Galanin Is Up-Regulated in Colon Adenocarcinoma

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Abstract

The early diagnosis of colorectal cancer and the early detection of recurrence are central to effective treatment, as prognosis is directly related to the stage of the disease. When colorectal cancer is diagnosed at an early, localized stage, 5-year survival is 90%. There is substantial interest in the identification of circulating human tumor-derived proteins in serum for the purposes of early cancer diagnosis. We have implemented an approach based on the analysis of microarray data for the identification of tumor proteins that may have utility as biomarkers in colon cancer. Expression analysis of microarray data obtained from a variety of 290 tumors and normal tissues revealed that galanin was maximally expressed in colon cancer. These findings were corroborated by real-time quantitative PCR, in which the colon cancer cell lines LOVO, HCT115, SW480, and SW620 cell lines showed significantly higher levels of galanin expression than did noncolorectal cancer cell lines. To evaluate galanin as a potential biomarker of colon cancer, a preliminary “training” set of serum from 40 healthy donors and 55 colon cancer patients was analyzed by ELISA. The data pattern was confirmed by an independent set of 90 masked serum samples: 24 from healthy donors and 66 from colon cancer patients. This result yielded a sensitivity of 69.7% (95% confidence interval (95% CI), 57.1-80.4), specificity of 75.0% (95% CI, 53.3-90.2), and positive predictive value of 88.5% (95% CI, 76.6-95.7). The galanin expression level was significantly increased with tumor size and tumor stage. These findings justify a prospective assessment of serum galanin protein as a screening tool for colon cancer.

Introduction

Colorectal cancer is the most common gastrointestinal cancer in the Western world and it is an important cause of cancer-related death (1, 2). An overall 5-year survival rate is ~50%. Most colorectal cancers develop slowly, beginning as small benign colorectal adenomas that progress over several decades to larger and more dysplastic lesions, eventually becoming malignant. This gradual progression provides ample opportunity for prevention and intervention. Diagnostic screening methods are at present suboptimal; therefore, new approaches are needed (3). In an effort to identify potential molecular markers of colorectal tumors, we have implemented an approach based on the analysis of microarray data for the identification of tumor proteins that may have utility as biomarkers in colon cancer. Expression analysis of microarray data obtained from a variety of 290 tumors and normal tissues revealed that galanin was maximally expressed in colon cancer.

Galanin is a 29-amino acid COOH-terminally, highly conserved but unique neuroendocrine peptide originally isolated from intestine (4). The first 14 amino acids are fully conserved in almost all species (5). Galanin is found in the brain and the gut. It modulates a variety of physiologic processes, including cognition, memory, sensor, pain processing, neurotransmitter, hormone secretion, and feeding behavior (6). Several NH2-terminally elongated or truncated, biologically active forms of galanin have also been isolated (7).

Although galanin has multiple effects on gut smooth muscle, the relationship between galanin and colon cancer has not been known. A few reports have indicated the presence of galanin in gliomas, pheochromocytomas, and pituitary and neuroblastic tumors (2, 8). There is much interest in identification of circulating tumor-derived proteins that may serve as a biomarker for the early detection of colon cancer. We show that galanin protein is expressed at higher levels in serum from colon cancer patients relative to serum from noncancer controls. As such, galanin may have utility as a biomarker for colon cancer.

Materials and Methods

Sera. Sera representing the predetermined training set were obtained at the time of diagnosis from 40 healthy individuals and 55 colon cancer patients following informed consent. For the independent test, 90 masked serum samples consisting of 24 from healthy donors and 66 from colon cancer patients were obtained in the same way. The experimental protocol was approved by NIH, Korea Institutional Review Board before serum collection.

Cell Lines and Cell Culture. The LOVO, HCT116, SW480, and SW620 colon adenocarcinoma cell lines, A549 lung carcinoma cell line, OVCAR3 and SKOV3 ovarian carcinoma cell lines, and the HS1 testis carcinoma cell line were each cultured (5% CO2, 37°C) in DMEM containing 10% fetal bovine serum, penicillin

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Microarray Analysis. Single isolates of tumor samples and cancer cells were homogenized in the presence of Trizol reagent (Invitrogen) and total cellular RNA was purified according to the manufacturer's procedures. RNA samples were further purified using RNeasy spin columns (Qiagen). RNA quality was assessed by 1% agarose gel electrophoresis in the presence of ethidium bromide. Samples that did not reveal intact and approximately equal 18S and 28S ribosomal bands were excluded from further study. This study used commercially available high-density microarrays (Affymetrix) that produced gene expression levels on 7,129 known genes and expressed sequence tags (HuGeneFL Array). Preparation of cRNA, hybridization, and scanning of the arrays were done according to the manufacturer's protocols. Briefly, 5 μg of the total RNA were used to generate double-stranded cDNA by reverse transcription using a cDNA synthesis kit (SuperScript Choice System, Life Technologies, Inc.) that uses an oligo(dT)24 primer containing a T7 RNA polymerase promoter 3' to the poly(T) (GenSet) followed by second-strand synthesis. Labeled cRNA was prepared from the double-stranded cDNA by in vitro transcription by T7 RNA polymerase in the presence of biotin-11-CTP and biotin-16-UTP (Enzo). The labeled cRNA was purified over RNeasy columns. cRNA (15 μg) was fragmented at 94°C for 35 min in 40 mM/L Tris-acetate (pH 8.1), 100 mM/L potassium acetate, and 30 mM/L magnesium acetate. The cRNA was then used to prepare 300 μL of hybridization cocktail (100 mM/L MES, 1 mM/L NaCl, 20 mM/L EDTA, 0.01% Tween 20) containing 0.1 mg/mL of herring sperm DNA (Promega) and 500 μg/mL of acetylated bovine serum albumin (Life Technologies). Before hybridization, the cocktails were heated to 94°C for 5 min, equilibrated at 45°C for 5 min, and then clarified by centrifugation (16,000 × g) at room temperature for 5 min. Aliquots of this hybridization cocktail containing 10 μg of fragmented cRNA were hybridized to HuGeneFL arrays at 45°C for 16 h in a rotisserie oven set at 60 rpm. The arrays were washed using nonstringent buffer (6× saline-sodium phosphate-EDTA) at 25°C followed by stringent buffer (100 mM/L MES (pH 6.7), 0.1 mM/L NaCl, 0.01% Tween 20) at 50°C. The arrays were stained with streptavidin-phycocerythrin (Molecular Probes), washed with 6× saline-sodium phosphate-EDTA buffer, incubated with biotinylated anti-streptavidin IgG, stained again with streptavidin-phycocerythrin, and washed again with 6× saline-sodium phosphate-EDTA. The arrays were scanned using the GeneArray scanner (Affymetrix). Image analysis was done with GeneChip software (Affymetrix).

Real-time Quantitative Reverse Transcription PCR. Total RNA was extracted from cells with Trizol reagent following the protocol that was provided. For each sample, 2 μg RNA was treated with DNase I (Roche) at 37°C for 30 min to remove contaminating DNA and then denatured in the presence of random hexamer primers (Promega). The samples were incubated with SuperScript II (Invitrogen) reverse transcriptase in the presence of 1.0 mM/L DTT and 1.0 mM/L each of dTTP, dGTP, dCTP, and cATP at 42°C for 40 min. The resulting cDNA was treated with RNase H (Roche) and subjected to PCR amplification. We relied on the Taqman assay (model 7700; Perkin-Elmer) to quantitate the amount of galanin mRNA. The forward and reverse primers and the FAM-tagged probe used for the galanin gene in the assay were 5'-AAGAATGGCCCTCACACCCAGAA, 5'-GAA-ACCAGGAAGCTTTGACAGG, and 5'-6FAM-AGCTG-CGGCCCGAGAGTACA-TAMRA, respectively. The forward and reverse primers and FAM-tagged probe used for the β-actin gene were 5'-AATTTGAATGATGTATGAAGGCTTTTGG, 5'-TTTTTTTTTTTTTTTTTTTTTTAAG, and 5'-6FAM-CAACT- GGTCTCAGTCAGTGACAGGCCCT-TAMRA, respectively. To assay the initial concentration of the reactants (the sequence abundance), the number of cycles at which the reaction crosses a threshold value was measured. This number varies directly with the initial sequence abundance. To measure the relative abundance of the galanin gene in any given RNA sample, the amplification value derived using the galanin sequence was divided by the amplification value using the β-actin sequence. Derivation of this fraction is independent of RNA sample concentration.

Two-Dimensional PAGE. Cultured cells were solubilized in lysis buffer containing 9.5 mol/L urea (Bio-Rad), 2% NP40, 2% carrier ampholytes (pH 4-8; Gallard/Schlessinger), 2% β-mercaptoethanol, and 10 mM/L phenylmethylsulfonyl fluoride. Protein concentrations were measured by the Bradford assay (Bio-Rad). Proteins (175 μg) were applied to isoelectric focusing gels. Isoelectric focusing was conducted with pH 4 to 8 carrier ampholytes at 700 V for 16 h followed by 1,000 V for an additional 2 h. The first-dimension gel was loaded on the second-dimension gel after equilibration in second-dimension sample buffer [125 mM/L Tris (pH 6.8) containing 10% glycerol, 2% SDS, 1% DTT, and bromophenol blue]. For the second-dimension separation, a gradient of 11% to 14% acrylamide (Serva, Crescent Chemical) was used. Proteins were transferred to an

![Figure 1](https://example.com/figure1.png)

**Figure 1.** DNA microarray analysis of galanin in tissues. Two hundred ninety tissues were analyzed using Affymetrix HuGeneFL GeneChip microarrays. A one-sided Wilcoxon signed-rank test of the individual oligonucleotide features for each probe set was used to determine whether a transcript was either present or absent in a given sample. The mean value of each group was expressed.
Immobilon-P polyvinylidene difluoride membrane (Millipore) or visualized by silver staining of the gels.

**Western Blotting.** After transfer, membranes were incubated for 2 h in blocking buffer containing 5% milk in 10 mmol/L Tris-HCl (pH 7.5), 2.5 mmol/L EDTA (pH 8), and 50 mmol/L NaCl. The membranes were incubated for 1 h at room temperature with a rabbit anti-galanin polyclonal antibody (ADI) at a 1:1,000 dilution. The membranes were then incubated for 1 h with horseradish peroxidase–conjugated anti-rabbit (Amersham) IgG antibodies at a dilution of 1:1,000. Immunodetection was accomplished by enhanced chemiluminescence (Amersham) followed by autoradiography on hyperfilm MP (Amersham). Patterns visualized after hybridization following revelation with patient’s sera were compared directly with Coomassie blue–stained blots from the same sample to determine correlations with proteins. β-Actin was used as a loading control.

**Competitive ELISA.** A 96-well Maxi-sorp microtiter plate (Nunc) was coated by incubation overnight at 4°C with purified galanin (10 μg/mL) in 0.1 mol/L carbonate buffer (pH 9.6). After washing in PBS (pH 7.4), any remaining protein binding sites in the microtiter plate were blocked by incubation with PBS/2% bovine serum albumin overnight at 4°C. Equal volumes of galanin antibody and serum competitor were mixed. The aliquots (100 μL) were added to the wells, in duplicate, after which the plate was incubated for 2 h at room temperature. After washing thrice in PBS/2% bovine serum albumin, 100 μL of horseradish peroxidase–conjugated goat anti-rabbit IgG (1:1,000 dilution) were added to each well and then incubated at room temperature for 1 h. After washing, bound peroxidase was determined using 3,3′,5,5′-tetramethylbenzidine as the substrate. The color reaction was developed for 30 min, stopped by the addition of 50 μL of 1 mol/L HCl to each well, and then read at 450 nm using a microtiter plate reader.

**Statistical Analysis.** Serum levels of galanin above 30 ng/mL were considered to be positive. Data were exported from a Microsoft Access database, formatted, and then loaded into Statistical Analysis System version 8.1 for analysis. An independent set of 90 serum samples had 95% power at the α = 0.05 level to reject an 80% sensitivity or specificity in favor of a true value of 95%, using an exact test for single proportions, with cutoff points for rejection based on the cumulative binomial distribution. Clinicopathologic variables associated with galanin expression were analyzed by either the χ² test or Fisher’s exact test. P < 0.05 was considered significant.

**Results**

**Expression of Galanin mRNA in Colon Cancer.** We profiled the gene expression of 290 tumors and normal tissue, consisting of 6 normal brains, 73 brain cancers, 15 normal colons, 51 colon adenocarcinomas, 10 normal lungs, 57 lung adenocarcinomas, 7 normal pancreas, 8 pancreatic adenocarcinomas, and 63 ovarian cancers by microarrays. To determine which genes were only expressed at high levels in colon cancer, we compared gene expression profiles from colon cancer with those from the other tissues analyzed. We found that galanin expression was ~10-fold higher in colon cancer than in any other tissue type analyzed (Fig. 1).

In addition to expression in tissues, expression of galanin mRNA was confirmed in colon cancer cells by microarray (Fig. 2). Galanin was expressed in all colon adenocarcinoma cells examined (LOVO, HCT116, SW480, and SW620 cells) but not in the A549 lung carcinoma cell line, OVCAR3 and SKOV3 ovarian carcinoma cell lines, or HSI testicular carcinoma cell line. Galanin expression in the colon cancer cells was confirmed by real-time PCR (Fig. 3). Galanin was expressed in all colon adenocarcinoma cells examined (LOVO, HCT15, SW480, and SW620 cells).

**Expression of Galanin Protein in Colon Cancer Cells.** We sought the galanin protein expression in colon cancer cells using two-dimensional Western blot. Colon cancer cell lysate was run in two-dimensional gel electrophoresis and stained in modified silver solutions, and

![Figure 2. DNA microarray analysis of galanin in cancer cells. The LOVO, HCT116, SW480, and SW620 colon adenocarcinoma cell lines, A549 lung carcinoma cell line, OVCAR3 and SKOV3 ovarian carcinoma cell lines, and the HSI testis carcinoma cell line were analyzed using Affymetrix HuGeneFL GeneChip microarrays. A one-sided Wilcoxon signed-rank test of the individual oligonucleotide features for each probe set was used to determine whether a transcript was either present or absent in a given sample. The mean value was expressed.](image)

![Figure 3. Real-time quantitative reverse transcription-PCR for galanin gene in different cancer cells. To measure the relative abundance of galanin gene in a given RNA sample, the amplification value derived using galanin gene was divided by the amplification value using the β-actin sequence.](image)
the gels were blotted on the nitrocellulose membrane and hybridized by galanin antibody. The equivalent spots to expected isoelectric point and molecular weight (5.8 and 12.5 kDa) were observed in colon cancer LOVO cell blot (Fig. 4). We confirmed the galanin expression in colon cancer cells by one-dimensional Western blot (Fig. 5A-D). We compared the levels of expression of galanin protein in colon cancer tissue and its normal counterpart from colon cancer patients and did Western blot (Fig. 5E-H).

Galanin Protein Expression in Colon Cancer Patient Serum. We have analyzed the level of serum galanin protein from normal controls and colon cancer patient using ELISA. Serum levels of galanin above 30 ng/mL were considered to be positive. After a preliminary “training” set of serum from 40 healthy donors and 55 colon cancer patients was analyzed, an independent set of 90 masked serum samples consisting of 24 from healthy donors and 66 from colon cancer patients was measured (Table 1). The average serum levels of galanin in healthy donors and colon cancer patients were 25.6 ± 14.5 and 41.4 ± 19.0 ng/mL, respectively. The average ages were 53.4 ± 9.3 and 63.9 ± 8.1 years old.

The galanin ELISA result yielded a sensitivity of 69.7% (95% confidence interval, 57.1-80.4), specificity of 75.0% (95% confidence interval, 53.3-90.2), and positive predictive value of 88.5% (95% confidence interval, 76.6-95.7). To determine the role of galanin expression in colon cancer, we examined the correlation of colon cancer expression scores with the clinicopathologic features. As shown in Table 2, the galanin expression score was significantly increased with tumor size (26.9 ± 14.6 ng/mL in tumor size <60 mm and 51.4 ± 14.8 ng/mL in tumor size ≥60 mm) and tumor stage (30.5 ± 18.3 ng/mL in Duke’s stage A, 31.4 ± 14.9 ng/mL in Duke’s stage B, 54.0 ± 16.3 ng/mL in Duke’s stage C, and 53.6 ± 12.9 ng/mL in Duke’s stage D). Age, sex, and metastasis were not related to galanin scores. There are statistically significant differences in galanin expression in healthy donors versus colon cancer Duke’s C and D as well as in tumors >60 mm. However, there is no statistically significant difference in galanin expression in healthy donors versus colon cancer Duke’s A and B as well as in tumors <60 mm. Galanin expression might be useful for screening of advanced colon cancer (Duke’s C and D) and tumors >60 mm in size but not early colon cancer. These findings justify a prospective assessment of serum galanin protein as a screening tool for colon cancer.

Discussion

Galanin mediates biological effects by interacting with high-affinity cell surface receptors. Three galanin receptors (GAL-R1, GAL-R2, and GAL-R3) have been cloned thus far. These receptors belong to the family of G protein–coupled receptor superfamily (2). Galanin was coexpressed with its receptors whatever the differentiation stage in neuroblastic tumors (8). The detection of galanin in the human colon cancer cells indicates that these epithelial cells play a key role in host defense of the bowel. In fact, our data show that human serum level for the galanin is high in colon cancer patients.

There are several explanations for a possible influence of the circulating levels of galanin on cancer growth. For instance, circulating levels may be influenced by cancer growth as a result of altered expression of galanin in cancer tissues. As galanin is an inhibitory factor in regulating cell proliferation (9-13), the protection mechanism would be increased with the cancer growth. Indeed, galanin expression was increased in testicular germ cell and neuroblastomas with only a direct influence on cancer proliferation (8, 14). Interestingly, cytolytic effects in tissues were occasionally correlated with the presence of galanin in the study. By contrast, mitogenic effects of galanin have been previously

![Lovo cell blot hybridized with galanin antibody](image)

Figure 4. Two-dimensional PAGE and Western blot analysis of LOVO cell proteins. Bottom, LOVO cell two-dimensional protein pattern after silver staining. An arrow points to the location of galanin, recognized by a polyclonal rabbit anti-galanin antiserum.

![Galanin Protein Expression in Colon Cancer Patient Serum](image)

Figure 5. Western blot of galanin expression in colon cancer cells (A, LOVO; B, HCT116; C, SW480; D, SW620 cells), normal cells (E and F), and cancer cells (G and H) from colon cancer patients. β-Actin was used as a loading control.
described on 235-1 clonal lactotroph cell (15-17). Thus, it is indeed plausible that galanin could contribute to cancer cell selection by promoting proliferation of a subset of malignant cells. At present, it is unknown whether such selection mechanisms could favor colon cancer cell subpopulations that produce galanin. Considering the existing literature together with the data presented in this article, it is interesting to speculate that galanin may contribute to host antitumor immunity or otherwise function as a tumor suppressor.

Screening based on a simple blood test that measures the concentrations of tumor-specific antigens would be far more likely to gain general acceptance among patients and cancer specialists. The most widely used tumor marker for colon cancer is carcinoembryonic antigen, which is a glycoprotein (18-20). However, the clinical applications of carcinoembryonic antigen have been limited to the detection of recurrence after colorectal surgery. Because carcinoembryonic antigen is significantly less sensitive for earlier-stage disease, it was abandoned as a screening marker for early cancer. Therefore, researchers are continually trying to develop new tumor markers with better diagnostic capabilities than carcinoembryonic antigen, which provide a simple and accurate test for early detection.

The sensitivity and specificity for the masked validation set for galanin was 69.7% and 75%, respectively. This result can be directly compared with a value of 40% to 45% and 88% to 90% for carcinoembryonic antigen, which provide a simple and accurate test for early detection.

Table 1. Classification of serum samples from masked validation set by galanin ELISA

<table>
<thead>
<tr>
<th>Classification by galanin ELISA</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>20</td>
<td>46</td>
<td>66</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>52</td>
<td>90</td>
</tr>
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</table>

In conclusion, galanin mRNA was maximally expressed in colon cancer. Colon cancer cell lines showed significantly higher levels of galanin expression than did non-colon cancer cell lines. The levels of galanin in the serum of colon cancer patients were significantly higher than that found in normal subject. Therefore, galanin may have utility in colon cancer screening and diagnosis. Larger-scale studies to establish the potential of these finding should be in progress.

References

Table 2. Clinicopathologic features and galanin expression in colon cancer

<table>
<thead>
<tr>
<th>Clinicopathologic feature</th>
<th>Variable</th>
<th>No. cases</th>
<th>Galanin expression (ng/mL)</th>
<th>P</th>
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<tbody>
<tr>
<td>Age (y)</td>
<td>&lt;60</td>
<td>25</td>
<td>39.7 ± 14.2</td>
<td>NS</td>
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<tr>
<td></td>
<td>≥60</td>
<td>41</td>
<td>42.4 ± 21.4</td>
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</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>26</td>
<td>41.0 ± 18.9</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>40</td>
<td>41.7 ± 19.1</td>
<td></td>
</tr>
<tr>
<td>Tumor size (mm)</td>
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<td>27</td>
<td>26.9 ± 14.6</td>
<td>&lt;60 and ≥60, &lt;0.001</td>
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<tr>
<td></td>
<td>≥60</td>
<td>41</td>
<td>51.4 ± 14.8</td>
<td>Control* and &lt;60, NS</td>
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<tr>
<td>Duke’s stage</td>
<td>A</td>
<td>13</td>
<td>30.5 ± 18.3</td>
<td>A and C, 0.002</td>
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<tr>
<td></td>
<td>B</td>
<td>23</td>
<td>31.4 ± 14.9</td>
<td>A and D, 0.005</td>
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<tr>
<td></td>
<td>C</td>
<td>11</td>
<td>54.0 ± 16.3</td>
<td>B and C, 0.007</td>
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<tr>
<td></td>
<td>D</td>
<td>19</td>
<td>53.6 ± 12.9</td>
<td>B and D, &lt;0.001</td>
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<tr>
<td>Metastasis</td>
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<td>47</td>
<td>40.6 ± 20.5</td>
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<tr>
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<td>M1</td>
<td>19</td>
<td>43.3 ± 14.5</td>
<td>Control* and B, NS</td>
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Abbreviation: NS, not significant.
*Galanin expression in control healthy donors is 25.6 ± 14.5.


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