumor necrosis factor (ligand) superfamily, member 10 | Induction of apoptosis. |
| DYRK2       | 202971_s_at   | Hs00705109_s1* | Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 | Induction of apoptosis. |
| SOCS3       | 227697_at     | Hs02330328_s1* | Suppressor of cytokine signaling 3 | Anti-apoptosis. Regulation of growth. Response to hypoxia. Aging. |
| IFI6        | 204415_at     | Hs00242571_m1* | Interferon, alpha-inducible protein 6 | Anti-apoptosis. |
| SERPINB9    | 242814_at     | Hs00244603_m1* | Serpin peptidase inhibitor, clade B (ovalbumin), member 9 | Anti-apoptosis. |

doi:10.1371/journal.pone.0074140.t003
We have analyzed and compared the proliferative and apoptotic activity in normal children, adult and CRC samples and tried to find the mRNA expression alterations in the background of controlled cellular proliferation in children and uncontrolled cellular proliferation in CRC. According to our immunohistochemical results, it has to be mentioned that cellular proliferation significantly decreases during physiological aging in histologically intact colorectal epithelium; moreover, the proliferative activity does not differ in normal adult and adenoma samples. However, the apoptotic activity is significantly lower in adenoma samples as compared to normal adult samples. Thus, we can assume that a decreased apoptosis has a major role in the misbalanced cell-renewal and cell-death of the adenomatous status. In colorectal cancer, both of the increased cellular proliferation and nearly absent apoptosis may contribute to the uncontrolled cellular growth.

As we are aware, this is the first study to investigate mRNA expression in children colorectal biopsy samples with particular focus on proliferation and apoptosis regulation. Furthermore, these results were correlated with in situ mitosis and apoptosis index in comparison with those of normal adult samples and colorectal cancer. The lack of similar data prevented us to compare ours with other data sets on Gene Expression Omnibus databank. Since routine colonoscopy in children is rarely performed, collection from children colonic samples was the bottleneck of this study. In most children biopsies available for us were obtained during the routine endoscopies performed in children colonic samples was the bottleneck of this study. In most children biopsies available for us were obtained during the routine endoscopies performed in children.

As we are aware, this is the first study to investigate mRNA expression in children colorectal biopsy samples with particular focus on proliferation and apoptosis regulation. Furthermore, these results were correlated with in situ mitosis and apoptosis index in comparison with those of normal adult samples and colorectal cancer. The lack of similar data prevented us to compare ours with other data sets on Gene Expression Omnibus databank. Since routine colonoscopy in children is rarely performed, collection from children colonic samples was the bottleneck of this study. In most children biopsies available for us were obtained during the routine endoscopies performed in children.

at children, Healthy adults (N) and CRCs (CRC) with ANOVA analysis.

| Gene symbol | Affymetrix ID | Children (Ch) | Healthy adults (N) | CRCs (CRC) | ANOVA on microarray |
|-------------|--------------|--------------|-------------------|------------|-------------------|
| CDKN2B      | 23631_at     | 10.13±0.45   | 11.64±0.61        | 7.74±2.55  | 2.90E-14          |
| MKI67       | 212022_s_at  | 9.02±0.95    | 7.94±0.78         | 9.97±1.35  | 3.67E-11          |
| CDC2/CDC1K | 210559_s_at  | 8.73±0.65    | 7.61±0.69         | 9.46±1.45  | 1.09E-09          |
| CCNE1       | 213523_at    | 6.89±0.39    | 5.71±0.40         | 6.74±0.84  | 4.96E-10          |
| ACVR1B      | 208223_s_at  | 4.75±0.65    | 5.79±0.80         | 4.72±1.09  | 1.08E-05          |
| TNFSF10     | 214329_x_at  | 9.87±0.47    | 10.97±0.52        | 9.89±0.88  | 6.05E-09          |
| DRYK2       | 202971_s_at  | 10.27±0.37   | 11.39±0.58        | 10.24±0.78 | 2.12E-10          |
| SOCS3       | 227697_at    | 8.2±1.04     | 6.44±1.21         | 10.27±1.86 | 2.20E-16          |
| IFI6        | 204415_at    | 7.07±1.66    | 5.92±0.95         | 7.97±1.49  | 4.18E-09          |
| SERPINB9    | 242814_at    | 5.43±1.36    | 4.3±0.78          | 5.39±1.82  | 2.20E-03          |

| Gene symbol | Taqman ID | Averages and standard deviations of normalized log2 intensities on microarray and RT-PCR, with ANOVA analysis. |
|-------------|----------|------------------------------------------------------------------------------------------------|
| CDKN2B      | Hs00365249_m1* | 14.13±0.37 | 14.37±0.22 | 13.96±0.44 | 2.19E-01          |
| MKI67       | Hs01032443_m1* | 22.94±0.51 | 22.24±0.58 | 22.63±0.55 | 1.10E-01          |
| CDC2/CDC1K | Hs00364293_m1 | 19.73±0.6  | 18.5±0.82  | 19.83±1.1  | 4.99E-03          |
| CCNE1       | Hs01026536_m1* | 20.01±0.41 | 19.43±0.51 | 19.98±0.8  | 7.05E-02          |
| ACVR1B      | Hs00923304_m1 | 15.5±0.93  | 15.45±1.02 | 16.08±2.52 | 4.90E-01          |
| TNFSF10     | Hs00234356_m1* | 22.4±0.26  | 22.32±0.22 | 22.55±0.77 | 3.22E-03          |
| DRYK2       | Hs00705109_s1* | 22.8±0.3   | 23.31±0.37 | 22.66±0.67 | 8.24E-03          |
| SOCS3       | Hs02330328_s1* | 21.85±1.09 | 20.29±0.55 | 22.72±1.41 | 1.90E-04          |
| IFI6        | Hs00242571_m1* | 21.35±1.38 | 20.42±0.52 | 22.37±1.23 | 8.94E-05          |
| SERPINB9    | Hs00244603_m1* | 20.57±0.47 | 20±0.42    | 20.61±0.75 | 2.30E-01          |

P-values of ANOVA analysis on microarray and RT-PCR represents in the last column. The significant different expression (p<0.05) is marked in bold.

doi:10.1371/journal.pone.0074140.t004

Table 4. Averages and standard deviation of normalized log2 intensities on microarray and RT-PCR, with ANOVA analysis.
Acknowledgments

We thank the co-workers at the Endoscopy Unit of 2nd Department of Internal Medicine and 1st Department of Pediatrics of Semmelweis University for their technical assistance. We thank László Herszényi, Emese Mihály, Pál Mihéller, Márk Juhász, Katalin Mullner, Anna Mária Németh, László Kónya, Hajnal Székely, Richárd Szmoda for their work with auscultoscopy and biopsic collection. Furthermore, we thank Gabriella Kónya (Cell Analysis Laboratory, 2nd Department of Medicine, Semmelweis University, Budapest, Hungary) for preparing immunostainings, Renáta Kis (3DHistech Ltd.) for performing slide digitalization and Nha Le for her work.

Author Contributions

Conceived and designed the experiments: BM ZT. Performed the experiments: KL OG TK G. Veres KT. Analyzed the data: FS TK BW. Contributed reagents/materials/analysis tools: AK ÁPV KT G. Valecz. Wrote the paper: KL.

References

1. Van der Flier LG, Cleven H (2009) Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol 71: 241-260.
2. Gordon JI, Hannon MJ (1998) Differentiation and self-renewal in the mouse gastrointestinal epithelium. Curr Opin Cell Biol 6: 795-803.
3. Lipkin M, Bell B, Sherlock P (1963) Cell proliferation kinetics in the gastrointestinal tract of man. J Cell Renew in colon and rectum. J Clin Invest 42: 767-776.
4. Hall PA, Coates PJ, Ansari B, Hopwood D (1994) Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. J Cell Sci 107: 5369-5377.
5. Leistner K, Galamb O, Sipos F, Spisák S, Tóth K, et al. (2010) The signs of aging in the gastrointestinal tract. Magy Belgyor Arch 63: 19-24.
6. Leistner K, Galamb O, Sipos F, Tóth K, Valicz G, et al. (2010) Age-related microscopic and macroscopic changes of the human colon, and their role in the development of colorectal cancer in elderly people. Orv Hetil 151: 885-892.
7. Mandir N, FitzGerald AJ, Goodlad RA (2005) Differences in the effects of age on intestinal proliferation, crypt fusion and apoptosis on the small intestine and the colon of the rat. Int J Exp Pathol 86: 125-130.
8. Xiao ZQ, Moragoda L, Jaszewski R, Hatfield JA, Fligiel SE, et al. (2001) Aging is associated with increased proliferation and decreased apoptosis in the colonic mucosa. Mech Ageing Dev 122: 1849-1864.
9. Moss SF, Scholes JV, Holt FR (1996) Abnormalities of epithelial apoptosis in multistep colorectal neoplasia demonstrated by terminal deoxynucleotid nick end labeling. Dig Dis Sci 41: 2238-2247.
10. Takano Y, Saegusa M, Ikenaga M, Mitomi H, Okayasu I (1996) J Cancer Res Clin Oncol 122: 166-170.
11. Kikuchi Y, Dinjens WN, Bosman FT (1997) Proliferation and apoptosis in colorectal cancer. Clin Oncol (Dordr) 11: 799-807.
12. Koornstra JJ, de Jong S, Hollema H, de Vries EG, Kleibeuker JH (2003) Multistep colorectal neoplasia demonstrated by terminal deoxynucleotid nick end labeling. Dig Dis Sci. 41: 2238–2247.
13. Diamond SJ, Enestvedt BK, Jiang Z, Holub JL, Gupta M, et al. (2011) Adenoma to carcinoma transition specific transcripts in colonic biopsy samples. PLoS One 7: e48547.
14. Kollig FT, Grapin A, Munte A, Wagner A, Mannsmann U, et al. (2011) Risk of advanced colorectal neoplasia according to age and gender. PLoS One 6: e20076.
15. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, et al. (1988) Genetic alterations during colorectal-tumor development. N Engl J Med 319: 525-535.
16. Kim HJ, Yu MH, Kim H, Byun J, Lee C (2008) Noninvasive molecular biomarkers for the detection of colorectal cancer. RMB Rep 41: 663-692.
17. Galamb O, Wichmann B, Sipos F, Spisák S, Krenacs T, et al. (2012) Dysplasia-carcinoma transition specific transcripts in colonic biopsy samples. PLoS One 7: e45547.
18. Carvalho B, Sillar-Hardebol AH, Postma C, Mongera S, Terhaar Sive Droste J, et al. (2012) Colorectal adenoma to carcinoma progression is accompanied by changes in gene expression associated with aging, chromosomal instability, and fatty acid metabolism. Cell Oncol (Dordr) 35: 53-63.
19. Arai T, Takubo K (2007) Clinicopathological and molecular characteristics of gastric and colorectal carcinomas in the elderly. Pathol Int 57: 301-314.
20. Ukraintseva SV, Yashina AI (2004) Cancer as “rejuvenescence”. Ann N Y Acad Sci 1019: 200-205.
21. Soussi T (2000) p53 Antibodies in the sera of patients with various types of cancer: a review. Cancer Res 60: 1777-1780.
22. Peter ME, Legembre P, Barnhart BC (2005) Does CD95 have tumor promoting activities? Biochim Biophys Acta 1735: 25-36.
23. Koju KS, Lehman JM (1995) Increased p53 protein associated with aging in human diploid fibroblasts. Exp Cell Res 217: 336-345.
24. Miyawaki T, Uehara T, Nibu R, Tsugi T, Yachie A, et al. (1992) Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. J Immunol 149: 3733-3738.
25. Aggarwal S, Gupta S (1998) Increased apoptosis of T-cell subsets in aging humans: altered expression of Fas (CD95). Fas ligand, Bcl-2, and Bax. J Immunol 160: 1627–1637.
26. Dean R, Kim SS, Delgado D (1986) Expression of c-myc oncogene in human fibroblasts during in vitro senescence. Biochim Biophys Acta Comm 135: 105–109.
27. Delgado D, Raymond L, Dean R (1986) Eras expression decreases during in vitro senescence in human fibroblasts. Biochim Biophys Acta Comm 137: 917-921.
28. Reenstra WR, Yaar M, Gilchrest BA (1996) Aging affects epidermal growth factor receptor phosphorylation and traffic kinetics. Exp Cell Res 227: 232-235.
29. Theodoulou NA, Tabin CJ (2003) Wnt signaling during development of the gastrointestinal tract. Dev Biol 259: 258–271.
30. Gregoiret A, Clevers H (2005) Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev 19: 877-890.
31. Galamb O, Spisák S, Sipos F, Tóth K, Solomosi N, et al. (2016) Reversal of gene expression changes in the colorectal normal-adenoma pathway by NS398 selective COX2 inhibitor. Br J Cancer 102: 763–773.
32. Galamb O, Sipos F, Solomosi N, Spisák S, Krenacs T, et al. (2008) Diagnostic mRNA expression patterns of inflamed, benign and malignant colorectal biopsy specimen and their correlation with peripheral blood results. Cancer Epidemiol Biomarkers Prev 17: 2833–2845.
33. Bingham S, Riboli E (2004) Diet and cancer-the European Prospective Investigation into Cancer and Nutrition. Nat Rev Cancer 4:206-215.
34. Lee HM, Greeley GH Jr, Enders GW, 2000) Changes in gene expression associated with aging, chromosomal instability, and fatty acid metabolism. Cell Oncol (Dordr) 35: 53-63.
35. Salh B, Bergman D, Marotta A, Pelech SL (1999) Differential cyclin-dependent kinase expression and activation in human colon cancer. Anticancer Res 19: 741-748.
36. Salh B, Bergman D, Marotta A, Pelech SL (1999) Differential cyclin-dependent kinase expression and activation in human colon cancer. Anticancer Res 19: 747–748.
37. Zhang T, Nanney LB, Luongo C, Lamps L, Heppner KJ, et al. (1997) Concurrent overexpression of cyclin D1 and cyclin-dependent kinase 4 (Cdk4) in intestinal adenomas from multiple intestinal neoplasia (Min) mice and human familial adenomatous polyposis patients. Cancer Res 57: 169-175.
38. Sahl B, Bergman D, Marotta A, Pelech SL (1999) Differential cyclin-dependent kinase expression and activation in human colon cancer. Anticancer Res 19: 741–748.
39. Antoniou and cancer: applying endocrinology to oncology. Cancer Biol Ther 3: 731-733.