Original Article

# Clinical Significance of Circulating Tumor Cells in Peripheral Blood From Patients With Gastric Cancer

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**BACKGROUND:** The authors hypothesized that circulating tumor cells (CTCs) in patients with gastric cancer are associated with prognosis and disease recurrence. In this study, they evaluated CTCs in gastric cancer and clarified the clinical impact of CTCs. **METHODS:** In total, 265 consecutive patients with gastric cancer were enrolled. Fourteen patients were excluded from the analysis, including 12 patients who another cancer and 2 patients who refused the treatment. The remaining 251 patients were divided into 2 groups: 148 patients who underwent gastrectomy (the resection group) and 103 patients who did not undergo gastrectomy (the nonresectable group). Peripheral blood samples were collected before gastrectomy or chemotherapy. A proprietary test for capturing, identifying, and counting CTCs in blood was used for the isolation and enumeration of CTCs. **RESULTS:** CTCs were detected in 16 patients (10.8%) from the resection group and in 62 patients (60.2%) from the nonresectable group. The overall survival rate for the entire cohort was significantly lower in patients with CTCs than in those without CTCs ($P<.0001$). In the resection group, relapse-free and overall survival in patients with CTCs was significantly lower than in patients without CTCs ($P<.0001$). It was noteworthy that the expression of CTCs was an independent factor for determining the overall survival of patients with gastric cancer in multivariate analysis ($P=.024$). In the nonresectable group, the overall survival rate was significantly lower in patients with CTCs than in those without CTCs ($P=.0044$). **CONCLUSIONS:** The evaluation of CTCs in peripheral blood may be a useful tool for predicting tumor progression, prognosis, and the effect of chemotherapy in patients with gastric cancer. *Cancer* 2013;119:3984-91. © *2013 American Cancer Society.*

**KEYWORDS:** circulating tumor cells, gastric cancer, prognosis, peritoneal dissemination, hematogenous recurrence.

## INTRODUCTION

The presence of circulating tumor cells (CTCs) has been evaluated in blood from patients with gastrointestinal cancers.[1-4] The early detection of CTCs has the possibility of providing useful information before the start of treatment, including surgery and/or systemic chemotherapy. Some patients develop recurrent disease after surgery, even after undergoing complete resection of their primary tumor. Currently, the prognosis for patients with gastric cancer has been improved by the development of new anticancer drugs However, if the presence of CTCs is confirmed before surgery, then the use of neoadjuvant chemotherapy may be indicated, and this may have an impact on the timing of surgical intervention. Furthermore, the presence of CTCs in patients with distant metastasis would be a useful parameter for evaluating the effect of chemotherapy. Various methods for detecting rare CTCs have been attempted using a molecular biologic approach, such as reverse transcriptase-polymerase chain reaction (RT-PCR) analysis and flow cytometry in gastric cancer.[4-7] Although CTCs have been evaluated in blood from patients with gastric cancer, the clinical significance of CTCs remain unclear. Several authors have reported that the detection of CTCs using RT-PCR in gastric cancer is useful for predicting prognosis.[8-11] The detection of CTCs in blood requires high sensitivity and reproducibility.

The CellSearch system (Veridex LLC, Warren, NJ) was developed to identify CTCs in blood, and its utility has been reported in patients with breast cancer and prostate cancer.[12,13] The presence of CTCs is correlated with shorter overall survival in patients with metastatic disease. However, there have been few reports regarding the evaluation of CTCs in patients with gastric cancer using the CellSearch system. We hypothesized that CTCs in patients with gastric cancers are

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associated with prognosis and the recurrence. In this study, we evaluated CTCs in patients with gastric cancer and explored the clinical impact of CTCs using the CellSearch system.

## MATERIALS AND METHODS

### Gastric Cancer Cell Line

To prepare for an examination of the CellSearch system, we used the KATO III gastric cancer cell line for the analysis. KATO III cells were cultured in RPMI 1640 (Nissui Pharmaceutical Company, Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (Mitsubishi Kasei, Tokyo, Japan), 100 U/mL penicillin, and 100 U/mL streptomycin. Cancer cells were grown at 37°C in a humidified atmosphere containing 5% $CO_2$, as previously described.

### Clinical Study Design

Patients with gastric cancer who received treatment at 2 medical centers (Kagoshima University Hospital and Jiaikai Imamura Hospital, Kagoshima, Japan) were analyzed using prospectively collected data. Informed consent was obtained from all patients in accordance with the ethical standards of the Committee on Human Experimentation of Kagoshima University Hospital and Jiaikai Imamura Hospital. We evaluated the usefulness of measuring CTC levels with regard to the overall survival of patients with gastric cancer. In total, 265 consecutive patients with gastric cancer were enrolled between February 2005 and December 2012 at 2 medical centers. Two hundred twenty-eight patients from Kagoshima University Hospital and 37 patients from Jiaikai Imamura Hospital were registered on the study. Fourteen patients were excluded from the analysis, including 12 patients who had another cancer, such as esophageal, colorectal, or prostate cancer, and 2 patients who refused the treatment for gastric cancer. The patients were divided into 2 groups; those who underwent gastrectomy (the resection group; N = 148) and those who did not undergo gastrectomy (the nonresectable group; N = 103) (Fig. 1). Patients in the resection group underwent gastrectomy with standard lymphadenectomy. Patients who had received any preoperative radiotherapy or chemotherapy were excluded from this study. Peripheral blood samples were collected before gastrectomy. Clinical stage was assigned according to the TNM classification.[14]

Patients in the nonresectable group did not undergo surgery because of the presence of distant metastasis or recurrence. Peripheral blood was collected before the beginning of chemotherapy in these patients. In the current study, various chemotherapy regimens were used and mainly included the oral fluoropyrimidine S-1, such as S-1 alone, S-1 plus cisplatin, S-1 plus paclitaxel, and so on.

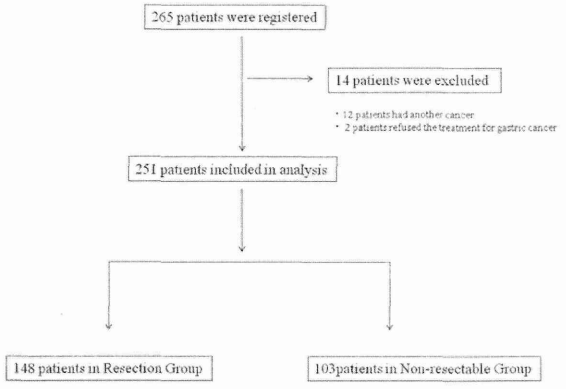


**Figure 1.** In total, 265 consecutive patients with gastric cancer were enrolled in the study. Fourteen patients were excluded from the analysis: 12 patients had another cancer, and 2 patients refused the treatment for gastric cancer. There were 148 patients with gastric cancer in the resection group and 103 patients who did not undergo gastrectomy in the nonresectable group.

All patients in the resection group were followed after discharge by physical examinations, routine blood tests, serum tumor marker tests (carcinoembryonic antigen [CEA] and cancer antigen 19-9 [CA 19-9]), and computed tomography scans every 3 to 6 months. Follow-up data after discharge were obtained for all patients, and the median follow-up was 31.6 months (range, 4-72 months). In the nonresectable group, patients were evaluated for chemotherapy every 2 to 3 months until death.

### Isolation and Enumeration of Circulating Tumor Cells

Ten-milliliter blood samples were drawn into CellSave Preservative Tubes (Veridex, LLC). The samples were maintained at room temperature and processed within 72 hours after collection. All evaluations were performed by technical assistants without knowledge of the clinical status of the patients. The CellSearch system was used to isolate and enumerate CTCs using 7.5 mL of the 10-mL samples. CellSearch is a semiautomated system for the preparation of a sample and is used with the CellSearch Epithelial Cell Kit. The procedure enriches the sample for cells that express epithelial cell adhesion molecule (EpCAM) with antibody-coated magnetic beads, and it labels the nucleus with the fluorescent nucleic acid dye 4,2-diamidino-2-phenylidole dihydrochloride (DAPI).

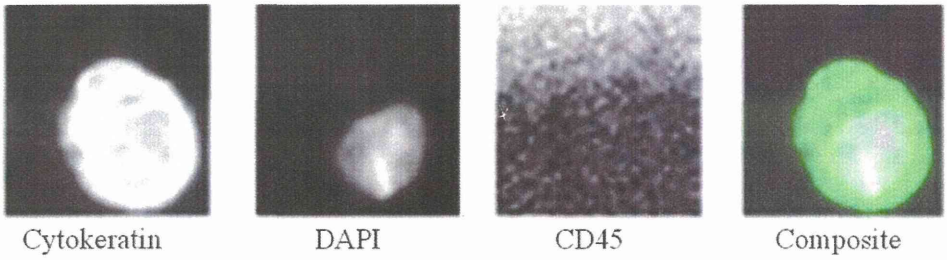


**Figure 2.** Circulating tumor cells were defined as nucleated cells that lacked allophycocyan (CD45) and expressed cytokeratin. DAPI indicates 4,2-diamidino-2-phenylidole dihydrochloride.

Fluorescently labeled monoclonal antibodies specific for leukocytes (CD45 allophycocyan) and epithelial cells (cytokeratin 8, cytokeratin 19, and 19-phycoerythrin) are used to distinguish epithelial cells from leukocytes. We identified and enumerated CTCs using the Celltracks analyzer II (Veridex, LLC), a semiautomated, fluorescence-based microscopy system that permits the computer-generated reconstruction of cellular images. CTCs were defined as nucleated cells that lacked CD45 and expressed cytokeratin (Fig. 2). Criteria used in the CellSearch system to define a tumor cell have been described previously. The results are expressed as the number of cells per 7.5 mL of whole blood.

Peripheral blood samples for use as a control group were obtained from 15 healthy volunteers who consented to participate. No volunteers had any illness or past history of cancer.

A spiking study was conducted to investigate the detectable limit of the CellSearch system. Therefore, the sensitivity and linearity of the CellSerach system was assessed by spiking a series of 10-fold serial dilutions of KATO III cells ($10^2$, 50, $10^1$, 5, $10^0$, and 0 cells) into whole blood from a normal healthy volunteer who did not have any cancer. This in vitro experiment was repeated 3 times for each series.

### *Statistical Analysis*

The chi-square test and the Fisher exact test were used to compare the status of CTCs with categorical clinicopathologic factors. The Kaplan-Meier method was used for survival analysis, and the differences in survival were examined using the log-rank test. Prognostic factors were assessed in univariate and multivariate analyses using Cox proportional hazards regression models. All statistical calculations were performed using SAS statistical software (SAS Institute, Inc., Cary, NC). A $P$ value $< .05$ was considered statistically significant.

## RESULTS

### *Patient Characteristics*

The 170 men and 81 women in the cohort ranged in age from 28 to 87 years (mean age, 64.4 years). Sixty-four percent of all patients remained alive at the time of this analysis.

In the resection group, 82 patients underwent distal gastrectomy, 13 patients underwent proximal gastrectomy, and 53 patients underwent total gastrectomy. The final pathologic findings indicated that all patients with disease greater than stage II had oral S-1 recommended as adjuvant chemotherapy for 1 year after surgery. Seventy-four patients (88.1%) were able to tolerate S-1; however, 10 patients (11.9%) were not able to tolerate S-1 because of anorexia and leucopenia. Twenty-six patients (17.6%) in the resection group had developed recurrent disease at the time of this analysis. These patients relapsed an average of 14.9 months after surgery.

In the nonresectable group, 72 patients had primary tumors of the stomach and distant metastasis, and 31 patients had recurrent distant metastasis after gastrectomy. Sixty-one patients had peritoneal dissemination, and 24 patients had para-aortic lymph node or Virchow lymph node swelling. Hematogenous distant metastases were identified in 24 patients. All patients in the nonresectable group received treatment with chemotherapy. The chemotherapy for gastric cancer consisted of S-1 plus cisplatin in 51 patients and S-1 plus paclitaxel in 52 patients.

CTCs were not identified in any samples from the healthy volunteers. In this study, the presence of $\geq 0$ CTCs per 7.5 mL of blood was considered a positive result.

### *Circulating Tumor Cells and Clinical Correlation*

Seventy-eight of 251 patients had CTCs detected. CTCs were detected in 16 patients (11.3%) from the resection

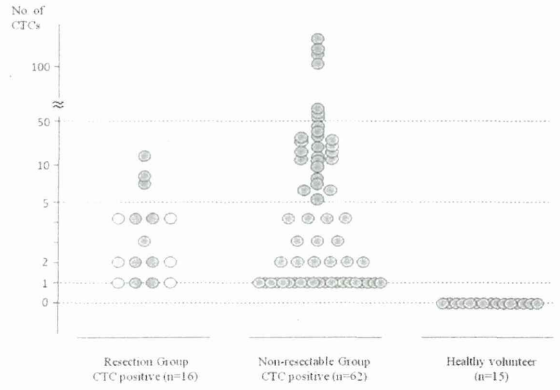


**Figure 3.** Circulating tumor cells (CTCs) were detected in 16 patients (10.8%) from the resection group and in 62 patients (60.2%) from the nonresectable group. The average number of CTCs was 3.5 in the resection group and 109.3 in the nonresectable group. CTCs were not observed in any samples from healthy volunteers.

group and in 62 patients (60.2%) from the nonresectable group. There was a significant difference in the positive rate between the 2 groups ($P < .0001$). Among those who had CTCs detected, the average count was 3.5 CTCs in patients from the resection group and 109.3 CTCs in patients from the nonresectable group (Fig. 3). The overall survival rate for all patients was significantly lower among those who had CTCs detected than among those who did not ($P < .0001$) (Fig. 4A).

In the resection group, CTCs were detected in 1 patient (1.6%) with a T1 tumor, in 2 patients (11.1%) with T2 tumors, in 6 patients (16.2%) with T3 tumors, and in 7 patients (23.3%) with T4 tumors. Clinicopathologic findings from the resection group are provided in Table 1. CTCs in patients who underwent gastrectomy were significantly correlated with the depth of tumor invasion, lymph node metastasis, distant metastasis, disease stage, vessel invasion, and lymphatic invasion. Although serum tumor markers like CEA and CA 19-9 were added to our analysis to be compared with CTCs, there was no significant correlation between CTCs and serum tumor markers.

Among 132 patients without CTCs, 14 patients (10.6%) had a recurrence after surgery. Eight patients had peritoneal dissemination, and 3 patients had hematogenous recurrences. Conversely, 12 of 16 patients (75%) with CTCs had a recurrence after surgery. The patients who had CTCs detected had a significantly higher relapse rate compared with patients who did not have CTCs detected ($P < .0001$). Two patients without recurrence on diagnostic imaging had transient elevation of serum CEA.

**TABLE 1.** Characteristics of Patients in the Resection Group

| Variable | CTCs: No. of Patients (%) | | |
|---|---|---|---|
| | Positive, n = 16 | Negative, n = 132 | *P* |
| Sex | | | |
| Men | 11 (68.8) | 88 (66.7) | .867 |
| Women | 5 (31.2) | 44 (33.3) | |
| Age, y | | | |
| <70 | 8 (50) | 83 (62.9) | .317 |
| >70 | 8 (50) | 49 (37.1) | |
| Tumor classification | | | |
| T1 | 1 (6.3) | 62 (47) | .009 |
| T2 | 2 (12.5) | 16 (12.1) | |
| T3 | 6 (37.5) | 31 (23.5) | |
| T4 | 7 (43.8) | 23 (17.4) | |
| Lymph node classification | | | |
| N0 | 2 (12.5) | 80 (60.6) | < .0001 |
| N1 | 0 (0) | 19 (14.4) | |
| N2 | 1 (6.3) | 17 (12.9) | |
| N3 | 13 (81.3) | 16 (12.1) | |
| Distant metastasis | | | |
| Yes | 3 (18.8) | 5 (3.8) | .012 |
| No | 13 (81.2) | 127 (96.2) | |
| Stage | | | |
| I | 1 (6.3) | 63 (47.7) | .0002 |
| II | 1 (6.3) | 25 (18.9) | |
| III | 11 (68.8) | 39 (29.5) | |
| IV | 3 (18.8) | 5 (3.8) | |
| Lymphatic invasion | | | |
| 0 | 1 (6.3) | 71 (53.8) | .0003 |
| 1 | 15 (93.7) | 61 (46.2) | |
| Vessel invasion | | | |
| 0 | 3 (18.8) | 73 (55.3) | .006 |
| 1 | 13 (81.2) | 59 (44.7) | |
| Histologic type | | | |
| Differentiated | 3 (18.8) | 39 (29.5) | 0.365 |
| Undifferentiated | 13 (81.2) | 93 (70.5) | — |

Abbreviations: CTCs, circulating tumor cells.

Peritoneal dissemination was the most common pattern of recurrence, and 5 patients had hematogenous recurrences (Table 2). There were no significant differences in the recurrence pattern between patients with and without CTCs. However, all patients who had CTCs detected, at the least, had either peritoneal dissemination or hematogenous distant metastases. The sensitivity and specificity for predicting recurrences were 46.2% and 96.7%, respectively.

When we analyzed relapse-free survival according to whether patients were positive for CTCs, relapse-free survival in patients who were positive for CTCs was significantly lower than in those who were negative ($P < .0001$) (Fig. 4B). Furthermore, the 5-year survival rate also was significantly lower in patients with CTCs than in those without CTCs ($P < .0001$) (Fig. 4C). Multivariate analysis demonstrated that the presence of CTCs was an

independent prognostic factor (Table 3). Factors that we included in this analysis were CTCs, tumor classification, lymph node classification, lymphatic invasion, and vessel invasion, all of which were considered to be significant characteristics in these patients. It is noteworthy that positive expression of CTCs in peripheral blood was identified as an independent factor for overall survival in patients with gastric cancer (hazard ratio, 1.73; 95% confidence interval, 1.08-2.77; $P = .024$).

All patients of the nonresectable group received chemotherapy. There was no significant correlation between the presence of CTCs and nonresectable factors (Table 4). In these 103 patients, the presence of CTCs was correlated with a lower survival rate ($P = .0044$) (Fig. 4D). The median survival was 248 days in patients with CTCs and 582 days in patients without CTCs.

**TABLE 2.** Recurrence Pattern of 16 Patients With Circulating Tumor Cells in the Resection Group

| Postgastrectomy | No. of Patients (%) |
|---|---|
| Recurrence pattern | 12 (75) |
| Peritoneal dissemination | 9 (56.3) |
| Liver metastasis | 2 (12.5) |
| Bone metastasis | 2 (12.5) |
| Adrenal gland metastasis | 1 (6.3) |
| Lymph node metastasis | 1 (6.3) |
| No recurrence | 4 (25) |

### *Sensitivity of the CellSearch System With Cell Line*

KATO III cells were used for the analysis of sensitivity and linearity of the CellSearch system. Representative results from the expected number of KATO III cells spiked into healthy donor samples plotted against the actual number of KATO III cells observed in the samples are illustrated in Figure 5. Regression analysis of the

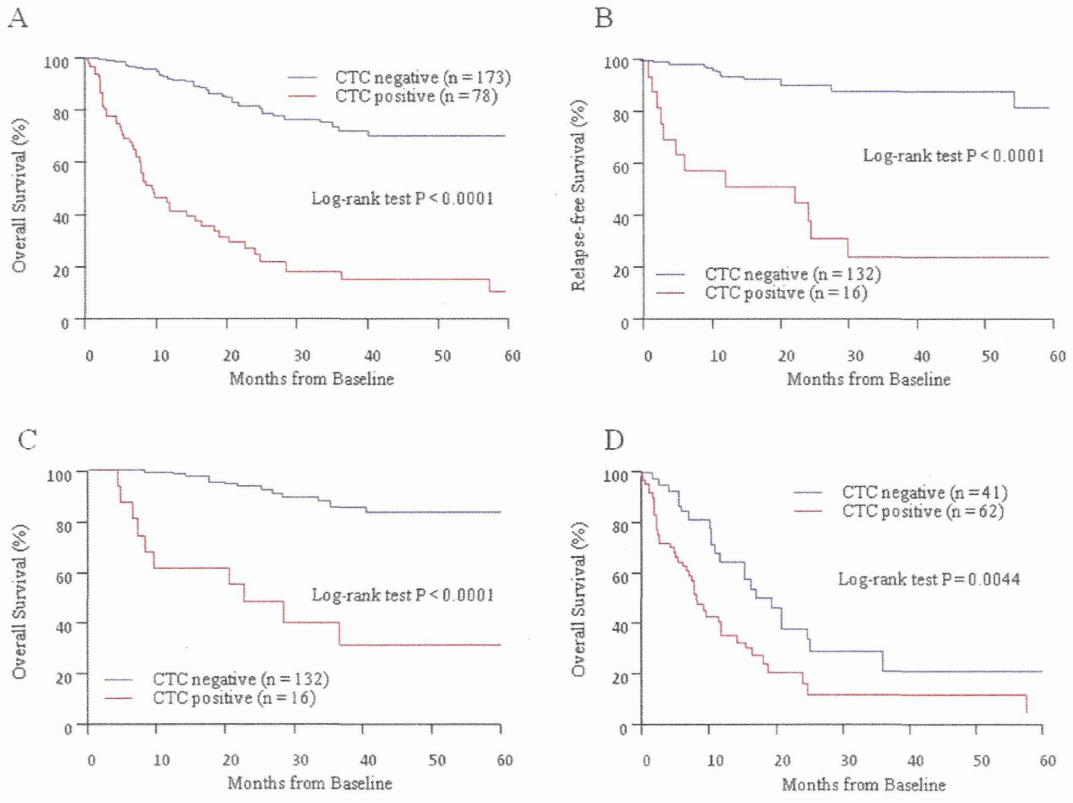


**Figure 4.** (A) Circulating tumor cells (CTCs) were detected in 78 of 251 patients. The 5-year survival rate was significantly lower in patients with CTCs than in those without CTCs ($P < .0001$). (B) In the resection group, the relapse-free survival rate was significantly lower in patients with CTCs than in those without CTCs ($P < .0001$). (C) The overall survival rate also was significantly lower in patients with CTCs than in those without CTCs ($P < .0001$). (D) In the nonresectable group, the overall survival rate was significantly lower in patients with CTCs than in those without CTCs ($P = .0044$). The median survival was 248 days in patients with CTCs and 582 days in patients without CTCs.

**TABLE 3.** Univariate and Multivariate Analysis in the Resection Group

| | Univariate Analysis | | Multivariate Analysis | |
|---|---|---|---|---|
| Independent Factor | HR (95% CI) | *P* | HR (95% CI) | *P* |
| Depth of tumor invasion | 2.33 (1.56-3.69) | < .0001 | 1.45 (0.82-2.69) | .204 |
| Lymph node metastasis | 2.60 (1.80-4.04) | < .0001 | 1.53 (0.91-2.69) | .114 |
| Distant metastasis | 3.85 (1.11-10.28) | .035 | 0.87 (0.23-2.59) | .807 |
| Lymphatic invasion | 10.41 (3.05-65.08) | < .0001 | 1.73 (0.33-13.90) | .541 |
| Vessel invasion | 7.53 (2.58-31.99) | < .0001 | 1.62 (0.39-9.07) | .526 |
| CTCs | 2.77 (1.81-4.18) | < .0001 | 1.73 (1.08-2.77) | .024 |

Abbreviations: CI, confidence interval; CTCs, circulating tumor cells; HR, hazard ratio.

**TABLE 4.** Nonresectable Factors in the Nonresectable Group

| | No. With CTCs | | |
|---|---|---|---|
| Nonresectable Factor | Positive | Negative | Incidence of CTCs, % |
| Liver metastasis | 9 | 7 | 56.3 |
| Lung metastasis | 0 | 2 | 0 |
| Bone metastasis | 4 | 1 | 80 |
| Brain metastasis | 2 | 0 | 100 |
| Peritoneal dissemination | 40 | 23 | 63.5 |
| Lymph node metastasis | 12 | 12 | 50 |
| Direct invasion to peripheral organs | 2 | 3 | 40 |

Abbreviations: CTCs, circulating tumor cells.

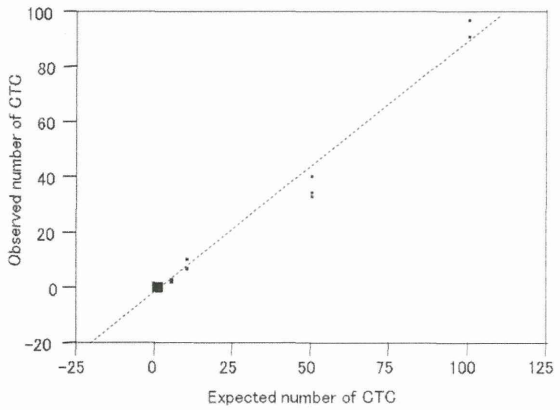


**Figure 5.** Regression analysis of the number of observed tumor cells versus the number of expected tumor cells produced a correlation coefficient ($R^2$) of 0.985. Even a single cell spiked into the samples was detected using this system.

number of observed tumor cells versus the number of expected tumor cells produced a correlation coefficient ($R^2$) of 0.985. Even a single cell spiked into the samples was detected using this system.

## DISCUSSION

CTCs measured with the CellSearch system and clinical correlation of the results were analyzed in 251 patients with gastric adenocarcinoma. The system was sensitive, and the results were correlated with relapse-free survival, the 5-year survival rate, and the overall survival rate in all patients, including the resection group and the nonresectable group. Although previous studies have reported the presence of CTCs determined by RT-PCR, reports of the morphologic detection of CTCs are few.[15-19] Furthermore, ours was a large-scale, prospective study that enrolled 265 patients with gastric cancer. To our knowledge, this is the first longitudinal analysis evaluating CTCs using the CellSearch system in such patients.

In Japan, almost all patients who have stage II or III tumors after undergoing gastrectomy receive adjuvant chemotherapy in the form of oral S-1 according to data from the Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer (ACTS-GC).[20] However, ≥60% of patients at this stage do not have a recurrence without adjuvant chemotherapy. If recurrence after surgery can be predicted, then information regarding CTCs could help patients avoid unnecessary adjuvant chemotherapy. This distinction is necessary to determine whether or not patients are eligible for curative resection. The measurement of CTCs in gastric cancer will be useful for determining treatment strategies if more accurate staging of the patient can be performed. Moreover, patients with gastric cancer who have CTCs should receive neoadjuvant chemotherapy before they undergo surgery.

Patients who had breast cancer, prostate cancer, or colorectal cancer with hematogenous metastasis had a high incidence of CTCs according to several reports.[12,13,21] However, no significant correlation between positive CTCs and hematogenous distant metastasis in gastric cancer has been demonstrated. In the current study, peritoneal dissemination was the most frequently observed pattern of recurrence. The detection of CTCs may be a useful diagnostic tool for predicting

peritoneal dissemination, which is difficult to detect on imaging studies, such as computed tomography, ultrasonography, and positron emission tomography.

The prognosis of patients in our nonresectable group also differed significantly according to the presence of CTCs. Some authors have reported that response to chemotherapy can be evaluated in several cancers with distant metastasis. Monitoring the number of CTCs may be more useful for evaluating chemotherapy.[22,23] It is an advantage that the measurement of CTCs using the CellSearch system is available at any time and is easily and noninvasively performed. An increase in the number of CTCs should lead to a change in chemotherapy regimen.

The CellSearch system is based on the enumeration of epithelial cells, which are separated from the blood by EpCAM antibody-coated magnetic beads and identified with the use of fluorescently labeled antibodies against cytokeratin and with a fluorescent nuclear stain. In the detection of CTCs by molecular techniques there is always the question of whether the result could be a false-positive. Although Sensitivity of RT-PCR is very high for CTCs identification, it is impossible to visually confirm cancer cells. Conversely, with the Cell Search system (Janssen Diagnostics, LLC, Raritan, NJ), the false-positive rate is extremely low, because it is possible to morphologically confirm the presence of cells. Conversely, some cases might be missed (false-negative) because some of the CTCs may not express these epithelial markers and may be undetectable by the CellSearch system.[24]

Most reports concerning the prognosis for patients with breast cancer and prostate cancer have used a cutoff of $\geq$5 CTCs to determine a positive test.[12,13] Conversely, some reports of other cancers use different cutoff values.[25] The question remains regarding the significance of the presence of a single CTC. For patients with gastric cancer who have undergone curative resection, this single cell may be consequential. Our criteria defined $\geq$1 CTC as a positive test. It is important to note that CTCs were not detected in healthy volunteers in our series. In fact, several patients in our cohort who had only 1 CTC relapsed, and the presence of any CTCs in peripheral blood was considered an independent prognostic factor for determining the overall survival of patients who underwent gastrectomy. It may become possible to more accurately estimate the prognosis for these patients if the presence of CTCs is added to the staging factors. In this study, many patients who were positive for CTCs had a recurrence, and several patients who did not have a recurrence had received chemotherapy because of up-regulated serum tumor marker levels during follow-up. The detection of CTCs is useful for predicting recurrence and prognosis. We conclude that the evaluation of CTCs in peripheral blood may be a useful tool for predicting tumor progression, prognosis, and the effect of chemotherapy in patients with gastric cancer.

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## CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

## REFERENCES

1. Yeh KH, Chen YC, Yeh SH, Chen CP, Lin JT, Cheng AL. Detection of circulating cancer cells by nested reverse transcription-polymerase chain reaction of cytokeratin-19 (K19)—possible clinical significance in advanced gastric cancer. *Anticancer Res.* 1998;18:1283-1286.
2. Noh YH, Kim JA, Lim GR, et al. Detection of circulating tumor cells in patients with gastrointestinal tract cancer using RT-PCR and its clinical implications. *Exp Mol Med.* 2001;33:8-14.
3. Vogel I, Kalthoff H. Disseminated tumour cells. Their detection and significance for prognosis of gastrointestinal and pancreatic carcinomas. *Virchows Arch.* 2001;439:109-117.
4. Ghossein RA, Bhattacharya S, Rosai J. Molecular detection of micrometastases and circulating tumor cells in solid tumors. *Clin Cancer Res.* 1999;5:1950-1960.
5. Nakashima S, Natsugoe S, Matsumoto M, et al. Clinical significance of circulating tumor cells in blood by molecular detection and tumor markers in esophageal cancer. *Surgery.* 2003;133:162-169.
6. Mimori K, Fukagawa T, Kosaka Y, et al. A large-scale study of MT1-MMP as a marker for isolated tumor cells in peripheral blood and bone marrow in gastric cancer cases. *Ann Surg Oncol.* 2008;15:2934-2942.
7. Bertazza L, Mocellin S, Marchet A, et al. Survivin gene levels in the peripheral blood of patients with gastric cancer independently predict survival [serial online]. *J Transl Med.* 2009;7:111.
8. Wu CH, Lin SR, Hsieh JS, et al. Molecular detection of disseminated tumor cells in the peripheral blood of patients with gastric cancer: evaluation of their prognostic significance. *Dis Markers.* 2006;22:103-109.
9. Mimori K, Fukagawa T, Kosaka Y, et al. Hematogenous metastasis in gastric cancer requires isolated tumor cells and expression of vascular endothelial growth factor receptor-1. *Clin Cancer Res.* 2008;14:2609-2616.
10. Koga T, Tokunaga E, Sumiyoshi Y, et al. Detection of circulating gastric cancer cells in peripheral blood using real time quantitative RT-PCR. *Hepatogastroenterology.* 2008;55:1131-1135.
11. Arigami T, Uenosono Y, Ishigami S, Hagihara T, Haraguchi N, Natsugoe S. Clinical significance of the B7-H4 coregulatory molecule as a novel prognostic marker in gastric cancer. *World J Surg.* 2011;35:2051-2057.
12. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med.* 2004;351:781-791.
13. Leversha MA, Han J, Asgari Z, et al. Fluorescence in situ hybridization analysis of circulating tumor cells in metastatic prostate cancer. *Clin Cancer Res.* 2009;15:2091-2097.
14. Sobin LH, Gospodarowicz MK, Wittekind C, eds. TNM Classification of Malignant Tumours. 7th ed. Oxford, United Kingdom: Wiley-Blackwell; 2010.

15. Nishida S, Kitamura K, Ichikawa D, Koike H, Tani N, Yamagishi H. Molecular detection of disseminated cancer cells in the peripheral blood of patients with gastric cancer. *Anticancer Res.* 2000;20:2155-2159.
16. Ikeguchi M, Ohro S, Maeda Y, et al. Detection of cancer cells in the peripheral blood of gastric cancer patients. *Int J Mol Med.* 2003; 11:217-221.
17. Miyazono F, Natsugoe S, Takao S, et al. Surgical maneuvers enhance molecular detection of circulating tumor cells during gastric cancer surgery. *Ann Surg.* 2001;233:189-194.
18. Arigami T, Uenosono Y, Hirata M, Yanagita S, Ishigami S, Natsugoe S. B7-H3 expression in gastric cancer: a novel molecular blood marker for detecting circulating tumor cells. *Cancer Sci.* 2011; 102:1019-1024.
19. Saad AA, Awed NM, Abd Elkerim NN, et al. Prognostic significance of E-cadherin expression and peripheral blood micrometastasis in gastric carcinoma patients. *Ann Surg. Oncol.* 2010;17:3059-3067.
20. Sakuramoto S, Sasako M, Yamaguchi T, et al. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med.* 2007;357:1810-1820.
21. Rahbari NN, Bork U, Kircher A, et al. Compartmental differences of circulating tumor cells in colorectal cancer. *Ann Surg Oncol.* 2012;19:2195-2202.
22. Budd GT, Cristofanilli M, Ellis MJ, et al. Circulating tumor cells versus imaging—predicting overall survival in metastatic breast cancer. *Clin Cancer Res.* 2006;12:6403-6409.
23. Nakamura S, Yagata H, Ohno S, et al. Multi-center study evaluating circulating tumor cells as a surrogate for response to treatment and overall survival in metastatic breast cancer. *Breast Cancer.* 2010;17:199-204.
24. Sakakura C, Hagiwara A, Nakanishi M, et al. Differential gene expression profiles of gastric cancer cells established from primary tumour and malignant ascites. *Br J Cancer.* 2002;87:1153-1161.
25. Hiraiwa K, Takeuchi H, Hasegawa H, et al. Clinical significance of circulating tumor cells in blood from patients with gastrointestinal cancers. *Ann Surg. Oncol.* 2008;15:3092-3100.

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# Cadmium-Free Sugar-Chain-Immobilized Fluorescent Nanoparticles Containing Low-Toxicity ZnS-$AgInS_2$ Cores for Probing Lectin and Cells

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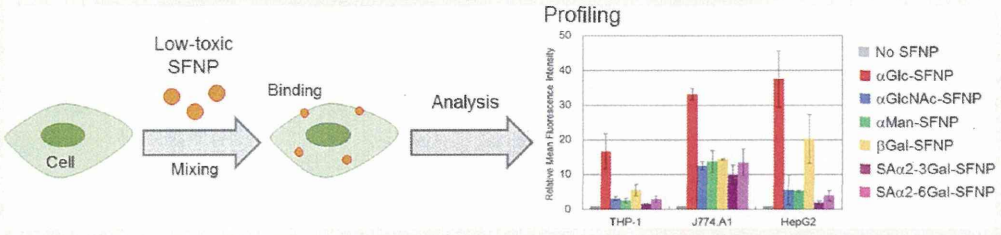


**ABSTRACT:** Sugar chains play a significant role in various biological processes through sugar chain−protein and sugar chain−sugar chain interactions. To date, various tools for analyzing sugar chains biofunctions have been developed. Fluorescent nanoparticles (FNPs) functionalized with carbohydrate, such as quantum dots (QDs), are an attractive imaging tool for analyzing carbohydrate biofunctions in vitro and in vivo. Most FNPs, however, consist of highly toxic elements such as cadmium, tellurium, selenium, and so on, causing problems in long-term bioimaging because of their cytotoxicity. In this study, we developed cadmium-free sugar-chain-immobilized fluorescent nanoparticles (SFNPs) using ZnS-$AgInS_2$ (ZAIS) solid solution nanoparticles (NPs) of low or negligible toxicity as core components, and investigated their bioavailability and cytotoxicity. SFNPs were prepared by mixing our originally developed sugar-chain-ligand conjugates with ZAIS/ZnS core/shell NPs. In binding experiments with lectin, the obtained ZAIS/ZnS SFNPs interacted with an appropriate lectin to give specific aggregates, and their binding interaction was visually and/or spectroscopically detected. In addition, these SFNPs were successfully utilized for cytometry analysis and cellular imaging in which the cell was found to possess different sugar-binding properties. The results of the cytotoxicity assay indicated that SFNPs containing ZAIS/ZnS have much lower toxicity than those containing cadmium. These data strongly suggest that our designed SFNPs can be widely utilized in various biosensing applications involved in carbohydrates.

## INTRODUCTION

Cell-surface sugar chains play a significant role in a variety of biological events, such as cell−cell recognition, proliferation, differentiation, immune response, signal transduction, and infection.[1,2] The specific binding interaction between sugar chains and proteins, and sometimes between sugar chains and sugar chains, is the initiating point of these events. Analysis of the binding interaction at the molecular level therefore leads us not only toward a better understanding of these biofunctions, but also toward new biological insights involving sugar chains. Numerous efforts have been devoted to sugar-chain structure−function analysis, and many techniques like sugar chain array, chips, fluorescent probes, and nanoparticles have been developed.[3−22]

Nowadays, sugar chains are considered new biomarkers for determining cell type,[1,2,23−25] similar to nucleic acids and proteins,[26−28] since the glycosylation pattern on the cell surface varies depending on the tissue and cell type. Glycomic analysis of the cell is therefore a valuable approach for knowing cell status. Lectin-based profiling is a useful method for determining cell status and has been investigated in various cells like embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, tumor cells, and other cultivated cell lines.[29−32] Sugar-chain-based profiling is a promising approach because the recognition ability of cells for sugar-chain changes depending on the cell conditions and provides information regarding the cell status, and is applicable to the evaluation of the metastatic property of tumor cells and quality control of cultivated cells.

Sugar-chain-immobilized fluorescent nanoparticles (SFNPs) are an attractive biosensing tool for the analysis of sugar chain

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biofunctions.[16−22] Quantum dots (QDs) are often utilized as core components and have been extensively investigated as a fluorescent probe for biomedical imaging and diagnosis.[33−37] Therefore, various SFNPs containing QDs have been synthesized and their biological applications explored. Most QDs contains cadmium ions, which is often problematic in in vivo and/or in vitro bioassays because of their cytotoxic activity.[38−42] In order to suppress the cytotoxicity of cadmium ions, coating with a low-toxicity ZnS shell or various polymers has been utilized. However, it is difficult to completely abrogate the cytotoxicity of cadmium ions because these ions are released from nanoparticles by lysosomal degradation processes, photolysis, and oxidation.[39,43−46]

In this paper, we report the synthesis of cadmium-free SFNP using low-toxicity ZnS-$AgInS_2$ (ZAIS)/ZnS NPs. Immobilization of the sugar chain moiety was accomplished by a simple ligand exchange reaction using our ligand conjugates, in which various sugar-chain structures were conjugated with a thioctic acid moiety via a linker molecule. Carbohydrate−lectin interaction using our SFNPs was detected spectroscopically or visually. Flow cytometry analysis and fluorescent imaging of cells was also performed, and the results clarified that the binding property of cell for a sugar chain is different depending on the cell type. Furthermore, in the cytotoxicity assay, SFNPs containing ZAIS/ZnS NPs were found to be much less toxic than those containing CdTe/CdS QDs.

## EXPERIMENTAL PROCEDURES

**Materials.** All chemical reagents were commercial grade except as noted. Thioglycolic acid (TGA, purity: 90%), 3-mercaptpropionic acid (3-MPA, purity: 98%), $NaBH_4$ (purity: >95.0%), KOH (purity: >85%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypan blue solution (0.5%) were purchased from Nacalai Tesque (Kyoto, Japan). Thioacetamide (purity: >99.0%), zinc acetate anhydrate (purity: 99.99%), RPMI1640 medium, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma Aldrich (St. Louis, MO, USA). Indium(III) nitrate trihydrate (purity: >98.0%), silver nitrate (purity: 99.9%), sodium *N,N*-diethyldithiocarbamate trihydrate (purity: >90%), and zinc nitrate hexahydrate (99.9%) were purchased from Wako (Osaka, Japan). Oleylamine (purity: >40%) was purchased from TCI (Tokyo, Japan). Concanavalin A (Con A), *Ricin communis* agglutinin I (RCA120), and bovine serum albumin (BSA) were purchased from Seikagaku Corporation (Tokyo, Japan), Vector Laboratories (Burlingame, CA, USA), and Nacalai Tesque, respectively. Fetal bovine serum (FBS) was purchased from Nichirei (Tokyo, Japan). Penicillin streptomycin (PS) was purchased from GIBCO (Carlsbad, CA, USA). Milli-Q water (18.2 MΩ $cm^{-1}$) was used in all experiments unless otherwise noted. Sugar chain−ligand conjugates[47,48] and SFNPs containing CdTe/CdS[49] were prepared using previously reported methods.

**Measurements.** UV/vis spectra and fluorescence spectra were measured using a V-650 spectrometer and an FP-6310 fluorescence spectrometer (JASCO, Tokyo, Japan), respectively. Mass spectra were measured using the Voyager-DE-PRO (Applied Biosystems, Foster City, CA, USA) or micrOTOF II (Bruker Daltonics, Billerica, MA, USA). Flow cytometry analyses were performed using the Cytomics FC 500 Cytometer (Beckman Coulter, Brea, CA, USA). Fluorescence imaging data were obtained using a Nikon A1si-90i (Nikon, Tokyo, Japan). Colorimetric MTT assay was performed using an Immuno Mini NJ-2300 (MICROTEC, Chiba, Japan). Stained cells were observed using an inverted microscope CKX-31 (Olympus, Tokyo, Japan).

**Preparation of Sugar-Chain−Ligand Conjugates.** Sugar-chain−ligand conjugates were prepared by the method reported previously.[47,48] Oligosaccharides used were commercially available or from a synthetic source. Briefly, a mixture of oligosaccharide and linker moiety in DMAc/$H_2O$/AcOH (10:10:1) was left at 40 °C. After 5 h, $NaBH_3CN$ was added to the mixture and the reaction mixture was left at 40 °C again. After 3 days, the reaction mixture was lyophilized. The residue was then purified by reversed-phase silica gel column chromatography (eluent: $H_2O$/MeOH gradient system) to yield the sugar-chain−ligand conjugate.

***N*-[3-[(2-Deoxy-2-acetamido-α-D-glucopyranosyl)-(1−4)-(1-deoxy-D-glucitol-1-yl)amino]phenyl]-DL-α-lipoamide (GlcNAcα1−6Glc-mono, 3).** $^1$H NMR (600 MHz, $D_2O$) $\delta$ 7.019 (1H, t, $J$ = 8.2 Hz, aromatic), 6.78 (1H, s, aromatic), 6.65 (1H, d, $J$ = 8.2 Hz, aromatic), 6.38 (1H, d, $J$ = 8.2 Hz, aromatic), 4.61 (1H, d, $J_{1',2'}$ = 3.4 Hz, H-1′), 3.76 (1H, ddd, $J_{2,3}$ = 6.8 Hz, $J_{2,1a}$ = 5.4 Hz, $J_{2,1b}$ = 4.8 Hz, H-2), 3.73−3.60 (5H, m, H-3, H-5, H-6a, H-2′, H-6a′), 3.55−3.49 (5H, m, H-4, H-3′, H-5′, H-6b′, -SSCH$_2$CH$_2$C<u>H</u>=), 3.39 (1H, d, $J_{6a,6b}$ = 8.2 Hz, H-6b), 3.23 (1H, dd, $J_{4',3'}$ = 8.8 Hz, $J_{4',5'}$ = 9.5 Hz, H-4′), 3.17 (1H, dd, $J_{1a,2}$ = 4.8 Hz, $J_{1a,1b}$ = 8.8 Hz, H-1a), 3.07 (1H, ddd, $J$ = 10.7 Hz, 6.1 Hz, 6.1 Hz, -SSC<u>H$_2$</u>CH$_2$CH=), 3.01 (1H, ddd, $J$ = 11.3 Hz, 6.8 Hz, 6.1 Hz, -SSC<u>H$_2$</u>CH$_2$CH=), 2.94 (1H, dd, $J_{1b,2}$ = 5.4 Hz, $J_{1b,1a}$ = 8.2 Hz, H-1b), 2.32 (1H, dddd, $J$ = 12.7 Hz, 6.1 Hz, 6.1 Hz, 6.1 Hz, -SSCH$_2$C<u>H$_2$</u>CH=), 2.22 (2H, t, $J$ = 7.48 Hz, -NHCOC<u>H$_2$</u>-), 1.83 (3H, s, −COC<u>H$_3$</u>), 1.82−1.78 (1H, m, -SSCH$_2$C<u>H$_2$</u>CH=), 1.64−1.59 (1H, m, -NHCOCH$_2$C<u>H$_2$</u>-), 1.55−1.44 (3H, m, -NHCOCH$_2$C<u>H$_2$</u>-, -NHCOCH$_2$CH$_2$CH$_2$C<u>H$_2$</u>-), 1.34−1.27 (2H, m, -NHCOCH$_2$CH$_2$C<u>H$_2$</u>-); HRMS (positive mode); Found: $m/z$ 686.2391 [(M+Na)$^+$], Calcd. for $C_{26}H_{42}N_2O_{11}S_2Na$: 686.2388.

***N*-[3-[(α-D-Mannopyranosyl)-(1−4)-(1-deoxy-D-glucitol-1-yl)amino]phenyl]-DL-α-lipoamide (Manα1−6Glc-mono, 4).** $^1$H NMR (600 MHz, $D_2O$) $\delta$ 7.20 (1H, t, $J$ = 8.2 Hz, aromatic), 6.95 (1H, s, aromatic), 6.85 (1H, $J$ = 7.5 Hz, d, aromatic), 6.58 (1H, d, $J$ = 7.5 Hz, aromatic), 4.82 (1H, s, H-1′), 3.97−3.92 (1H, ddd, $J_{2,3}$ = 7.7 Hz, $J_{2,1a}$ = 4.1 Hz, $J_{2,1b}$ = 4.1 Hz, H-2), 3.92−3.88 (1H, m, H-2′), 3.88−3.80 (4H, m, H-3, H-6a, H-6a′, H-6b′), 3.80−3.76 (1H, dd, $J_{3',4'}$ = 6.1 Hz, $J_{3',2'}$ = 3.4 Hz, H-3′), 3.73−3.59 (6H, m, H-4, H-5, H-6b, H-4′, H-5′, -SSCH$_2$CH$_2$C<u>H</u>=), 3.35 (1H, dd, $J_{1a,2}$ = 4.1 Hz, $J_{1a,1b}$ = 9.5, H-1a), 3.25 (1H, ddd, $J$ = 10.1 Hz, 6.8 Hz, 6.8 Hz, -SSC<u>H$_2$</u>CH$_2$CH=), 3.19 (1H, ddd, $J$ = 10.1 Hz, 6.8 Hz, 6.8 Hz, -SSC<u>H$_2$</u>CH$_2$CH=), 3.16−3.11 (1H, dd, $J_{1b,1a}$ = 8.2 Hz, $J_{1b,2}$ = 5.4 Hz, H-1b), 2.50 (1H, dddd, $J$ = 12.3 Hz, 6.1 Hz, 6.1 Hz, 6.1 Hz, -SSCH$_2$C<u>H$_2$</u>CH=), 2.40 (2H, t, $J$ = 6.8 Hz, -NHCOC<u>H$_2$</u>-), 2.00 (1H, dddd, J = 12.3 Hz, 6.1 Hz, 6.1 Hz, 6.1 Hz, -SSCH$_2$C<u>H$_2$</u>CH=), 1.82−1.76 (1H, m, -NHCOCH$_2$C<u>H$_2$</u>-), 1.76−1.64 (3H, m, -NHCOCH$_2$C<u>H$_2$</u>-, -NHCOCH$_2$CH$_2$CH$_2$C<u>H$_2$</u>-), 1.53−1.47 (2H, m, -NHCOCH$_2$CH$_2$C<u>H$_2$</u>-); HRMS (positive mode); Found: $m/z$ 645.2126 [(M+Na)$^+$], Calcd. for $C_{26}H_{42}N_2O_{11}S_2Na$: 645.2122.

***N*-[3-[(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-galacto-2-nonulopyranosidonic acid)-(2−3)-(α-D-galactopyranosyl)-(1−4)-(1-deoxy-D-glucitol-1-yl)amino]phenyl]-DL-α-lipoamide (SAα2−3Galβ1−4Glc-mono, 5).** $^1$H NMR (600 MHz, $D_2O$) $\delta$ 7.40 (1H, t, $J$ = 8.2 Hz, aromatic), 7.34


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