# Decreased Expression of α2,8 Sialyltransferase and Increased Expression of β1,4 *N*-Acetylgalactosaminyltransferase in Gastrointestinal Cancers

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**Gangliosides such as GD3, GM2, and GD2 are abundantly expressed on the cell surfaces of various malignant cells, suggesting the potential for anti-ganglioside antibody therapy for tumors. Anti-ganglioside GD2 antibody treatment is currently undergoing clinical trials for melanoma and neuroblastoma. We previously reported high *in vivo* antitumor effects of anti-GM2 ganglioside antibody against lung cancer. To determine whether anti-GM2 antibody may be clinically indicated for gastrointestinal cancers, we evaluated the mRNA expression of α2,8 sialyltransferase, a GD3 synthase, and β1,4 *N*-acetylgalactosaminyltransferase (β1,4 GalNAc-T), a GM2/GD2 synthase, in gastrointestinal cancers. We performed modified semi-quantitative RT-PCR, which reduces complexity incidental to radiolabeling on samples taken from small surgically removed clinical specimens. Stomach (19/22) and colorectal (21/30) cancers showed decreased expression of α2,8 sialyltransferase as compared with respective normal tissues ($P < 0.05$). In contrast, increased expression of β1,4 GalNAc-T was detected in both types of tumors. Clinicopathological analysis revealed significantly higher expression level of α2,8 sialyltransferase in the poorly differentiated than in the well-differentiated stomach cancer group ($P < 0.05$). Furthermore, the expression level of α2,8 sialyltransferase was significantly decreased in male as compared with female colorectal cancer patients ($P < 0.05$). These results suggest that expression level of GM2 ganglioside is elevated in gastrointestinal cancer, and that anti-GM2 antibody may be applicable to its treatment.**

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**Key words:** gastrointestinal cancer; ganglioside; β1,4 *N*-acetylgalactosaminyltransferase; α2,8 sialyltransferase

Some tumors of neuroectodermal origin, such as malignant melanoma, glioblastoma, and neuroblastoma, show higher ganglioside expression than normal tissues (1, 2). This has led to the testing of anti-ganglioside antibodies as cancer treatments. Phase I trials testing anti-GD2 antibody against melanoma or neuroblastoma are currently underway (3–6). GM2 is one of the major cell-surface gangliosides expressed in human lung cancer cell lines (7), and has received attention as a target molecule for specific immunotherapy. We previously reported high GM2 expression in adriamycin-resistant cell lines accompanying increased expression level of β1,4 GalNAc-T mRNA, and showed that anti-GM2 antibody treatment overcame resistance to adriamycin both *in vitro* and *in vivo* (8, 9). Determination of the level of ganglioside expression in clinical samples may facilitate clarification of indications for anti-ganglioside antibodies. Gastrointestinal cancer is a major cause of death in adults (10, 11), and most patients with gastrointestinal cancer are not highly responsive to chemotherapy. Therefore, surgical resection in the early clinical stage has been regarded as the best treatment. Adjuvant and neoadjuant chemotherapy have been attempted, but the results remain unclear (12, 13). Anti-ganglioside antibody is a potential alternative therapy for gastrointestinal cancer. To

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determine whether anti-ganglioside antibody might be applicable to gastrointestinal cancer treatment, we examined the expression of two major synthases in normal and tumor tissues.

The synthetic pathways of gangliosides are regulated mainly by two synthetic enzymes; α2,8 sialyltransferase (14) and β1,4 GalNAc-T (15–17). α2,8 sialyltransferase synthesizes GD3 from GM3 and β1,4 GalNAc-T synthesizes GM2/GD2 from GM3 and GD3. It was recently reported that the levels of α2,8 sialyltransferase and β1,4 GalNAc-T mRNA expression correlate with their enzyme activities (18), but determination of both mRNA and protein levels by Northern and Western blot analysis using limited amounts of clinical samples remains difficult. We investigated the expression levels of these two enzymes at the mRNA level using a semi-quantitative RT-PCR method, with some modifications which facilitated analysis of small clinical samples, in both normal and tumor tissues.

## Materials and Methods

**Patients and Preparation of Clinical Samples.** Clinical samples were obtained from 30 patients with colorectal cancer and 22 patients with stomach cancer. Patient characteristics are presented in Table I. Paired specimens of cancer and normal tissues from each patient were obtained from surgically resected gastric or colorectal specimens. All patients had been treated for colorectal or stomach cancer in the 2nd Department of Surgery of Wakayama Medical School. All patients provided written informed consent according to the protocol submitted and approved by local and regional ethical committees. Tissue samples obtained at the time of operation were frozen in liquid nitrogen and stored at −80°C until use. They were broken into pieces and homogenized in ISOGEN (Nippon Gene, Tokyo, Japan). RNA was then extracted following the manufacturer's instructions.

**Table I.** Patient Characteristics

| | Colorectal cancer patient (N = 30) | | Stomach cancer patient (N = 22) | |
|---|---|---|---|---|
| | No. | % | No. | % |
| Age (years) | | | | |
| <65 | 10 (52.2 ± 7.3)[b] | | 12 (52.2 ± 6.9) | |
| ≧65 | 20 (75.0 ± 6.0) | | 10 (70.7 ± 4.8) | |
| Sex | | | | |
| Male | 18 | 60 | 17 | 77 |
| Female | 12 | 40 | 5 | 23 |
| Stage of original diagnosis[a] | | | | |
| I | 9 | 30 | 1 | 5 |
| II | 11 | 37 | 2 | 9 |
| III | 10 | 33 | 6 | 27 |
| IV | 0 | 0 | 13 | 59 |
| Differentiation | | | | |
| Well | 23 | 77 | 8 | 36 |
| Moderate | 4 | 13 | 0 | 0 |
| Poor | 2 | 7 | 13 | 59 |
| Others | 1 | 3 | 1 | 5 |

[a] Stage of original diagnosis was determined by TNM clinical classification.
[b] Mean age ± SD (yrs).

**Gene Expression by Semi-quantitative RT-PCR.** Samples of 1 μg of RNA were subjected to reverse transcription, separately with random hexamer and oligo $d(T)^{16}$ as a primer. cDNAs were synthesized using extracted RNA, and semi-quantitative PCR was performed using a modification of the method of Kinoshita *et al.* (19). PCR was carried out for each gene using the following primers: for a 749-bp fragment of human α2,8 sialyltransferase: 5′-GGTATGACGGGGAGTTTTTAT-3′ (sense primer) and 5′-AGTGGGCTGGAGTGAGGTATC-3′ (antisense primer), for a 925-bp fragment of human β1,4 GalNAc-T: 5′-ACTCGAAGACCGAAATTTTGCCGCTGCCTTAG-3′ (sense primer) and 5′-GATGAGAGCCCGTAGCCGATCATA-3′ (antisense primer), and for a 578-bp fragment of human β-actin: 5′-CCCCATGCCATCCTGCGTCTG-3′ (sense primer) and 5′-TCGTCATACTCCTGCTTGCTG-3′ (antisense primer). To quantitate the expression ofα2,8 sialyltransferase and β1,4 GalNAc-T, β-actin was used as an internal control. In short, the β-actin oligonucleotide primer was added to the same reaction tube and every two cycles from cycle 28 to cycle 40, 5 μl of the PCR product was removed. PCR conditions were 38 or 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Each removed sample was subjected to electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining (Fig. 1A).

**Quantitative Analysis and Statistics.** To quantitate each PCR product, we performed image analysis using a Kodak Digital Science 1D (Eastman-Kodak, New Haven, CT). After quantitation of each PCR product, we plotted the amplification curves of the gene of interest and β-actin from each sample. The difference in the cycle in the logarithmic phase of the amplification curves of the gene of interest and β-actin in each sample was defined as Δ-cycle (Fig. 1B). Δ-cycle correlates linearly with the initial amount of the gene of interest. We calculated the Δ-cycle difference between tumor and normal tissues (T-N) from each patient. The paired *t* test or Mann-Whitney's U test was used to compare expression levels among the patients. All reported *P* values are based on two-sided tests. Statistical analyses were implemented using Stat View software (Abacus Concepts, Berkeley, CA)

## Results

Clinical samples from 30 patients with colorectal cancer and 22 patients with stomach cancer were examined using a semiquantitative RT-PCR method. Δ-cycle values of each clinical sample are shown in Table II. A total of 21 of 30 patients (70%) with colorectal cancer and 19 of 22 (86%) with stomach cancer had decreased α2,8 sialyltransferase expression in cancer tissue as compared with normal tissue (Fig. 2A). This suggested GM2/GM3 expression to be

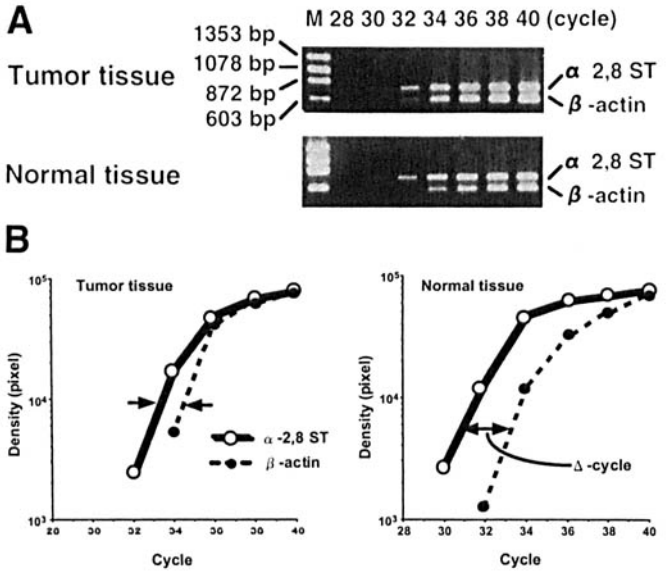


**Figure 1.** Semiquantitative RT-PCR. (A) β-actin oligonucleotide primer was added to the same reaction tube, and every two cycles from cycles 28 to 40, 5 μl of the PCR product was removed. Each sample was subjected to electrophoresis on a 2% agarose gel and was visualized by ethidium bromide staining. (B) After quantitation of the intensity of the band by densitometry (Kodak Digital Science 1D), we plotted the amplification curves of the gene of interest and β-actin in each sample. The cycle difference between the logarithmic phase of the amplification curves of the gene of interest and β-actin in each sample was defined as Δ-cycle. α2, 8-ST: α2, 8-sialyltransferase.

higher than GD2/GD3 expression in tumor tissue. A statistically significant decrease in α2,8 sialyltransferase expression was observed in both colorectal and stomach cancer patients ($P < 0.05$).

A total of 17 of 30 patients (57%) with colorectal cancer and 12 of 22 (55%) with stomach cancer had increased expression of β1,4 GalNAc-T in cancer tissue as compared with normal tissue. Stomach cancer patients had significantly increased expression of β1,4 GalNAc-T in cancer tissue as compared with normal tissue ($P < 0.05$), whereas colorectal cancer patients only showed a tendency for increased expression in cancer tissue (Fig. 2B). These results suggested expression of GM2/GD2 ganglioside to be higher than that of GM3/GD3 in tumor tissue.

Overall, these results suggested that decreased expression of α2,8 sialyltransferase and increased expression of β1,4 GalNAc-T might result in accumulation of GM2 in stomach and colorectal tumors.

Clinicopathologic factors such as age, sex, clinical stage, and types of differentiation were analyzed (Table III). The expression level of α2,8 sialyltransferase was significantly higher in the poorly-differentiated than in the well-differentiated stomach cancer group ($P < 0.05$). Furthermore, a significant correlation was observed between the expression level of α2,8 sialyltransferase and gender in colorectal cancer. Male patients with colorectal cancer had significantly lower expression of α2,8 sialyltransferase than female patients ($P < 0.05$).

**Table II.** Results of Semi-quantitative PCR

| Colorectal cancer T-N (Δ-cycle) | | | Stomach cancer T-N (Δ-cycle) | | |
|---|---|---|---|---|---|
| Patient | α2,8-ST[a] | β1,4-GNT[b] | Patient | α2,8-ST | β1,4-GNT |
| 1 | −2.0 | 2.0 | 1 | 3.0 | 0.0 |
| 2 | −0.4 | 2.8 | 2 | −3.0 | 0.0 |
| 3 | −0.3 | 0.4 | 3 | −0.2 | −3.0 |
| 4 | −0.4 | −0.3 | 4 | −3.7 | 1.5 |
| 5 | 4.3 | 0.0 | 5 | −6.3 | 1.8 |
| 6 | −0.8 | −0.2 | 6 | 2.2 | −0.2 |
| 7 | −0.1 | −0.8 | 7 | −2.1 | 0.0 |
| 8 | −11.0 | 4.2 | 8 | 0.4 | 4.3 |
| 9 | 0.5 | 1.4 | 9 | −1.9 | −1.7 |
| 10 | −0.7 | 3.0 | 10 | −1.5 | −4.2 |
| 11 | −6.7 | 0.0 | 11 | −2.2 | 1.5 |
| 12 | 0.4 | 1.4 | 12 | −0.1 | 0.3 |
| 13 | −2.8 | 0.0 | 13 | −0.7 | 3.5 |
| 14 | −7.4 | 3.2 | 14 | −2.9 | 5.8 |
| 15 | 0.4 | 0.6 | 15 | −2.3 | 0.8 |
| 16 | −2.6 | 1.5 | 16 | −0.9 | 3.0 |
| 17 | −4.0 | 0.8 | 17 | −5.2 | 3.0 |
| 18 | −1.4 | 1.6 | 18 | −1.0 | 2.2 |
| 19 | 0.4 | 4.2 | 19 | −5.4 | −1.2 |
| 20 | −0.8 | −0.2 | 20 | −3.0 | 0.0 |
| 21 | −8.4 | −0.8 | 21 | −0.5 | 2.4 |
| 22 | −5.4 | 0.0 | 22 | −3.5 | 0.0 |
| 23 | 0.1 | 0.0 | | | |
| 24 | −1.2 | 2.7 | | | |
| 25 | 0.3 | 0.0 | | | |
| 26 | 0.3 | 1.3 | | | |
| 27 | −2.7 | 1.8 | | | |
| 28 | −2.7 | 6.6 | | | |
| 29 | 0.4 | 0.0 | | | |
| 30 | −1.3 | 0.0 | | | |

*Note.* T-N (Δ-cycle) means the difference in expression level of targeted genes between tumor and normal tissues.
[a] α2,8 sialyltransferase.
[b] β1,4 GalNAc-T.

## Discussion

In general, glycolipids including gangliosides synthesized by α2,8 sialyltransferase or β1,4 GalNAc-T are difficult to detect immunohistochemically. This is especially true of clinical samples, which are often preserved under improper conditions. Therefore, we attempted to estimate the expression of these gangliosides by measuring the expression of the enzymes that synthesize them. Northern blot analysis has been widely employed to detect expression at the transcriptional level, though there are a few problems with clinical specimens. In most cases, total amounts of clinical samples, especially tissue samples, are not sufficient for Northern or Western blot analysis. Gangliosides synthesized by β1,4 GalNAc-T are usually expressed at very low levels, and are therefore particularly difficult to detect in tissue samples (20). Kinoshita *et al.* (19) established a semiquantitative method of measuring mRNA expression by using β-actin as an internal control in RT-PCR. We employed essentially the same method in the present study, but used ethidium bromide staining instead of radiolabeled probe.

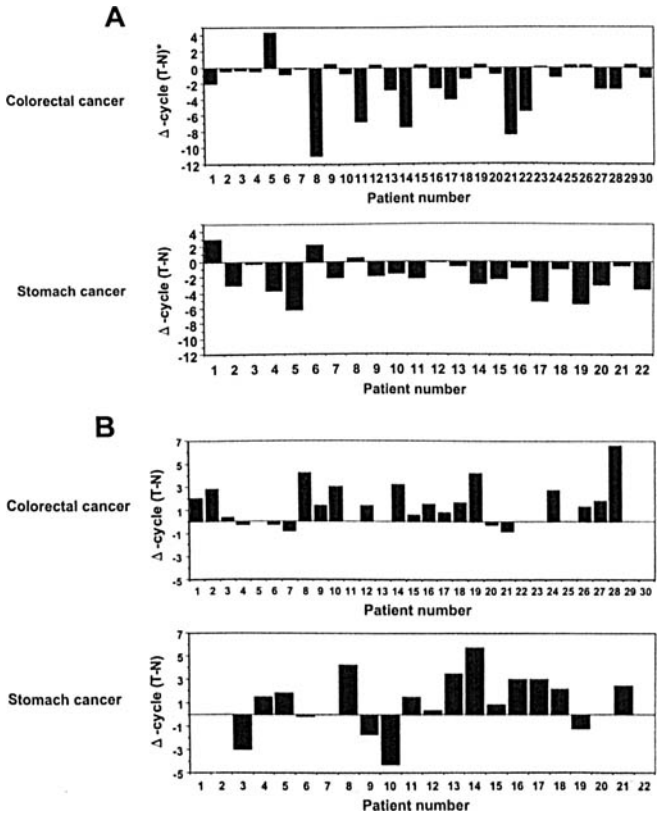


**Figure 2.** Expression of two major ganglioside synthases. (A) Expression of α2, 8-sialyltransferase. A total of 21 of 30 colorectal cancer patients and 19 of 22 stomach cancer patients showed decreased expression in tumor compared with normal tissue. A significant decrease in α2, 8-sialyltransferase expression was observed in both colorectal and stomach cancer patients ($P < 0.05$). (B) Expression of β1, 4 GalNAc-T. A total of 17 out of 30 colorectal cancer patients and 12 of 22 stomach cancer patients had higher expression of β1, 4 GalNAc-T in tumor than normal tissue. Stomach cancer patients had significantly higher expression of β1, 4 GalNAc-T in cancer tissue ($P < 0.05$) than in colorectal cancer patients. However, the latter also showed increased expression in tumor compared with normal tissue. *(T-N) indicates the Δ-cycle difference between tumor and normal tissue.

We found α2,8 sialyltransferase mRNA expression to be decreased in tumor tissues from both colorectal and stomach cancer patients. No evidence on α2,8 sialyltransferase expression has been reported in gastrointestinal cancers using clinical samples. This enzyme determines whether the synthetic pathway proceeds to production of the GM3/GM2 or the GD3/GD2 series. Our results suggested the ganglioside synthase pathway to proceed mainly to production of the GM3/GM2 series. In clinicopathologic analysis, poorly-differentiated stomach cancers showed significantly higher expression level of α2,8 sialyltransferase than well-differentiated stomach cancers. This suggested α2,8 sialyltransferase might possibly be attributed to histologic tumor differentiation. We also found significantly decreased expression of α2,8 sialyltransferase in male patients as compared with female patients. Ganglioside expression is reportedly modulated by gender difference, and castration of male rats induced an increase in sialyltransferase activity (20). Administration of testosterone to castrated male rats increased the b-series gangliosides contents, especially that of GD3 ganglioside, but did not significantly affect the level of other ganglioside proteins. Our results were also consistent with a sex-related difference in sialyltransferase activity. We also found β1,4 GalNAc-T mRNA expression to be increased in colorectal and stomach cancer tissues as compared with respective normal tissues from the same patients. This result is consistent with previous reports and indicates the possibility of GM2/GD2 ganglioside accumulation in these tumors (21). Together with the increased expression level of β1,4 GalNAc-T, we strongly suggest that decreased α2,8 sialyltransferase expression may have a pivotal role in enhanced GM2 content in gastrointestinal cancers, and that anti-GM2 antibody may be applicable to gastrointestinal cancer treatment.

Anti-ganglioside GM2 antibody was found to mediate complement-dependent cytotoxicity (CDC) and antibody-

**Table III.** Statistical Correlation

| | Colorectal cancer mean value of T-N (Δ-cycle) | | Stomach cancer mean value of T-N (Δ-cycle) | |
|---|---|---|---|---|
| | α2,8-ST | β1,4-GNT | α2,8-ST | β1,4-GNT |
| Age (years) | | | | |
| <65 | −2.0 ± 3.4 NS[a] | 1.1 ± 1.4 NS[a] | −2.1 ± 2.5 NS[a] | 0.2 ± 2.0 NS[a] |
| ≧65 | −1.8 ± 3.1 | 1.5 ± 2.3 | −1.5 ± 2.1 | 1.7 ± 2.5 |
| Sex | | $P < 0.05$[c] | | |
| Male | −2.2 ± 3.5 $P < 0.05$[b] | 1.5 ± 2.0 NS | −1.6 ± 2.5 NS | 0.8 ± 2.3 NS |
| Female | −1.2 ± 2.6 | 0.8 ± 1.2 | −2.7 ± 0.7 | 1.1 ± 2.9 |
| Stage of original diagnosis | | | | |
| I, II | −1.2 ± 3.0 NS | 0.9 ± 1.4 NS | −1.1 ± 4.2 NS | 0.6 ± 2.2 NS |
| III, IV | −3.2 ± 3.2 | 2.0 ± 2.2 | −2.0 ± 2.0 | 0.9 ± 2.4 |
| Differentiation | | | | |
| Well | −2.0 ± 2.8 NS | 1.0 ± 1.7 NS | −3.3 ± 1.7 $P < 0.05$[c] | −0.4 ± 2.0 NS |
| Poor | −0.9 ± 0.7 | 0.7 ± 1.3 | −1.3 ± 2.1 | 1.8 ± 2.3 |

[a] Not significant.
[b] Male patients with colorectal cancer had significantly lower expression of α2,8 sialyltransferase than female patients.
[c] Expression level of α2,8 sialyltransferase was significantly higher in the poorly-differentiated than in the well-differentiated stomach cancer group.

dependent cellular cytotoxicity (ADCC) (7). Gangliosides have been implicated in apoptotic pathways, and GM2 in particular may function in signal transduction pathways that regulate cell growth and cell division (22–24). It was recently reported that anti-GM2 antibody treatment resulted in apoptosis-like morphological changes in a multicellular heterospheroid model, which more closely mimics the *in vivo* state than monolayer cell culture, independent of effector functions (25). Biological conditions differ between *in vitro* and *in vivo* states and this may have a critical impact, especially on the mechanisms of action of anti-ganglioside antibody. Thus, it is apparently very important to determine *in vivo* status, especially in human tissues. In conclusion, our results obtained in human tissues support the potential application of anti-GM2 antibody to the treatment of patients with gastrointestinal cancers.


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