Differential Expression of Tight Junctions and Cell Polarity Genes in Human Colon Cancer

Themistoklis Kourkoumpetis1,2, Kathryn E. Royse3, Liang Chen1,2,3, Milan Ravishankar1,2, Michael Ittmann2,4, Hashem B. El-Serag1,2,3 and Li Jiao1,2,3*

1Section of Gastroenterology and Hepatology, Baylor College of Medicine, Houston, USA; 2Michael E. DeBakey VA Medical Center, Houston, Texas, USA; 3Section of Health Service Research, Department of Medicine, Baylor College of Medicine, Houston, USA; 4Department of Pathology and Immunology, Baylor College of Medicine, Houston, USA

Abstract

Background and objectives: Tight junction and cell polarity proteins are paramount to epithelial cell polarity and transport. In vivo studies have shown the differential expression of tight junctions in colorectal cancer, with little to no human data to corroborate those findings. We investigated whether tight junction genes were differentially expressed in human colon cancer versus normal controls.

Methods: Total RNA was extracted from fresh frozen tumor tissues and normal adjacent tissues of 6 patients who were diagnosed with primary colon cancer as well as from normal mucosa of 5 unrelated polyp-free individuals. We used the Qiagen RT² Profiler PCR array to determine the expression of 84 genes in the tight junction pathway. Student’s t-test was used to compare gene expression levels between cancer tissues and normal mucosa using normalized gene expression data.

Results: Compared with normal mucosa, significant up-regulation of the claudin 1 (CLDN1) gene (fold-change = 16, p = 0.001) but down-regulation of the AMOTL1, CLDN5, JAM2 and TIAM1 genes (fold-change > 2, p < 0.05) were seen in colon cancer tissue.

Conclusions: We observed the differential expression of CLDN, AMOTL1, JAM2 and TIAM1 in colon cancer versus normal mucosa. Further larger studies are warranted to investigate the role of tight junction and cell polarity proteins in the progression of human colon tumors.

Introduction

The intestinal epithelium is a selective semipermeable barrier for luminal molecules and microbes. It maintains its complexity through a diverse family of molecules that promote cell-to-cell anchorage and appropriate individual cell polarity, mainly being represented by the adherens junctions and tight junctions. Tight junctions are a diverse family of molecules which form an essential barrier between the internal and external environment and maintain its complexity. Cell-to-cell adhesion is paramount to the maintenance of normal epithelial tissue architecture, and disruption of this anchorage is believed to aid to tumor progression and subsequent invasion to deeper tissues as well as distant metastasis. Intestinal barrier dysfunction is also an essential contributor to intestinal inflammation. Structural disruption of colonic epithelial cells might impact early tumorigenesis or progression to colorectal cancer. Changes in cell-to-cell junction molecules can lead to cellular migration, invasion and metastasis of cancer cells in a tissue-dependent manner. Tight junctions are protein complexes near the apical surface of the epithelial cell and have been found to promote cellular polarity, epithelial barrier maintenance and paracellular transport. Tight junctions are compiled by multiple building blocks, including claudins, which play a vital role in tight junction anchorage onto the cellular membrane.
We extracted total RNA from fresh frozen tissue biopsies of 17 RNA extraction and gene expression analysis specimens for research before the surgery procedure. Each unrelated control was matched to each case per age, sex, race/ethnicity, and colonic segment. Analysis. The resected tumor tissue and normal adjacent tissue of 5 cancer patients was not ideal for gene expression analysis. We hypothesized that gene expression among the tight junction pathway is significantly altered in colon cancer compared to normal mucosa.

Table 1. Basic characteristics of cases with colon cancer and controls

<table>
<thead>
<tr>
<th>Characteristic, mean (SD) or n (%)</th>
<th>Cases, n = 6</th>
<th>Controls, n = 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years</td>
<td>61.3 (5.3)</td>
<td>62.4 (9.1)</td>
</tr>
<tr>
<td>Sex, male</td>
<td>6 (100)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Race, Caucasian</td>
<td>6 (100)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>3 (50)</td>
<td>NA</td>
</tr>
<tr>
<td>Late</td>
<td>3 (50)</td>
<td>NA</td>
</tr>
<tr>
<td>Colon segment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sigmoid</td>
<td>2 (33.3)</td>
<td>2 (40.0)</td>
</tr>
<tr>
<td>Right colon</td>
<td>4 (66.7)</td>
<td>3 (60.0)</td>
</tr>
</tbody>
</table>

Methods

Study participants and sample collection

Our study consisted of 6 patients who were diagnosed with colon cancer and underwent total curative surgical excision at the affiliated hospitals of Baylor College of Medicine between 1999 and 2013. All study subjects had no personal history of malignancy and did not receive preoperative neoadjuvant chemotherapy. The research coordinators collected the tissue samples during the surgical procedure. The resected tumor tissue and normal adjacent mucosa samples were snap-frozen in liquid nitrogen and stored at −80 °C within 10 minutes from obtainment. The pathological report confirmed the diagnosis of colon adenocarcinoma, and the tumor volume for all was >60%. We defined the stage of cancer using the TNM definition of the American Joint Committee on Cancer. Early-stage colon cancer was defined by no regional lymph node metastasis and no distant metastasis.

In a colonoscopy-based case-control study, we also obtained colon mucosa samples from 5 unrelated individuals who underwent screening colonoscopy and were found to have a normal colon at the Michael E. DeBakey Veterans Affairs Medical Center, an affiliated hospital of Baylor College of Medicine. For each, the collected normal biopsy was immediately placed on the dry ice and transferred to a −80 °C freezer within 20 minutes. These samples served as the control tissues when the RNA quality of normal adjacent tissue of 5 cancer patients was not ideal for gene expression analysis. Each unrelated control was matched to each case per age (±5 years), sex, race/ethnicity, and colonic segment. The patients provided written informed consent to allow their specimens for research before the surgery procedure.

RNA extraction and gene expression analysis

We extracted total RNA from fresh frozen tissue biopsies of 17 samples (6 biopsies from excised cancerous tissues, 6 biopsies from adjacent normal colon mucosa, and 5 from unrelated normal control mucosa) in the genomic core lab at the Texas Digestive Disease Center using the NucleoSpin RNA isolation kit (MACHEREY-NAGEL Inc, Bethlehem, PA, USA). We determined the nucleic acid 260:280 ratio to confirm the RNA purity of samples, which was >2.0 for all. However, the RNA integrity number was <6.0 for 5 normal adjacent mucosa samples. These RNA samples were deemed not adequate for gene expression analysis and thus were excluded from the gene expression experiment. As a result, the gene expression analysis was restricted to 6 resected colon cancers from 6 patients, 1 normal adjacent mucosa from 1 patient, and 5 normal mucosal biopsies from unrelated healthy controls.

We used the Qiagen RT² Profiler PCR Array (Hilden, Germany) to determine the expression of 84 genes in the tight junction pathway. The array includes all claudin genes, and genes encoding OCLN, cell adhesion proteins (ESAM1, ICAM1, ICAM2 and PECAM1), junction adhesion proteins (F11R, IGSF5, JAM2 and alpha actins), catenins (ACTN1-4, CTNNA1-3 and CTNNB1), junction interacting proteins (ACTN1-4, AMOT1, AMOTL1, GGN, YBX3, CTN, EPB41, HCLS1, INADL, MAG1, MAG2, MLLT4, MPDZ, PAR3, SYMPK, TJP1, TJP2-3, VAPA and ZAK), cytoskeleton regulation proteins (AMOT1, ASH1L, YBX3, CTN, LGI1, LGI2, PARD3, PARD6A, PARD6B, SPTA1, SPTAN1, SPTB, SMURF1 and TIM1), G protein signaling proteins (ARHGFE2, CDC42, CDK4, GNAI1, RAC1 and RHOA), and protein kinases (CASK, CSNK2A1, CSNK2A2, CSNK2B, ILK, MAG1, MAG2, MARK1, MPP5, MPP6, PRKCI, PRKCK and PTEN). The assays were conducted at the core facility at Baylor College of Medicine using the Bio-Rad LightCycler RealTime PCR System (Hercules, CA, USA). After the gene expression analysis, the data from 1 unrelated normal control showed poor signal and we eliminated the data from the analysis. Therefore, we included 6 colon cancer tissue and 5 control tissues in this analysis.

The institutional review board of Baylor College of Medicine and Michael E. DeBakey Veterans Affairs Medical Center approved the study protocol.

Statistical analysis

We used the RT² Profiler PCR Array Data Analysis Software, version 3.5, for data analysis. Student’s t-test was used to compare the expression levels between cancer and normal mucosa using normalized gene expression data against ACTB, HPRT1 and RPLP0 as the housekeeping genes. We also exploratorily conducted the
analysis by cancer stage, i.e. early-stage cancer or late-stage cancer. We calculated fold-change/fold-regulation using the ΔΔCt method, in which delta Ct was calculated between the gene of interest and an average of reference housekeeping genes, followed by the ΔΔCt calculation: ΔCt (cancer group) − ΔCt (control group). Fold-change was then calculated using the $2^{\Delta \Delta C_t}$ formula.

The scatter plot compares the normalized expression of every gene on the array between two groups by plotting them against each other. The central line indicates the constant gene expression. The dotted lines indicate the selected fold-regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold-regulation threshold. Multiple testing for 84 genes was adjusted using false discovery rate. Raw $p$-value of <0.01 was indicative of statistical significance in this study.

Gene Set Enrichment Analysis was performed using WebGestalt (http://www.webgestalt.org) for Kyoto Encyclopedia of Genes and Genomes pathway analysis to determine the enrichment scores and functional significance of all genes in the panel following log2 conversion of their fold-changes.7

**Results**

The 6 cancer patients were all non-Hispanic Caucasian men. The age range was 55 to 75 years. Three patients had an early-stage tumor and three patients had a late-stage tumor (Table 1).

When colon cancer was compared with healthy controls, the differential expression of multiple genes was observed (Fig. 1). The CLDN1 gene was significantly up-regulated, with fold-change of 16 ($p<0.001$) (Table 2). Four genes were down-regulated after multiple testing adjustments, including AMOTL1, CLDN5, JAM2 and TIAM1. The differential expression was persistently seen in early-stage as well as late-stage tumors. The down-regulation of CTNNA3, JAM3, MPP5 and PTEN was not statistically significant after adjustment for multiple testing. CLDN2 was up-regulated by >25-fold and CLDN8 was down-regulated >100-fold in tumor tissue compared with control mucosa. However, the difference was

| Table 2. Differential expression of genes in human colon cancer tissue and normal control tissue |

<table>
<thead>
<tr>
<th>Gene</th>
<th>All cases versus controls, fold-change</th>
<th>Crude $p$</th>
<th>Early stage cancer versus controls, fold-change</th>
<th>Crude $p$</th>
<th>Late stage cancer versus controls, fold-change</th>
<th>Crude $p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMOTL1</td>
<td>3.13</td>
<td>0.007</td>
<td>2.74</td>
<td>0.07</td>
<td>3.57</td>
<td>0.03</td>
</tr>
<tr>
<td>CLDN1</td>
<td>16.62</td>
<td>0.008</td>
<td>23.14</td>
<td>&lt;0.0001</td>
<td>11.94</td>
<td>0.10</td>
</tr>
<tr>
<td>CLDN5</td>
<td>−4.89</td>
<td>0.004</td>
<td>−3.63</td>
<td>0.05</td>
<td>−6.60</td>
<td>0.03</td>
</tr>
<tr>
<td>CTNNA3</td>
<td>3.24</td>
<td>0.010</td>
<td>0.54</td>
<td>0.03</td>
<td>3.04</td>
<td>0.09</td>
</tr>
<tr>
<td>JAM2</td>
<td>−7.18</td>
<td>0.002</td>
<td>−5.36</td>
<td>0.03</td>
<td>−9.60</td>
<td>0.02</td>
</tr>
<tr>
<td>JAM3</td>
<td>2.94</td>
<td>0.012</td>
<td>2.29</td>
<td>0.11</td>
<td>−3.77</td>
<td>0.04</td>
</tr>
<tr>
<td>MPP5</td>
<td>2.18</td>
<td>0.015</td>
<td>2.43</td>
<td>0.08</td>
<td>−1.96</td>
<td>0.08</td>
</tr>
<tr>
<td>PAR6A</td>
<td>1.67</td>
<td>0.092</td>
<td>2.54</td>
<td>0.004</td>
<td>1.09</td>
<td>0.64</td>
</tr>
<tr>
<td>PTEN</td>
<td>−2.36</td>
<td>0.013</td>
<td>−2.75</td>
<td>0.07</td>
<td>−2.02</td>
<td>0.08</td>
</tr>
<tr>
<td>TIAM1</td>
<td>−4.77</td>
<td>0.008</td>
<td>−5.27</td>
<td>0.06</td>
<td>−4.32</td>
<td>0.04</td>
</tr>
<tr>
<td>CGN</td>
<td>−2.09</td>
<td>0.039</td>
<td>−1.04</td>
<td>0.28</td>
<td>−4.19</td>
<td>0.05</td>
</tr>
</tbody>
</table>
not statistically significant (unadjusted $p > 0.05$). Overall, gene signatures and pathway analysis showed down-regulation of tight junction genes in colon cancer compared with normal mucosa (Supplemental Table S1).

We identified three significantly positively enriched Kyoto Encyclopedia of Genes and Genomes pathways (false discovery rate $p < 0.001$), including cell adhesion molecules, leukocyte transendothelial migration and tight junction. Significant contributors to all three sets were members of the claudin family. The primary differentially enriched genes of the tight junction pathway were those related to cell polarity and proliferation and junction assembly (Fig. 2).

**Discussion**

Our pilot findings suggested an altered expression of multiple tight junctions and cell polarity genes in colon cancer compared with normal colonic tissues. Specifically, AMOTL1, CLDN5, JAM2 and TIAM1 were the genes significantly down-regulated and CLDN1 was the only gene significantly up-regulated in colon cancer compared with normal mucosa.

Claudins are a diverse family of transmembrane proteins that aid in the adhesion of tight junction complexes to the intracellular cytoskeleton. At least 24 subtypes have been described, with some of them having a specific tissue predominance and differential expression in specific malignancies. In our study, CLDN1 was up-regulated in colon cancer compared with normal mucosa. It has been proposed that increased CLDN1 expression could promote malignant cell behavior via a decrease in E-cadherin by increasing expression of ZEB-1, beta-catenin nuclear translocation and WNT signaling. Our findings were consistent with previous findings in premalignant conditions in humans. Weber et al. have shown CLDN1 to be significantly up-regulated in active inflammatory bowel disease, colorectal adenomas and inflammatory bowel disease-associated dysplasia. Bujko et al. found increased CLDN1 expression in 26 samples of colorectal cancer, and 42 adenomas compared to normal colon tissue. In contrast, Ersoz et al. and Suren et al. have shown that CLDN1 expression was significantly down-regulated in a series of colon cancer tissues and this lower expression was linked to a higher proportion of lymph node metastasis or aggressive behavior of colorectal carcinoma. This difference might be explained by the different gene expression approach used. We evaluated the gene expression at the mRNA levels, whereas Suren et al. and Matsuoka et al. used immunohistochemical testing.

We found CLDN5 was significantly down-regulated and CLDN2 was insignificantly up-regulated in cancer tissues compared with control mucosa. CLDN2 is a pore-forming protein and CLDN5 is a seal-forming protein, and increased expression of CLDN2 and decreased expression of CLDN5 are consistent with epithelial dysfunction, as characterized by reduced barrier resistance. CLDN5 was only shown to be down-regulated in one human study and its role in colorectal tumorigenesis has not been well elucidated. We did not find significant differential expression of other CLDN genes between cancer and normal mucosa, although CLDN6, 7 and 8 were all non-significantly down-regulated in colon cancer tissue. Nakayama et al. have shown that CLDN7 expression was significantly down-regulated in human
node metastasis, earlier recurrence and worse prognosis. This molecular mechanism of claudins and tight junction disruption and its clinical significance in epithelial to mesenchymal transition may provide novel insight into colorectal carcinogenesis. In conclusion, in colon cancer, the expression of multiple genes related to tight junctions and cell polarity was found to be significantly altered compared with controls. Understanding the molecular mechanism of claudins and tight junction disruption and its clinical significance in epithelial to mesenchymal transition may provide novel insights into colorectal carcinogenesis.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study design (LJ), sample acquisition (MI, HBE), data analysis (KER, LC), data interpretation and manuscript writing (TK, KER, MR), critical intellectual input (TK, KER, LC, MR, MI, HBE, LJ).

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/ERHM.2017.00036.

Supplemental Table S1. Differential expression of genes in human colon cancer tissue (n = 6) and normal control tissues (n = 5).

References

Kourkoumpetis T. et al: Tight junction and colorectal cancer


