Demethylation of the Synuclein γ Gene CpG Island in Primary Gastric Cancers and Gastric Cancer Cell Lines

Naoki Yanagawa,¹ Gen Tamura,¹
Teiichiro Honda,¹ Makoto Endoh,¹
Satoshi Nishizuka,² and Teiichi Motoyama¹
¹Department of Pathology, Yamagata University School of Medicine, Yamagata, Japan, and ²Laboratory of Molecular Pharmacology, National Cancer Institute, NIH, Bethesda, Maryland

ABSTRACT

Purpose: Whereas synuclein γ (SNCG) gene expression is usually highly tissue-specific and restricted to the nervous system, SNCG is expressed in advanced-stage breast and ovarian cancers. When overexpressed, SNCG stimulates cancer cell proliferation and metastasis. It is thought that the molecular mechanism of CpG island demethylation may underlie aberrant SNCG expression. To determine whether aberrant SNCG expression and demethylation play a role in gastric carcinogenesis, we examined the expression and methylation status of SNCG in primary gastric cancers, gastric cancer cell lines, and non-neoplastic gastric mucosal tissues.

Experimental Design: Ten gastric cancer cell lines, 10 primary gastric cancers, and 10 non-neoplastic gastric mucosal tissues were examined. SNCG expression and methylation status were examined by reverse transcription-PCR and bisulfite-single-strand conformational polymorphism followed by direct sequencing, respectively. The relationship between SNCG methylation status and various clinicopathological factors of the primary gastric cancers was then analyzed.

Results: SNCG mRNA expression was observed in 5 of 10 cell lines. Analysis of cell lines positive for SNCG expression revealed that most of the SNCG CpGs were demethylated. SNCG mRNA was not expressed in the 10 non-neoplastic gastric mucosal tissues, although several CpGs were demethylated. Of the 105 primary gastric cancers, 40 (38.1%) showed apparent SNCG demethylation, similar to the result obtained using cell lines. SNCG demethylation was more frequent in primary gastric cancers positive for lymph node metastasis (51%; 26 of 51) than in cancers without lymph node involvement (26%; 14 of 54; P < 0.05), and also more common in stage II-IV (48%; 27 of 56) than in stage I (27%; 13 of 49) cancers (P < 0.05).

Conclusions: Aberrant SNCG gene expression can occur via CpG island demethylation, and tends to occur during the more progressive stages of gastric carcinogenesis.

INTRODUCTION

DNA methylation at CpG dinucleotides has been recognized as an important mechanism for the regulation of gene expression in mammalian cells (1, 2). Epigenetic alterations, including hypermethylation of promoter CpG islands, histone deacetylation of tumor suppressor and tumor-related genes (1–5), and global DNA hypomethylation (6–8), have been recognized as important contributors to carcinogenesis in humans. Global DNA hypomethylation has been observed in carcinomas of the breast, liver, and colon, and is thought to occur during the early stages of tumor development (9–13). However, little is known about hypomethylation of specific gene promoters, such as promoters for oncogenes and growth-related genes, with the exception of the correlation between demethylation and increased expression of c-abl, c-myc, c-Ha-ras, and raf (14–17).

The synuclein γ (SNCG) gene, also known as the breast cancer specific gene 1 (BCSG1), is a member of the synuclein neuronal protein family along with synuclein α (SNCA) and synuclein β (SNCB; Refs. 18–20). Although the function of synuclein proteins remains largely unknown, SNCA aggregation may be important in the etiology and pathogenesis of neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease (21). SNCG is located on chromosome 10q23, transcribed as a 1Kb mRNA, and encodes a 127-amino acid polypeptide (19). SNCG protein is highly tissue-specific, and is transcribed as a 1Kb mRNA, and encodes a 127-amino acid polypeptide (19). SNCG protein is highly tissue-specific, and is expressed at presynaptic terminals in the brain and peripheral nervous system (19, 20). However, this tissue specificity appears to be lost during breast and ovarian cancer disease progression. Whereas SNCG expression is normally silent in the breast and ovary, it becomes abundantly expressed in the vast majority of advanced-stage breast and ovarian cancers (22). SNCG expression is activated by demethylation of the SNCG CpG island in a similar manner to the oncogenes and growth-related genes described above (14–17, 22). These findings suggest that SNCG might act as a proto-oncogene, and play a positive role in the process of invasion and metastasis in breast and ovarian cancer (22). To determine whether aberrant expression and demethylation of SNCG is involved in gastric carcinogenesis, we examined the expression and methylation status of SNCG in primary gastric cancers, gastric cancer cell lines, and non-neoplastic gastric mucosal tissues.

MATERIALS AND METHODS

Cell Lines. Ten gastric cancer cell lines with different histologies were cultured under appropriate conditions in our laboratory, MKN1, an adenosquamous cell carcinoma; MKN7,
Table 1  Correlation between methylation status and clinicopathological factors of primary gastric cancers

| Clinicopathological factors (n) | Methylation status |  |
|-------------------------------|--------------------|
|                              | Demethylated (40)  | Methylated (65) |
| Sex                           |                    |                |
| Male (80)                     | 29                 | 51             |
| Female (25)                   | 11                 | 14             |
| Age (years)                   | 68.4 ± 1.6         | 66.6 ± 1.2     | NS
| Location                      |                    |                |
| Upper third (20)              | 10                 | 10             |
| Middle third (25)             | 11                 | 24             |
| Lower third (44)              | 18                 | 26             |
| Unknown (6)                   | 1                  | 5              |
| Histological types            |                    |                |
| Differentiated (69)           | 28                 | 41             |
| Undifferentiated (36)         | 12                 | 24             |
| Stage                         |                    |                |
| I (49)                        | 13                 | 16             |
| II-IV (56)                    | 27                 | 29             |
| Lymph node metastasis         |                    |                |
| Positive (51)                 | 26                 | 25             |
| Negative (54)                 | 14                 | 40             |

* NS, not significant.

a well-differentiated adenocarcinoma; MKN28 and MKN74, moderately differentiated adenocarcinomas; MKN45 and KWS-I, poorly differentiated adenocarcinomas; KATO-III, a signet ring cell carcinoma; ECC10 and ECC12, endocrine cell carcinomas; and TSG11, a hepatoid carcinoma. Genomic DNA and mRNA were extracted from each cell line using standard procedures.

Gastric Cancer Patients and Surgical Specimens. One hundred and five gastric cancer samples were obtained at surgery. Patients ranged in age from 43 to 89 years (mean, 67 years). All of the patients received a median of 37.5 months of follow-up care (range, 3–77 months). All of the samples were snap-frozen and stored at −80°C until processing. The tumors consisted of 40 early and 65 advanced tumors, and were identified as 69 differentiated and 36 undifferentiated tumor samples. Lymph node metastasis was positive in 51 cases, and 49 cases were stage I (Table 1). Genomic DNA was extracted from each sample using standard procedures.

Non-Neoplastic Gastric Mucosal Tissues. Ten non-neoplastic gastric mucosal tissues were obtained at autopsy. The autopsy specimens consisted of 6 males and 4 females, ranging in age from 8 to 86 years (mean, 62.7 years). Genomic DNA and mRNA were extracted from the tissues using standard procedures.

Reverse Transcription-PCR. RNA isolated from cell lines and non-neoplastic gastric mucosal tissues was reverse transcribed and amplified using the ONE-STEP reverse transcription-PCR System (Life Technologies, Inc., Gaithersburg, MD). Primer sequences used were sense 5′-CAA GAA GGG CCT CTC CAT CGC CAA GG-3′ and antisense 5′-CCT TCT TGG ATG CCA CAC CC-3′ for SNCG (318 bp; Ref. 22). A 587-bp β-actin fragment was amplified as a control. After initial activation of the enzyme at 94°C for 2 min, PCR reactions were exposed to 38 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

5-Aza-2′-Deoxycytidine Treatment. To examine SNCG expression in response to treatment with 5-aza-2′-deoxycytidine (Sigma), cell lines were incubated for 96 h with 0.2 or 1 μM 5-aza-2′-deoxycytidine, and then harvested for RNA extraction and reverse transcription-PCR.

Bisulfite-Single-Strand Conformation Polymorphism (SSCP) and Sequencing Analyses. DNA samples were treated with bisulfite to convert all of the unmethylated cytosines to uracils, while leaving methylated cytosines unaffected. Briefly, 2 μg of aliquots of genomic DNA were denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, WI), treated with NaOH, precipitated with ethanol, and resuspended in 30 μl. DNA methylation status was determined by bisulfite-SSCP with some modifications (23). After treatment of DNA with sodium bisulfite, a 415-bp fragment encompassing the CpG island, and exon 1 of the SNCG gene was amplified using primers 5′-GTT GTT AGT AGG AGT TTA-3′ and 5′-CCT ACC ATA CCC CAC TTA CCC-3′ (22). The PCR mix contained 1× PCR buffer (15 mM Tris-HCl (pH 8.0) and 50 mM KCl), 1.5 mM MgCl2, 0.2 mM deoxynucleoside triphos-
Fig. 3 Sequencing histograms of SNCG exons 1 using an antisense primer. Bisulfite treatment of the MKN 74 DNA converted all cytosines to thymines (A), cytosines remained as cytosines in TSG11 (B). Several cytosines were converted to thymines and others remained as cytosines in a non-neoplastic gastric mucosal tissue obtained at autopsy (C). *, converted thymines.

![Sequencing histograms of SNCG exons 1](image)

Fig. 4 Comparison of SNCG mRNA expression before (−) and after (+) 5 aza-dC treatment. Treatment with 5 aza-dC restored SNCG mRNA expression in MKN1 and MKN7, but did not affect SNCG expression level in MKN28.

![Comparison of SNCG mRNA expression](image)

MKN1 MKN7 MKN28

5-aza-dC − + − + − SM

-318 bp

Fig. 5 Overall survival of 105 gastric cancer patients. There was a tendency for patients with poor prognosis to show SNCG demethylation compared with patients with normal methylation ($P = 0.076$).

![Overall survival of gastric cancer patients](image)
exhibited mobility-shifted bands by bisulfite-SSCP (Fig. 2A). Sequencing of the SSCP bands revealed that whereas the SNCG exon 1 CpGs were mostly demethylated in the cell lines positive for SNCG mRNA (Fig. 3A), the CpGs remained methylated in the remaining 5 cell lines (Fig. 3B), and the latter cell lines expressed SNCG mRNA after 5-aza-2‘-deoxycytidine treatment (Fig. 4).

**Methylation Status in Primary Gastric Cancers.** Of the 105 primary gastric cancer samples examined, 40 (38.1%) showed band mobility shifts by SSCP. Sequencing of the shifted bands demonstrated demethylated CpGs, similar to the results obtained for the gastric cancer cell lines that expressed SNCG mRNA (Fig. 2B).

**mRNA Expression and Methylation Status of SNCG in Non-Neoplastic Gastric Mucosal Tissues.** SNCG mRNA was not expressed in any of the 10 non-neoplastic gastric mucosal tissues. However, a few tissues exhibited faint bands with mobility shifts by bisulfite-SSCP (data not shown), and sequencing of these bands revealed partial demethylation of SNCG CpGs (Fig. 3C).

**Correlation between Methylation Status and Clinico-pathological Factors of Primary Gastric Cancers.** Clinico-pathological features of primary gastric cancers showing SNCG demethylation were analyzed. Demethylation of SNCG was more frequent in lymph node metastasis-positive primary gastric cancers (51%; 26 of 51) than in metastasis-negative cancers (26%; 14 of 54; P < 0.05), and in stage II-IV cancers (48%; 27 of 56) than in stage I cancers (27%; 13 of 49; P < 0.05; Table 1).

**SNCG Demethylation and Patient Prognosis.** Kaplan-Meier estimates were used to examine the relationship between SNCG demethylation and patient survival. Although not statistically significant, there was a tendency toward patients with a poor prognosis showing SNCG demethylation (P = 0.076; Fig. 5).

**DISCUSSION**

In the present study, we examined the expression and/or methylation status of SNCG in gastric cancer cell lines, primary gastric cancers, and non-neoplastic gastric mucosal tissues. For the gastric cancer cell lines, SNCG mRNA expression strongly correlated with demethylation of SNCG exon 1 CpG islands. Whereas SNCG was not expressed in non-neoplastic gastric mucosal tissues obtained at autopsy, partial demethylation was present in these tissues. We also examined SNCG methylation status in several non-neoplastic gastric mucosal tissues from gastric cancer patients. Although demethylation of SNCG was observed frequently in these tissues, demethylation only affected a few CpGs, similar to the autopsy samples (data not shown). Similar findings have also been reported for normal breast tissue (22). These results suggest that demethylation occurs before malignant transformation and that only partial demethylation does not result in up-regulated SNCG mRNA expression. Thus, it appears that partial SNCG demethylation can occur in normal gastric mucosa, which then extends in some cases to become to fully demethylated, resulting in up-regulated SNCG mRNA expression. At present, the normal cellular functions of SNCG are largely unknown. Future studies are required to clearly define the function of this protein in neurons as well as in neoplasms, and will provide insight into the molecular mechanisms that contribute to human carcinogenesis.

We also analyzed the clinico-pathological features of gastric cancers that showed SNCG demethylation. Previous studies have suggested that SNCG acts as an oncogene in breast and ovarian cancer cells (22, 24, 25). A stage-specific expression pattern of SNCG mRNA has been demonstrated by in situ hybridization that varied from virtually no detectable expression in normal or benign breast tissues to low level or partial expression in low-grade ductal carcinoma in situ, through to high expression in advanced infiltrating carcinomas (24). Similarly, immunohistochemical studies have shown that SNCG protein is not expressed in normal breast tissues but is expressed in a high percentage of stage III/IV breast carcinomas (25). SNCG is also expressed in ovarian cancers (25). In the present study, SNCG demethylation was more frequent in primary gastric cancers positive for lymph node metastasis (51%; 26 of 51) than in metastasis-negative cancers (26%; 14 of 54; P < 0.05), and was also more frequent in stage II-IV cancers (48%; 27 of 56) than in stage I cancers (27%; 13 of 49; P < 0.05). Our results are in agreement with those obtained for breast and ovarian cancers (25). Although not statistically significant, there was an increased tendency for gastric cancer patients with poor prognoses to show SNCG demethylation compared with gastric cancer patients with normal methylation (P = 0.076).

Although additional studies on gene methylation, protein expression, and localization in normal and transformed tissues are required to establish that the methylation status of synuclein γ CpG islands regulates mRNA, and ultimately protein expression, a distinct correlation between mRNA levels and CpG methylation is established by the present study. Our study indicated that SNCG was frequently expressed, via CpG demethylation, during the more progressive stages of gastric carcinogenesis. SNCG, a potential oncoprotein, may play a role in the progression and metastasis of common human malignancies.

**REFERENCES**


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