Expression of CD44 variant 9 induces chemoresistance of gastric cancer by controlling intracellular reactive oxygen spices accumulation

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Original Article

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Abstract

Background: CD44 variant 9 (CD44v9) has been reported to suppress reactive oxygen spices (ROS) in association with antioxidant factors such as glutathione (GSH) and glutathione peroxidase 2 (GPx2), resulting in promoted tumor growth.

Methods: CD44v9 and GPx2 expression were investigated by immunohistochemistry in resected specimens from 193 gastric cancer (GC) patients without preoperative chemotherapy and in pretreatment biopsy specimens from 29 GC patients with preoperative chemotherapy. We analyzed the relationship between CD44v9 expression and clinicopathological factors, prognosis, and pathological response to chemotherapy. In GC cell lines, we examined the relationship between CD44v9 expression and chemotherapeutic sensitivity.

Results: In patients without preoperative chemotherapy, CD44v9 expression was significantly associated with depth of invasion, lymphatic permeation, vascular invasion, distant metastasis and GPx2 expression. In multivariate analysis, CD44v9 expression was an independent poor prognosis factor for overall survival and recurrence-free survival. In patients with preoperative chemotherapy, CD44v9 expression was significantly associated with worse pathological response and GPx2 expression. In GC cell lines, downregulation of CD44v9 expression enhanced chemotherapeutic sensitivity to 5-fluorouracil with changing GSH and ROS levels.

Conclusions: CD44v9-positive expression was associated with chemotherapeutic resistance by controlling intracellular accumulated ROS, suggesting that CD44v9 may be a predictive biomarker for chemotherapy in GC.

Keywords:

1 CD44 variant 9, cancer stem cells, gastric cancer, reactive oxygen spices, glutathione
2 peroxidase 2
3

Introduction

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer-related death in the world.[1] GC shows the highest estimated mortality rates in Eastern Asia and is one of the most common neoplasms in Japan. Early detection and resection of GC with gastrointestinal endoscopy and the development of various anti-cancer drugs have improved the survival rates of GC. However, the treatment outcome of advanced GC is still unsatisfactory. The reason is because some early and advanced GC patients show recurrence and chemotherapy resistance, leading to poor prognosis. Therefore, investigation of poor prognostic biomarkers and predictive biomarkers for the response to chemotherapy in GC is crucial.

CD44 is a cell surface marker that is associated with cancer stem cells (CSC) in various solid tumors.[2-5] CD44 variant 9 (CD44v9), a splicing variant of CD44, has been reported to stabilize a glutamate-cystine transporter (xCT) at the cell surface and promote the uptake of cystine required for intracellular glutathione (GSH) synthesis.[6] Glutathione peroxidase 2 (GPx2), the gastrointestinal form of glutathione peroxidases, is an antioxidant enzyme that catalyzes the reduction of intracellular reactive oxygen spices (ROS) using GSH as a reductant.[7, 8] These mechanisms suggest that CD44v9 has a specific function in the regulation of intracellular accumulated ROS. The regulation of redox balance in cancer cells is reported to be an important factor in tumor development and the response to anticancer therapies.[8, 9]

In GC patients, CD44v9-positive expression was recently reported to be significantly associated with clinicopathological findings such as depth of invasion, lymph node metastases, tumor-node-metastasis (TNM) stage,[10] higher risk of recurrence[11] and worse prognosis.[12] These findings indicated that the high CD44v9 expression in GC was

associated with promoting tumor growth. However, no studies have evaluated the relationship between the regulation of intracellularly accumulated ROS in CD44v9-postitive cancer cells and chemotherapeutic sensitivity in clinical specimens. Therefore, in this study, we investigated whether the regulation of redox balance by CD44v9 expression was associated with prognosis and chemotherapeutic efficacy in GC clinical specimens and cell lines.

Methods

Patients and specimens

The study flow for patient selection is show in Figure 1. We initially included 596 GC patients who underwent surgery between 2006 and 2016 at the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University. From this patient group, we obtained one set of samples as resected specimens from 193 primary GC patients who underwent surgery with negative (R0) or microscopically positive (R1) margins without preoperative chemotherapy between January 2008 and December 2012. We obtained pretreatment biopsy specimens from the remaining 29 primary GC patients who underwent surgery after chemotherapy between January 2006 and December 2016 as the second sample set. TNM staging and pathological classification were defined according to the Japanese Gastric Cancer Association (JGCA) staging system (14th edition).[13] In the 193 primary GC patients who underwent surgery without preoperative chemotherapy, postoperative adjuvant chemotherapy was completely performed to 36 patients with pathological stage II–III. Among these 36 patients, 32 were treated with S-1 alone, one was treated with tegafur and uracil alone, and three were treated with capecitabine plus oxaliplatin. Among the 29 patients who underwent surgery after chemotherapy, six were treated with S-1 alone, nine were treated with S-1 plus cisplatin, four were treated with capecitabine plus cisplatin (and plus trastuzumab), three were treated with S-1 plus oxaliplatin, and seven were treated with S-1 plus docetaxel. In resected specimens of these patients, histological evaluation criteria of tumor response after preoperative chemotherapy were judged according to the JGCA staging system: Grade 0 (no effect); Grade 1a (very slight effect); Grade 1b (slight effect); Grade 2 (considerable effect); and Grade 3 (complete response).[13]

This study protocol was approved by the ethics committees of Kyushu University (Number 29-384).

Immunohistochemistry

CD44v9 and GPx2 immunohistochemistry were performed using a rat monoclonal anti-CD44v9 antibody (LKG-M001, COSMO BIO CO LTD, Tokyo, Japan) at 1:5000 dilution and a rabbit polyclonal anti-GPx2 antibody (ab137431, Abcam, Cambridge, UK) at 1:1000 dilution, respectively.

CD44v9 expression is mainly localized in the cell membrane, and GPx2 is mainly localized in the cytoplasm. CD44v9 staining was scored as described previously.[14] The proportion of stained carcinoma cells was semi-quantitatively analyzed in whole-tumor tissue in low-power fields (×40). The proportion scores were defined as follows: 0, 0% (no positive cells); 1, 1% – 25%; 2, 26% – 75%; and 3, 76% – 100%. The intensity scores were defined as follows: −1, no or weak staining homogeneously; 0, intermediate or strong staining heterogeneously; and 1, strong staining homogeneously. The total score was calculated as the sum of the proportion and intensity score of positively stained carcinoma cells. Samples with scores from −1 – 1 were categorized as CD44v9-negative and samples with scores from 2 – 4 were categorized as CD44v9-positive. The GPx2 staining was scored as described previously.[15] The expression rate was quantified from 0% to 100%. The intensity score of positively stained carcinoma cells was scored as follows: 0, no staining; 1, weak staining; 2, intermediate staining; and 3, strong staining. Total scores were determined by multiplying the expression rate and intensity scores. Samples with scores less than 0.5

were defined as GPx2-negative, and those with scores more than 0.5 were defined as GPx2-positive.

Cell culture

Human GC cell lines (MKN45, MKN74, NUGC4, KATOIII, SNU-1) were obtained from the Japanese Collection of Research Bioresources Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition, Japan. The human colon cancer cell line HCT116, which was purchased as above and reported to have CD44v9 high expression,[16] was used as positive control.

Quantitative RT- PCR

Total RNA was separated from cells using Maxwell RSC simplyRNA Tissue KitRNeasy (Promega, Madison, WI, USA) and reverse transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using StepOnePlus (Applied Biosystems, Foster City, CA, USA). We determined mRNA expression with TaqMan qPCR using TaqMan probe Hs01081475_m1 (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA expression levels were measured in triplicate for each sample. β-actin mRNA level was used as an internal control to normalize the mRNA levels.

Western blotting

Proteins were separated from cell lines using ice-cold RIPA Buffer (Nacalai Tesque, Kyoto, Japan). Western blotting was performed using anti-CD44v9 (LKG-M001, COSMO BIO LTD) at 1:1000 dilution and anti-β-actin (#4970, Cell Signaling

Technologies, Danvers, MA, USA) at 1:1000 dilution as primary antibodies by iBind Western Systems (Thermo Fisher Scientific). The signals were visualized by Amersham Imager600 (GE Healthcare, Little Chalfont, UK).

siRNA transfection

CD44v9 and negative control were obtained from Thermo Fisher Scientific, Inc. siRNA sequences were as follows: CD44v9 siRNA sense, 5′-CUA CUU UAC UGG AAG GUU Att-3′ and antisense, 5′-UAA CCU UCC AGU AAA GUA Gtt-3′.[17] Silencer Select Negative Control siRNA was used as a non-targeting siRNA. Cells seeded in a 6-well plate (1×10^5 cells per well) were reverse-transfected with 10 nmol CD44v9 siRNA with Lipofectamine RNAimax reagent (Thermo Fisher Scientific). mRNA knockdown and downregulated protein expression were verified by qRT-PCR and western blotting, respectively, at three time points, 48 h, 72 h, and 96 h.

Cell viability assays

Cells transfected with CD44v9 or negative control siRNA were seeded into a 96-well plate (2×10^3 cells per well) and cultured overnight. On the next day, 5-fluorouracil (5-FU; Sigma-Aldrich, St. Louis, MO, USA) was added at various concentrations and cells were incubated for 72 h. Cell viability was measured using CellTiter-Glo Luminescent Cell Viability Assay kit (Promega). Luminescence was measured using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, Tokyo, Japan). IC_{50} values were calculated using XLfit (ID Business Solutions Ltd.).

Measurement of GSH levels

Intracellular GSH levels were evaluated using GSH-Glo Glutathione Assay Kit (Promega). Cells transfected by CD44v9 or negative control siRNA were seeded into a 96-well plate (2×10^3 cells per well), and GSH measurement was performed 48 h later using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek).

Measurement of ROS levels

The intracellular ROS levels under normal and stress conditions were detected using DCFDA/H2DCFDA-Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, UK). Cells transfected with CD44v9 or negative control siRNA were seeded into a 96-well plate (2×10^3 cells per well) and incubated for 24 h. Various concentrations of 5-FU were added, and cells were incubated for 72 h. Next, 20 μM DCFDA was added and cells incubated for 30–45 min at 37°C in the dark. Fluorescence intensity was immediately measured using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek).

Statistical analysis

All statistical analyses were performed using JMP software version 13.0 (SAS Institute Inc., Cary, NC, USA). Between-group differences were analyzed using chi-squared test, Fisher's exact test, or Mann-Whitney U test, as appropriate. Kaplan–Meier curves were constructed for Overall survival (OS) and recurrence-free survival (RFS) using log-rank test. Univariate and multivariate analyses were performed using Cox proportional hazards model. A p-value of < 0.05 was considered significant.

Results

CD44v9 expression in the resected specimens and clinicopathological factors in the patients who underwent surgery without preoperative chemotherapy

Representative CD44v9 and GPx2 immunohistochemical staining patterns are shown in Figure 2. Some cases showed heterogeneous expression of CD44v9 and GPx2 regardless of the infiltration of cancer cells. Positive CD44v9 staining in resected specimens was observed in 51 (26.4 %) of the 193 cases who underwent surgery without preoperative chemotherapy. Association between CD44v9 expression and clinicopathological factors in these GC patients is shown in Table 1. In patients without preoperative chemotherapy, CD44v9 expression was significantly associated with sex ($p = 0.0154$), depth of invasion ($p = 0.0088$), lymphatic permeation ($p = 0.0012$), vascular invasion ($p = 0.0470$), and distant metastasis ($p = 0.0114$) (Table 1). CD44v9 expression was also strongly correlated with GPx2 expression ($p < 0.0001$). In addition, the association between CD44v9 expression and Lauren classification in 177 GC patients with diagnoses other than pathologically solid-type poorly differentiated adenocarcinoma (por1) and mucinous adenocarcinoma is shown in Supplementary Table 1.

CD44v9 expression and patient outcomes in patients who underwent surgery without preoperative chemotherapy

We next evaluated the prognostic potential of CD44v9-positive cells. Kaplan–Meier survival curves according to the expression of CD44v9 are shown in Figure 3. Patients with CD44v9-positive expression showed significantly poorer OS and RFS than those with negative expression (OS: hazard ratio (HR) = 2.904, $p = 0.0034$; RFS: HR = 2.644,

p = 0.0027) (Figure 3a, 3b). Furthermore, there were significant differences in OS and RFS among four groups of patients: patients with CD44v9-negative and GPx2-negative expression (n = 85), patients with CD44v9-negative and GPx2-positive expression (n = 57), patients with CD44v9-positive and GPx2-negative expression (n = 12), and patients with CD44v9-positive and GPx2-positive expression (n = 39) (OS: p = 0.0350, RFS: p = 0.0139). The double-positive group in CD44v9 and GPx2 expression showed relatively poor outcomes in RFS (Supplementary Figure 1a, 1b). In multivariate analysis, in all patients who underwent surgery without preoperative chemotherapy, pStage III/IV and CD44v9-positive expression were independent poor prognosis factors for OS (HR = 18.898; 95% confidence interval (CI), 6.441–55.447; p < 0.0001, HR = 2.393; 95% CI, 1.110–5.159; p = 0.0259, respectively), and pStage III/IV and CD44v9-positive expression were also independent poor prognosis factors for RFS (HR = 13.830; 95% CI, 5.731–35.375; p < 0.0001, HR = 2.395; 95% CI, 1.216–4.714; p = 0.0115, respectively) (Table 2).

In addition, to evaluate the correlation of expression of CD44v9 and chemotherapeutic effect, we analyzed the prognosis of the patients with postoperative adjuvant chemotherapy. CD44v9-positive patients treated with completed postoperative adjuvant chemotherapy (pStage II-III) showed significantly poorer prognosis (OS; p = 0.0297, RFS; p = 0.0012) than those with negative expression (Supplementary Figure 2), whereas CD44v9 expression did not have any prognostic impact for the patients without postoperative adjuvant chemotherapy (OS; p = 0.8080, RFS; p = 0.5726) (Supplementary Figure 3).

CD44v9 expression in pretreatment biopsy specimens and clinicopathological factors in patients who underwent surgery with preoperative chemotherapy

Positive staining of CD44v9 in the pretreatment biopsy specimens was observed in 14 (48.3 %) of 29 cases who underwent surgery with preoperative chemotherapy (Table 3). In patients with preoperative chemotherapy, CD44v9 expression in biopsy specimens was significantly associated with differentiation ($p = 0.0209$), lymph node metastasis ($p = 0.0352$), and tumor response grade after preoperative chemotherapy ($p = 0.0253$) (Table 3). In addition, CD44v9 expression in the biopsy specimens was also strongly correlated with GPx2 expression ($p = 0.0078$). Supplementary Figure 4 shows a representative immunohistochemical image from a patient in CD44v9- and GPx2-positive expression cases in the same biopsy specimens.

Relationship between CD44v9 expression with chemoresistance to 5-fluorouracil in GC cell lines

We found that CD44v9-positive expression was associated with resistance to chemotherapy in GC in clinical specimens. We speculated that the acquisition of antioxidant capacity through CD44v9 was related to chemotherapeutic sensitivity. We therefore explored this possibility in GC cell lines. We first evaluated CD44v9 expression in MKN45, MKN74, NUGC4, KATOIII, and SNU-1, by qRT-PCR and used HCT116 as a positive control.[16] MKN45 and NUGC4 cells showed high CD44v9 mRNA expression (Supplementary Figure 5a). Western blot analysis of CD44v9 expression in MKN45 and NUGC4 cell lines corroborated the qRT-PCR results (Supplementary Figure 5b). Supplementary Figure 5c and 5d confirms the efficacy of

CD44v9 siRNA on downregulating CD44v9 mRNA and protein expression in qRT-PCR and western blotting, respectively.

We next examined the association of CD44v9 expression in GC cell lines with chemotherapeutic sensitivity to 5-FU, using the negative control siRNA transfected cells (control) and CD44v9 knockdown cells (CD44v9 siRNA). Although no significant difference was observed, CD44v9 siRNA cells showed a tendency to increase chemotherapeutic sensitivity to 5-FU compared with controls in MKN45 and NUGC4 cells. The IC50 values of MKN45 control and CD44v9 siRNA cells were 8.02 and 3.83 μg/ml, respectively ($p = 0.4329$). The IC50 values of NUGC4 control and CD44v9 siRNA cells were 8.91 and 4.50 μg/ml, respectively ($p = 0.1362$) (Figure 4).

Effect of siRNA-mediated knockdown of CD44v9 on intracellular GSH levels and ROS levels in GC cell lines

To determine the molecular mechanism responsible for chemotherapeutic resistance to 5-FU in CD44v9-positive cells, we next investigated whether knockdown of CD44v9 changed intracellular GSH levels and ROS levels. CD44v9 siRNA transfection significantly reduced intracellular GSH levels in MKN45 and NUGC4 cells compared with controls (Figure 5a, $p \leq 0.001$ and $p \leq 0.05$). In addition, CD44v9 siRNA transfection significantly increased intracellular ROS levels by administration of 5-FU in MKN45 and NUGC4 cells (Figure 5b, $p \leq 0.001$ and $p \leq 0.01$).

Discussion

In this study, we demonstrated that CD44v9 expression was associated with poor clinicopathological factors and prognosis and chemoresistance in GC clinical specimens. Furthermore, in GC cell lines, CD44v9 was associated with chemoresistance to 5-FU and controlled intracellular GSH and ROS levels. These findings may suggest that the regulation of intracellular accumulated ROS by CD44v9 expression was associated with tumor aggressiveness, prognosis and chemotherapeutic sensitivity in GC.

Recent studies have identified CSC as one of the causes of chemotherapy resistance in cancers,[18,19] and CD44 is one of the cell surface markers associated with CSC in various solid tumors.[2-5] CD44, a major adhesion molecule for the extracellular matrix, is a cell surface receptor for hyaluronic acid and involved in various biological processes such as lymphocyte activation and homing, tissue remodeling and cell migration.[20, 21] *CD44* gene transcripts undergo complex alternative splicing, which results in many functionally distinct isoforms, such as CD44 standard isoform (CD44s) and CD44 variant isoform (CD44v).[22] CD44v is highly expressed in a number of carcinoma cells and related to tumor progression and metastatic potential.[19, 22-26]

Among the various CD44 isoforms, we focused on CD44v9 in this study because CD44v9-positive expression was recently reported to be significantly associated with poor clinicopathological findings and prognosis in GC patients.[10, 11] CD44v9 stabilizes xCT and promotes the uptake of cystine required for intracellular GSH synthesis.[6] GSH is the most abundant non-enzymatic antioxidant molecule in cells and acts directly on eliminating intracellular ROS. GPx2, the gastrointestinal form of glutathione peroxidases, is an antioxidant enzyme that catalyzes the reduction of intracellular ROS such as H_2O_2 or hydroperoxide to water or the corresponding alcohols using GSH as

reductant.[7, 8] This regulation of intracellularly accumulated ROS in cancer cells is reported to be an important factor in tumor development and the response to anticancer therapies.[8, 9] CD44v9 is also a key molecule that promotes tumor development through the regulation of redox balance.

We showed that the presence of CD44v9-positive cells was significantly associated with not only poor clinicopathological factors and prognosis, but also poor response to chemotherapy such as worse treatment response after preoperative chemotherapy and poor prognosis after postoperative adjuvant chemotherapy in GC patients. These results indicated that CD44v9-positive GC patients showed chemotherapeutic resistance. We performed an analysis comparing pretreatment biopsy specimens and resected specimens in patients who received preoperative chemotherapy. If CD44v9-positive cells are resistant to chemotherapy, then CD44v9-positive cells are expected to increase in the resected specimens after preoperative chemotherapy. However, CD44v9-positive cells were not increased in the resected specimens. It may have been difficult to evaluate tumor cells because the resected specimens after chemotherapy were highly fibrotic and had undergone therapy-induced changes.

In this study, CD44v9 expression and GPx2 expression were strongly correlated in clinical specimens, and GC patients with high expression of both indicated the relatively poor prognosis in RFS. These results suggest that some common upstream factors may regulate both CD44v9 and GPx2 expression. A previous study reported a metabolomic analysis, which revealed that glutathione disulfide (GSSG) levels were significantly lower and reduced GSH/ GSSG ratio was significantly higher in CD44v9-positive tumors than in CD44v9-negative tumors, suggesting that CD44v9 may enhance pentose phosphate pathway flux and maintain GSH levels in cancer cells.[27] Other studies

reported that the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is most important regulator of the gene expression of various antioxidants elements such as GPx, GSH, and xCT. However, CD44v gene expression is regulated by epithelial splicing regulatory protein 1 (ESRP1), which controls CD44 isoform switching from CD44s to CD44v.[21, 28, 29] It is still unknown how these factors or other upstream factors regulate control CD44v9 and GPx2 at the same time, and the discovery of these expression regulators may lead to the development of new therapies.

We further investigated that CD44v9 was associated with chemoresistance to 5-FU and controlled intracellular GSH and ROS levels using GC cell lines. In MKN45 and NUGC4 cells, CD44v9 siRNA-transfected cells showed significantly reduced intracellular GSH levels and increased intracellular ROS levels in response to 5-FU than control cells. Previous studies showed that 5-FU inhibits thymidylate synthetase and/or incorporates into RNA and DNA, resulting in an intracellular increase in ROS levels.[30] Similarly, in our study, both MKN45 and NUGC4 cells showed elevated intracellular ROS levels after exposure to 5-FU. Furthermore, CD44v9 siRNA-transfected MKN45 and NUGC4 cells showed elevated intracellular ROS levels compared with control cells. Interestingly, in these cell lines, an increase in ROS was observed only by adding CD44v9 siRNA with DMSO, respectively. Thus, we speculated that the reason for these results was because CD44v9-positive cells could regulate intracellular redox balance.

For clinical application, an anti-CD44v9 targeting therapy is expected to be developed. Sulfasalazine (SSZ), which has been used to for inflammatory diseases such as rheumatoid arthritis and ulcerative colitis, is a specific inhibitor of xCT-mediated cystine transport and has been shown to selectively suppress the proliferation of CD44v-positive cancer cells.[31] In addition, SSZ was reported to induce the

phosphorylation of p38 mitogen-activated protein kinase, an indicator of increased intracellular ROS levels, and to give oxidative cytotoxicity in CD44v-positive gastric cancer cells.[6] In Japan, based on these findings, several clinical studies have evaluated the treatment effects of SSZ for advanced GC and non-small cell lung cancer.[32-34] From our results of chemoresistance in CD44v9-positive GC, the further development of novel treatment strategies related to an anti-CD44v9 targeting therapy is required for managing patients with GC.

The present study has several limitations. First, this was a retrospective study at a single institution and not a trial-based correlative study. Thus, the possibility of bias cannot be ruled out. In particular, the sub-analyses were conducted in small populations. In fact, the number of the pStage III patients who received postoperative adjuvant chemotherapy was greater than that of the patients who did not receive postoperative adjuvant chemotherapy. Therefore, we think that postoperative adjuvant chemotherapy was not the only cause of poor outcomes of CD44v9-positive patients. Second, we evaluated CD44v9 and GPx2 immunohistochemical staining in whole-tumor tissue. Some cases showed heterogeneous expression of CD44v9 and GPx2 regardless of the infiltration of cancer cells. However, several recent studies showed that CD44v9-positive cells located at the tumor invasive front (TIF) were important because of the association between CD44v9 and the epithelial-mesenchymal transition (EMT).[26, 35] Thus, we think that it is necessary to evaluate CD44v9 expression at the TIF, focusing on the intratumoral heterogeneity and the relationship between intracellular accumulated ROS and EMT. Furthermore, in a future study, we will investigate a second cohort to validate the findings of the current study.

In conclusion, we demonstrated CD44v9 expression was associated with chemoresistance in GC by the regulation of intracellularly accumulated ROS. These findings suggest that CD44v9 may be a not only a prognostic but also predictive biomarker for the response to chemotherapy in GC patients.

Additional Information

Author contributions:

TJ performed the experiments, assisted in the statistical analysis and wrote the manuscript. EO designed the experiments and supervised the manuscript. TJ and YO analyzed the immunohistochemically stained samples. Other co-authors assisted in the experimental process and helped to write the manuscript. EO, YO, and YM organized the writing of the manuscript. All authors contributed to the discussion and revision of the manuscript and have approved the final manuscript.

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Data availability:

All data generated or analyzed during this study are included in this published article and its supplementary files.

Ethics approval and consent to participate:

All procedures followed in this study were in accordance with the Declaration of Helsinki of 1964 and later versions and the Japanese Ethical Guidelines for Medical and Health Research Involving Human Subjects. Informed consent for it was obtained from all patients for their being included in the stud included in the study in the form of opt-out on the web-site.

Conflict of interest:

The authors declare no conflicts of interest in association with this study.

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Figure legends

Figure 1. Flowchart depicting the patient selection process. This study included 596 primary GC patients who underwent surgery between January 2006 and December 2016. Among these patients, 193 GC patients were treated without preoperative chemotherapy from 2008 to 2012 and 29 GC patients received preoperative chemotherapy from 2006 to 2016. Among the 69 GC patients with pathological stage II–III without preoperative chemotherapy, 36 patients completed postoperative adjuvant chemotherapy, 12 patients were treated with non-completed postoperative adjuvant chemotherapy, and 21 patients did not receive postoperative adjuvant chemotherapy. Abbreviations: GC = gastric cancer; CD44v9 = CD44 variant 9.

Figure 2. CD44v9 and GPx2 expression in the resected specimens in GC patients. Representative immunohistochemical staining of CD44v9 and GPx2 in resected specimens of primary GC. CD44v9 intensity score (a) -1, no staining; (b) -1, weak staining homogeneously; (c) 0, intermediate staining heterogeneously; (d) 1, strong staining homogeneously. Gpx2 intensity score (e) 0, no staining; (f) 1, weak staining; (g) 2, intermediate staining; (h) 3, strong staining. (a–h, high-power view of square, ×20 objective lens, scar bar 100 μm). Abbreviations: CD44v9 = CD44 variant 9; GPx2 = glutathione peroxidase 2; GC = gastric cancer.

Figure 3. Overall survival and recurrence-free survival in GC patients who underwent surgery without preoperative chemotherapy. Patients who underwent surgery without preoperative chemotherapy with CD44v9-positive expression exhibited significantly poorer prognosis than those with CD44v9-negative expression in (a) OS

and (b) RFS. Abbreviations: GC = gastric cancer; CD44v9 = CD44 variant 9; OS = overall survival, RFS = recurrence-free survival.

Figure 4. The relationship between CD44v9 expression and chemotherapeutic sensitivity to 5-FU in MKN45 and NUGC4 cells. Cell viability was measured after treatment with different concentrations of 5-FU or DMSO (refer to Materials and Methods for details) for 72 h in MKN45 and NUGC4 cells transfected with CD44v9 or control siRNA. CD44v9 siRNA cells exhibited higher chemotherapeutic sensitivity to 5-FU than control cells in MKN45 and NUGC4 lines. Data are means ± standard deviation from three independent experiments. Abbreviations: CD44v9 = CD44 variant 9; 5-FU = 5-fluorouracil.

Figure 5. CD44v9-knockdown results in altered intracellular GSH levels and ROS levels in MKN45 and NUGC4 cells. Knockdown of CD44v9 results in (a) significantly reduced intracellular GSH levels and (b) significantly increased ROS levels by administration of 5-FU in MKN45 and NUGC4 cells. Data are means ± standard deviation from three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001. Abbreviations: CD44v9 = CD44 variant 9; GSH = glutathione; ROS = reactive oxygen spices; GC = gastric cancer; 5-FU = 5-fluorouracil.

Supplementary Figure 1. Overall survival and recurrence-free survival in GC patients who underwent surgery without preoperative chemotherapy.
Patients who underwent surgery without preoperative chemotherapy were distinguished to four groups depending on the combination of CD44v9 and GPx2 expression. There

were significant differences in (a) OS and (b) RFS among the four groups of patients, and the double-positive group in CD44v9 and GPx2 expression showed the worst outcome in RFS. Abbreviations: GC = gastric cancer; CD44v9 = CD44 variant 9; OS = overall survival, RFS = recurrence-free survival.

Supplementary Figure 2. Overall survival and recurrence-free survival in GC patients who underwent surgery with postoperative adjuvant chemotherapy (pStage II–III). Patients who underwent surgery with postoperative adjuvant chemotherapy with CD44v9-positive expression exhibited significantly poorer prognosis than those with CD44v9-negative expression in (a) OS and (b) RFS. Abbreviations: GC = gastric cancer; CD44v9 = CD44 variant 9; OS = overall survival, RFS = recurrence-free survival.

Supplementary Figure 3. Overall survival and recurrence-free survival in GC patients who underwent surgery without postoperative adjuvant chemotherapy (pStage II–III). CD44v9 expression did not have any prognostic impact for patients without postoperative adjuvant chemotherapy in (a) OS and (b) RFS. Abbreviations: GC = gastric cancer; CD44v9 = CD44 variant 9; OS = overall survival, RFS = recurrence-free survival.

Supplementary Figure 4. CD44v9 and GPx2 expression in the same biopsy specimens in a GC patient. Representative immunohistochemical staining of CD44v9 and GPx2 in the same biopsy specimen in primary GC from a patient: (a) hematoxylin-eosin staining, (b) CD44v9-positive expression, and (c) GPx2-positive

expression. (a,b,c, high-power view of square, ×20 objective lens, scar bar 100 μm). Abbreviations: CD44v9 = CD44 variant 9; GPx2 = glutathione peroxidase 2; GC = gastric cancer.

Supplementary Figure 5. CD44v9 expression in GC cell lines. (a) qRT-PCR and (b) western blotting results showing CD44v9 expression in various GC cell lines (c) qRT-PCR and (d) western blotting results showing CD44v9 knockdown efficiency following CD44v9 siRNA transfection in MKN45 and NUGC4 cells. Abbreviations: CD44v9 = CD44 variant 9; GC = gastric cancer.

Figure 1

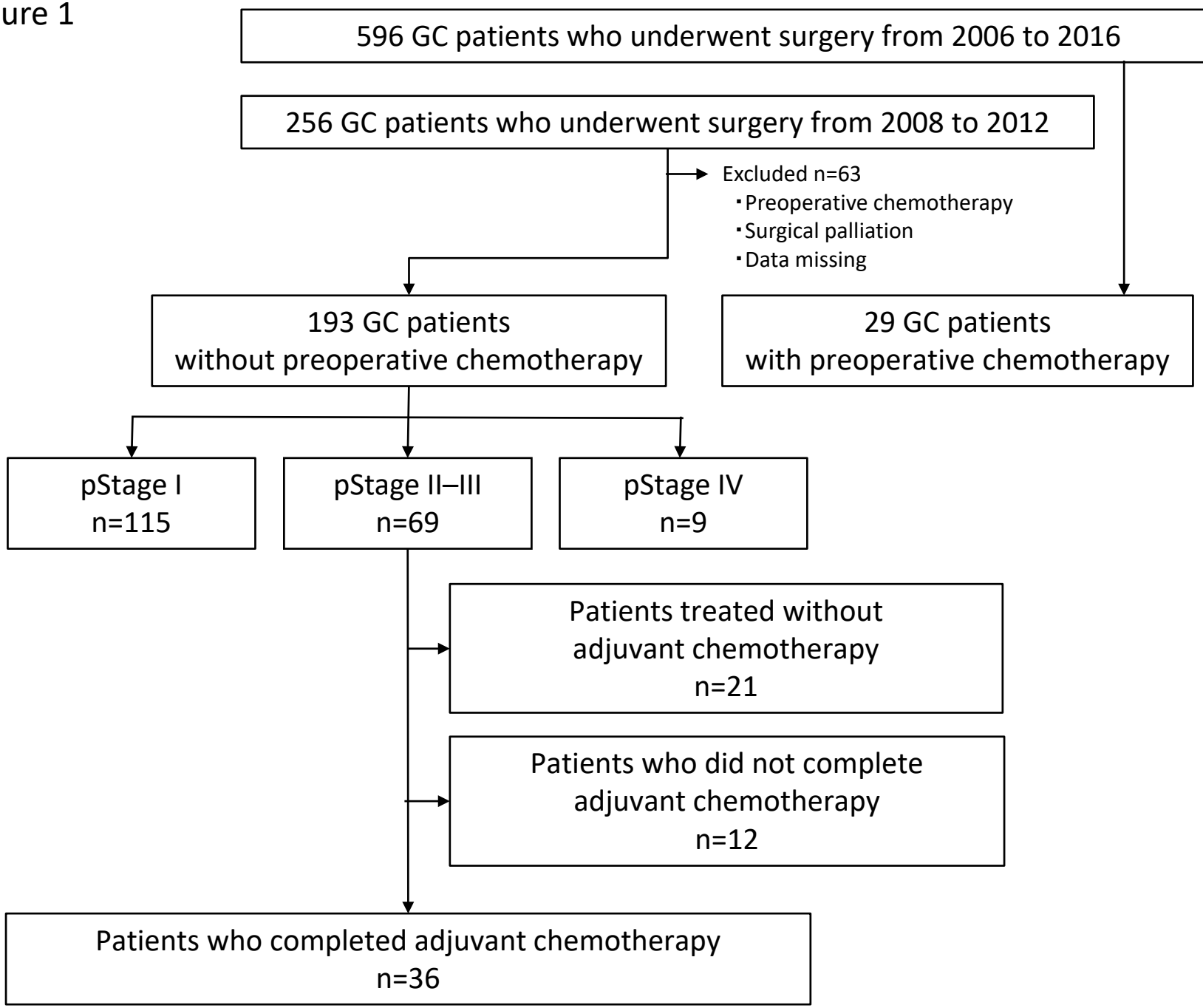

596 GC patients who underwent surgery from 2006 to 2016
256 GC patients who underwent surgery from 2008 to 2012
Excluded n=63
・Preoperative chemotherapy
・Surgical palliation
・Data missing
193 GC patients
without preoperative chemotherapy
29 GC patients
with preoperative chemotherapy
pStage I
n=115
pStage II–III
n=69
pStage IV
n=9
Patients treated without
adjuvant chemotherapy
n=21
Patients who did not complete
adjuvant chemotherapy
n=12
Patients who completed adjuvant chemotherapy
n=36

Figure 2

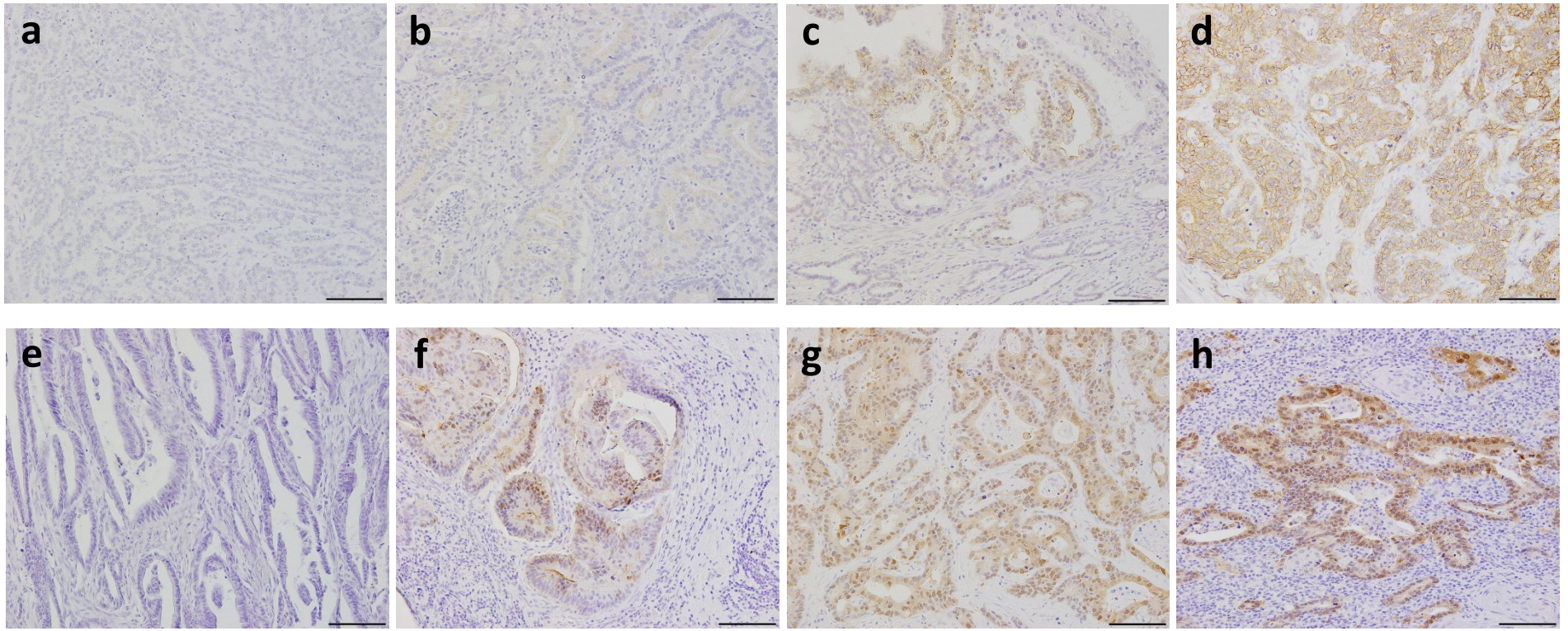
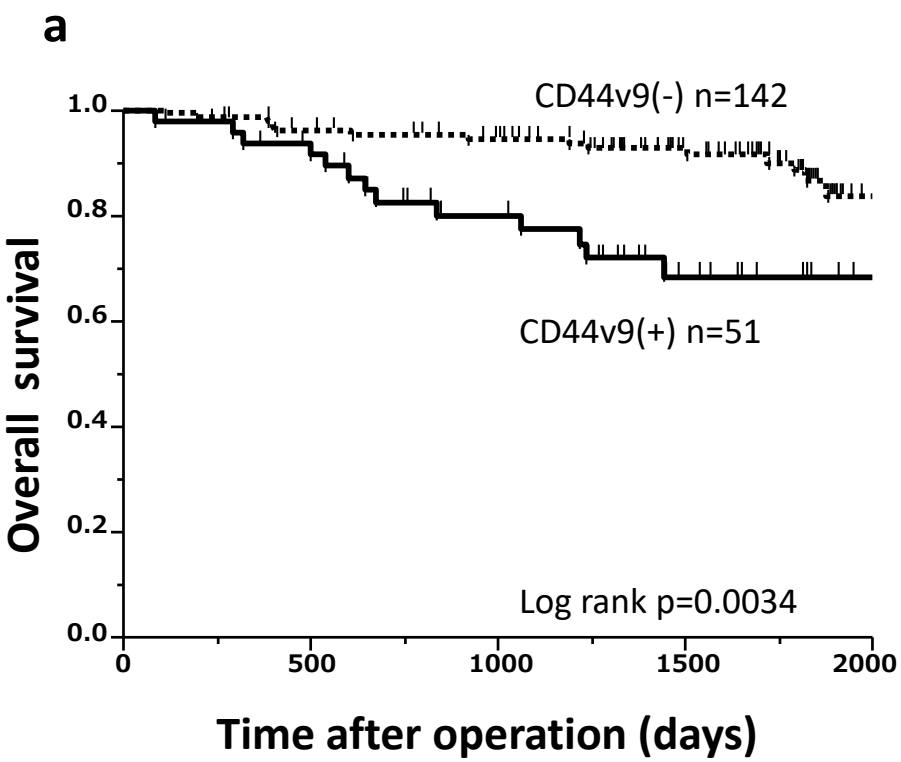


Figure 3


a
CD44v9(-) n=142
CD44v9(+) n=51
Log rank p=0.0034
Overall survival
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
Time after operation (days)

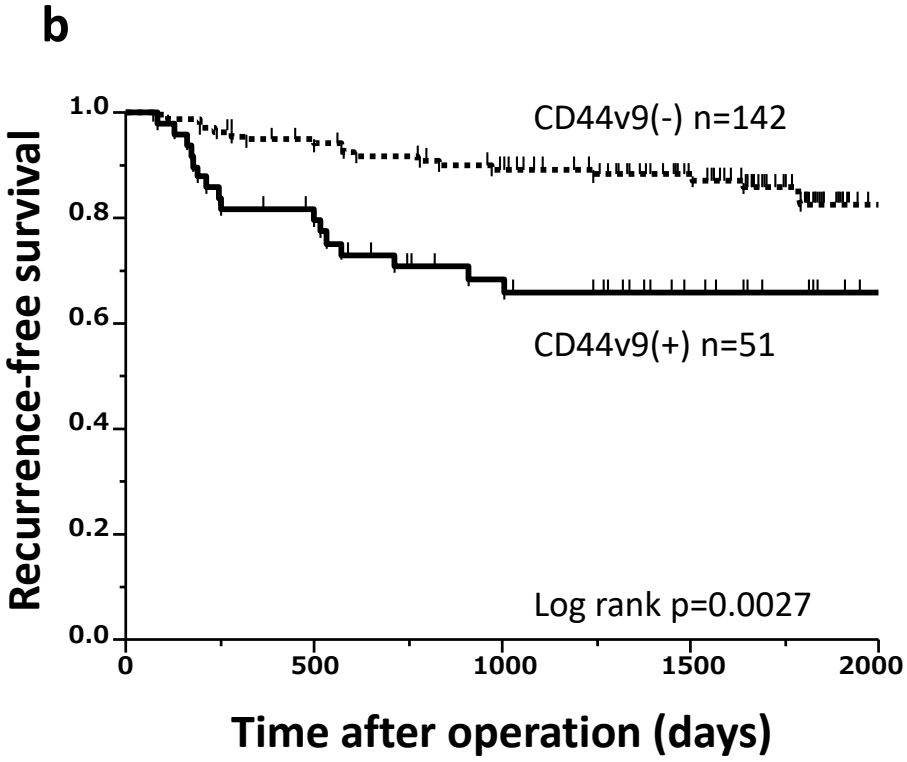

b
CD44v9(-) n=142
CD44v9(+) n=51
Log rank p=0.0027
Recurrence-free survival
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
Time after operation (days)

Figure 4

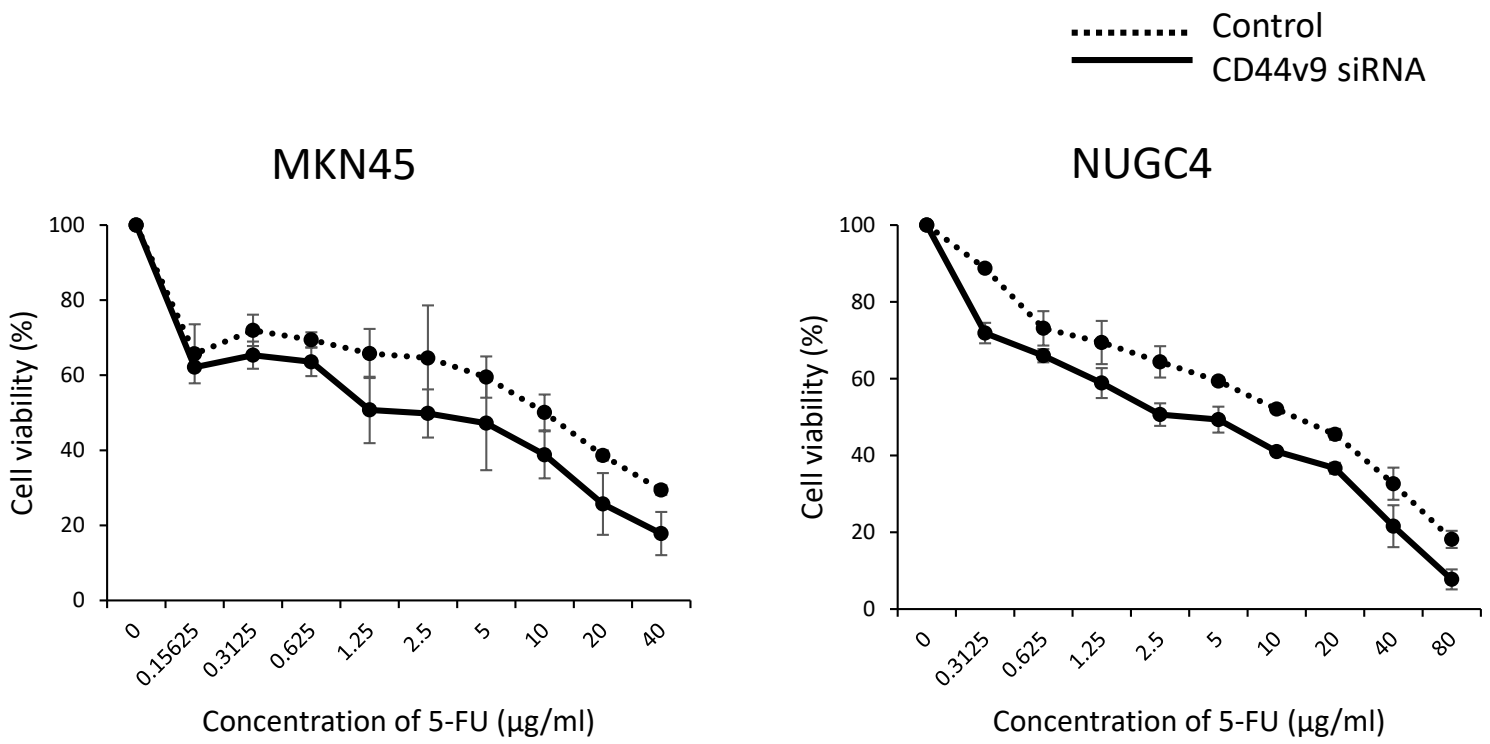
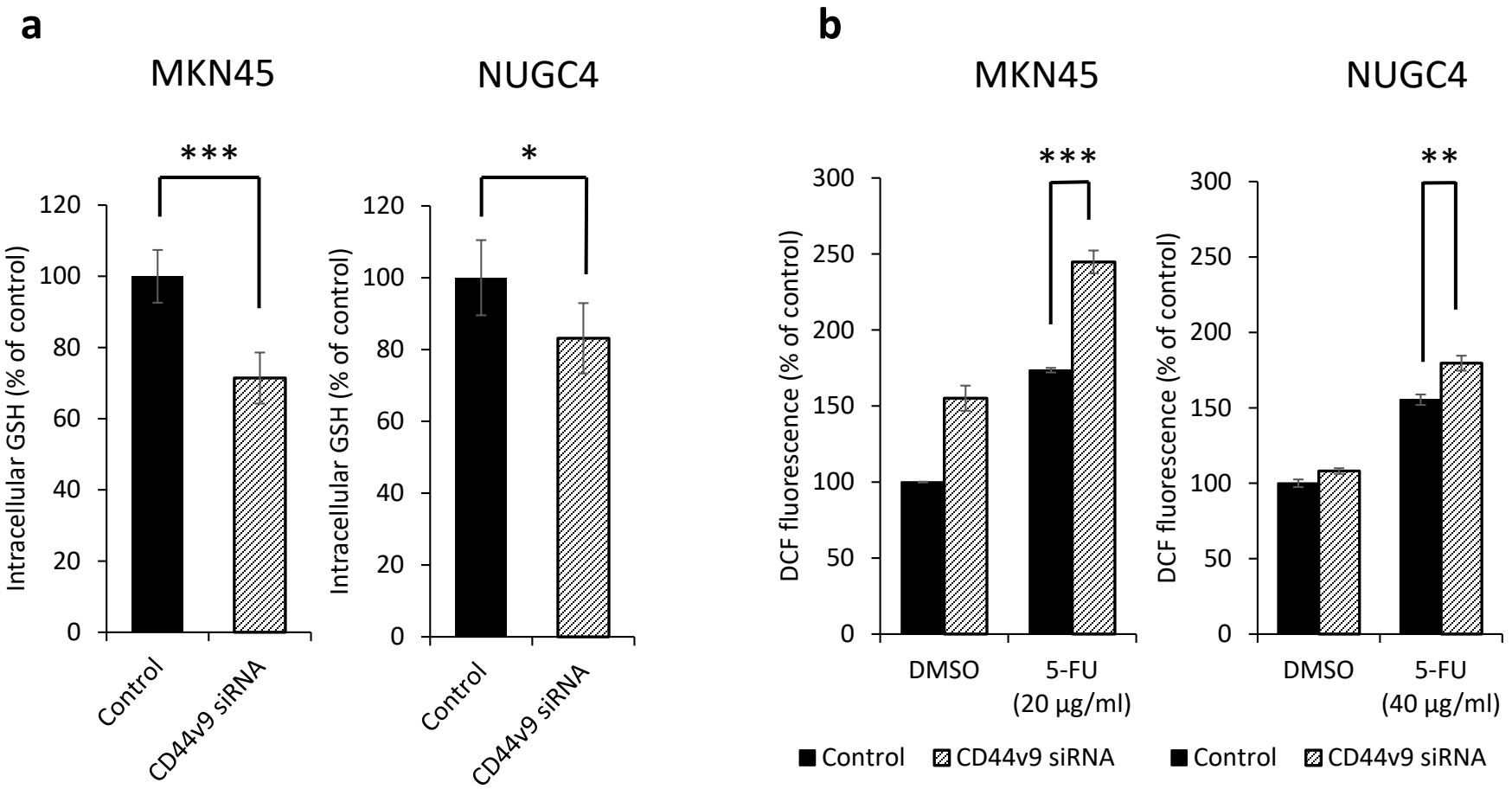
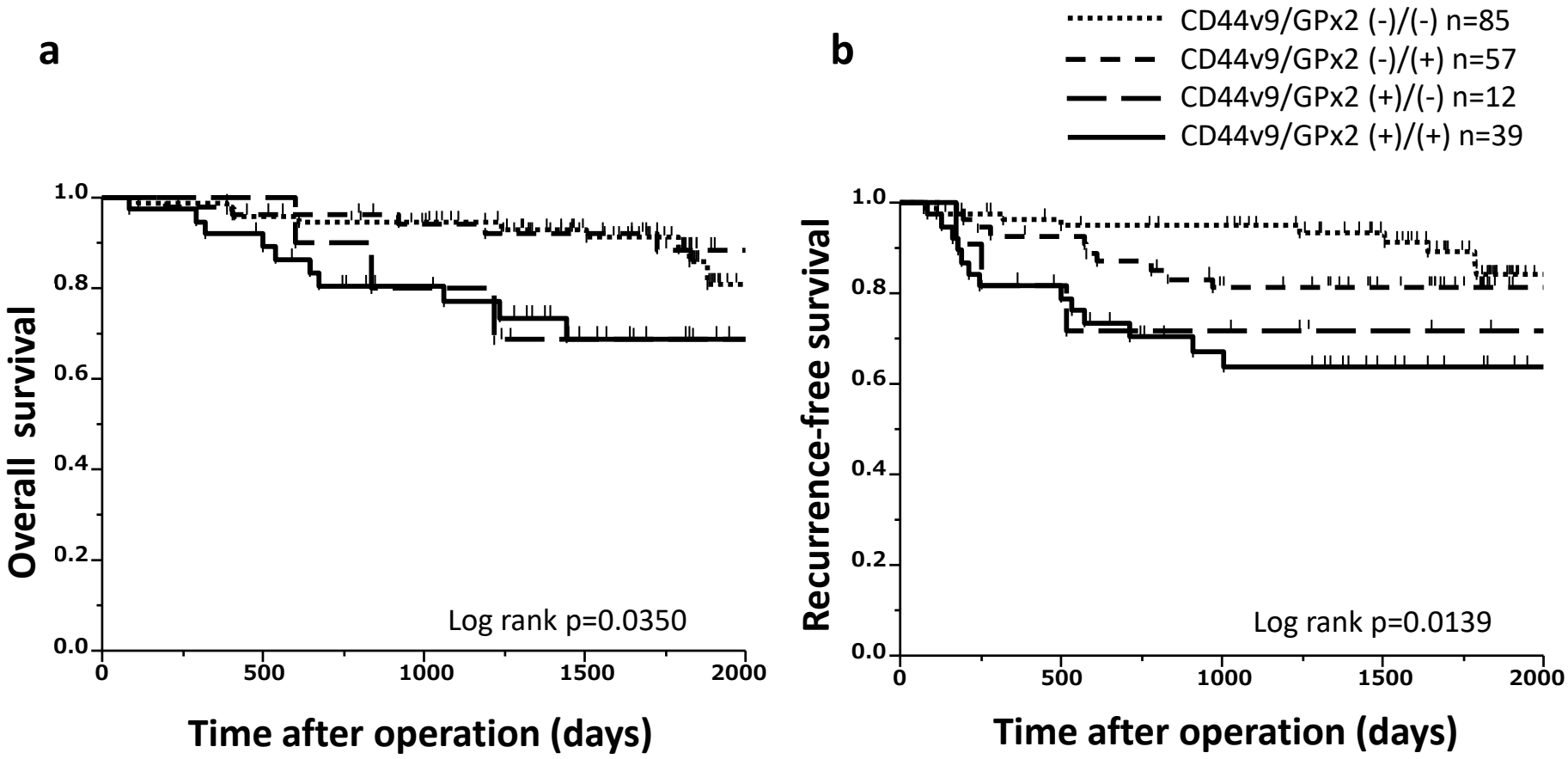

Control
CD44v9 siRNA
MKN45
NUGC4
Cell viability (%)
Concentration of 5-FU (μg/ml)
0
20
40
60
80
100
0
0.15625
0.3125
0.625
1.25
2.5
5
10
20
40
80

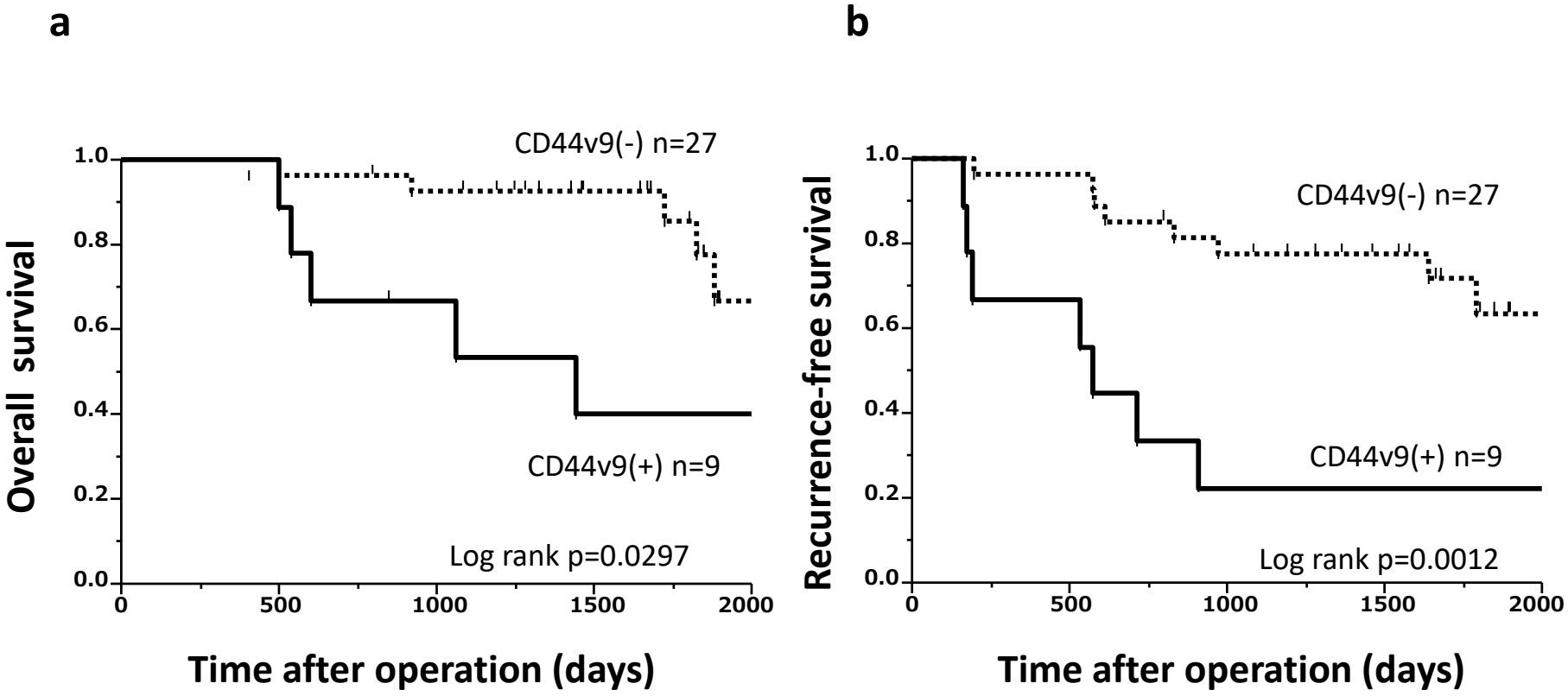
Figure 5


a
MKN45
NUGC4
***
*
Intracellular GSH (% of control)
120
100
80
60
40
20
0
Control
CD44v9 siRNA
b
MKN45
NUGC4
***
**
DCF fluorescence (% of control)
300
250
200
150
100
50
0
DMSO
5-FU (20 μg/ml)
5-FU (40 μg/ml)
Control
CD44v9 siRNA

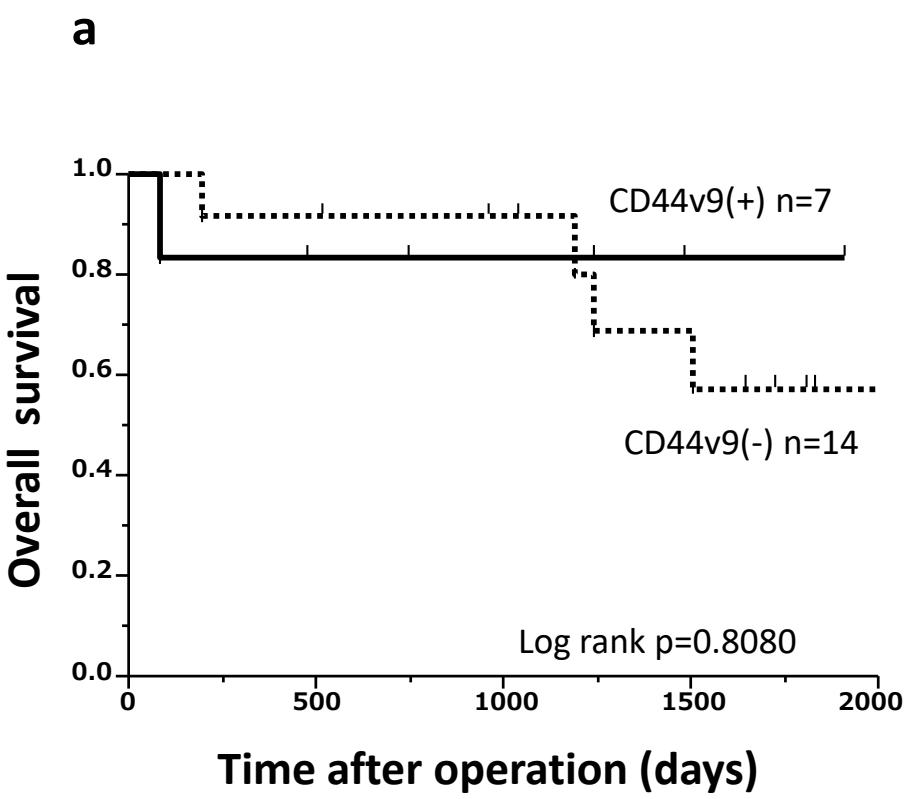
Supplementary Figure 1

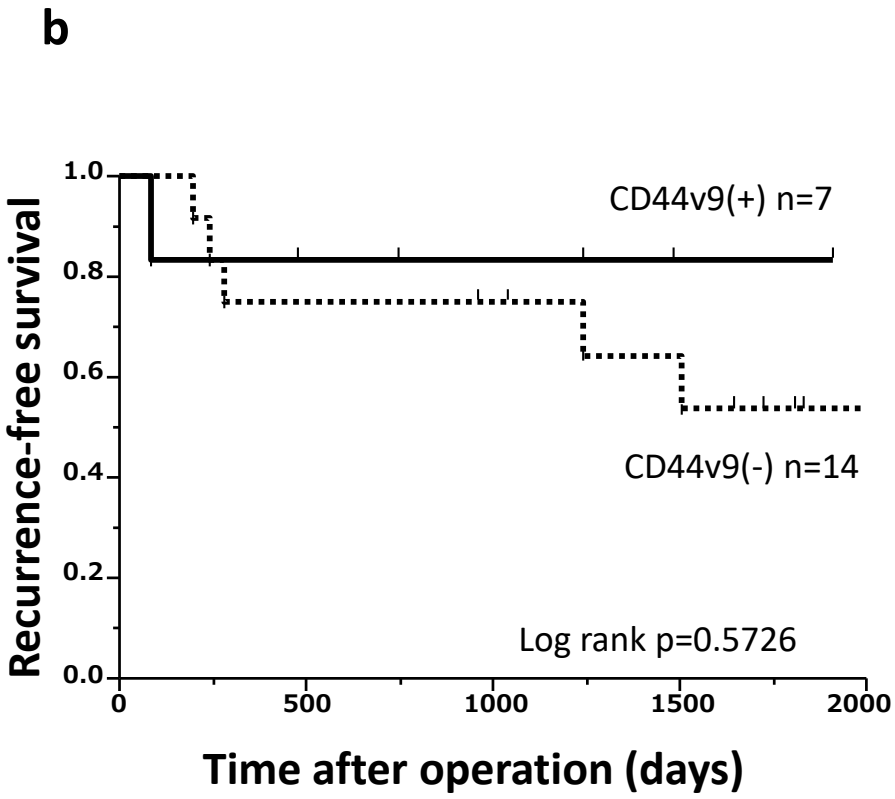


Supplementary Figure 2

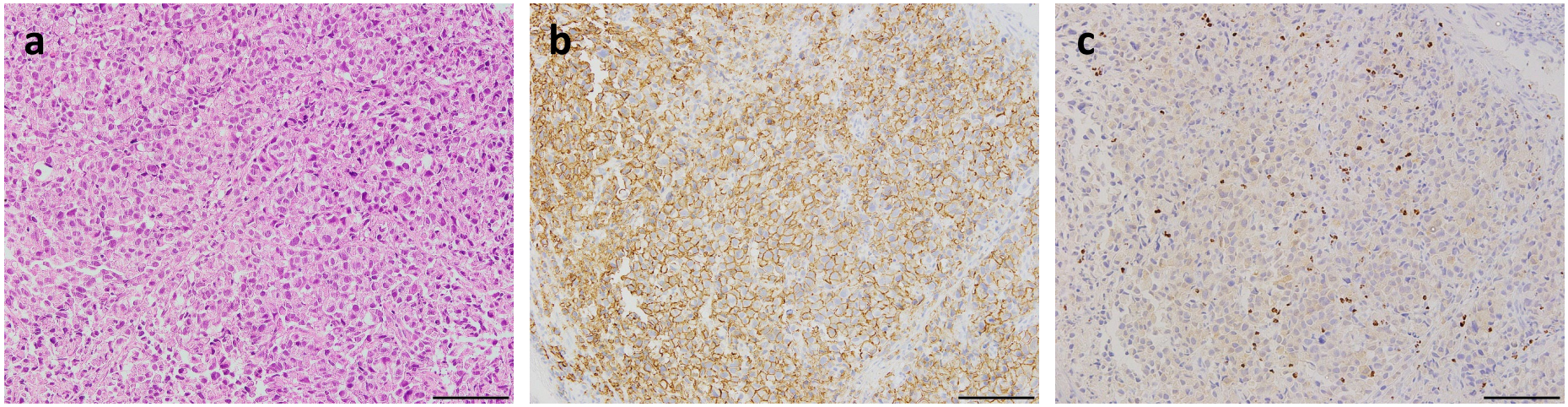


Supplementary Figure 3





Supplementary Figure 4



Supplementary Figure 5

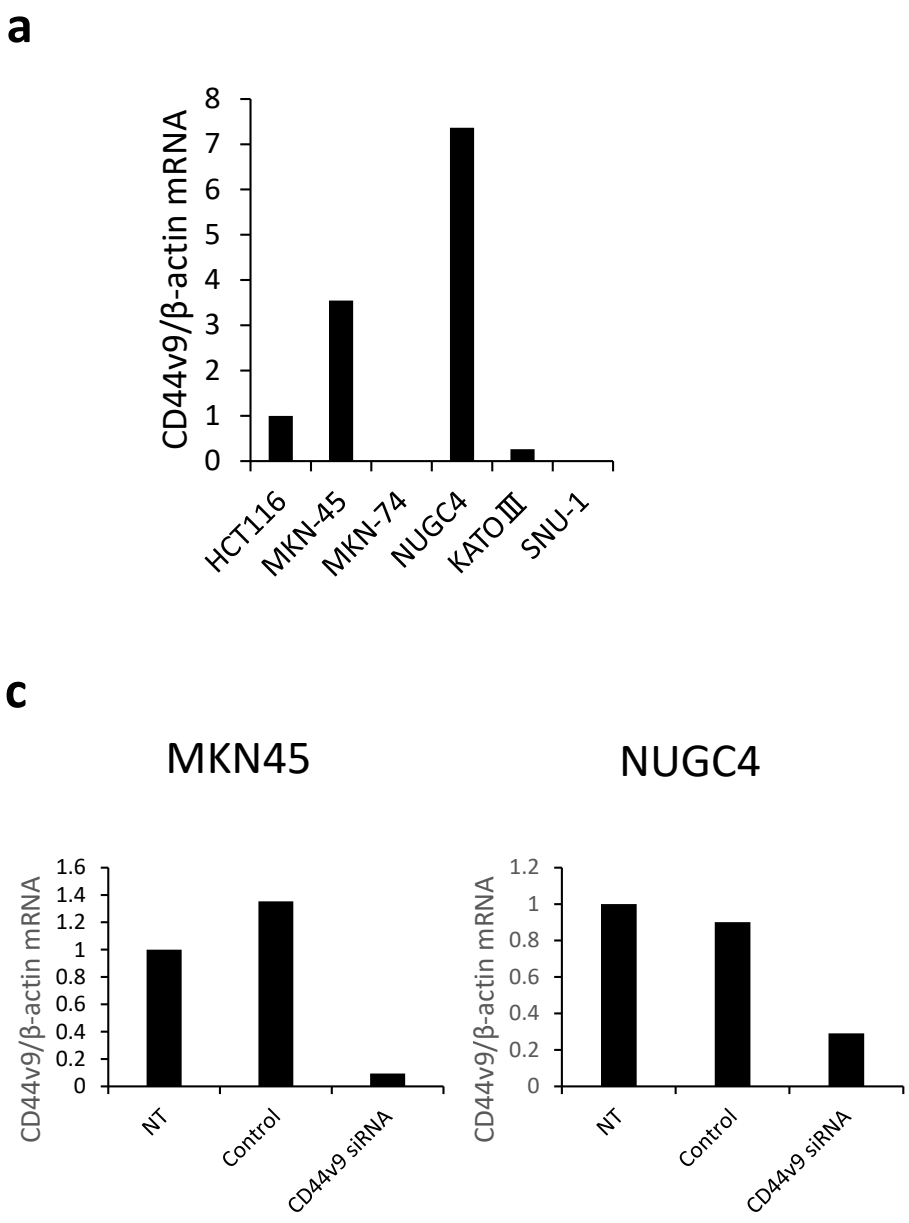


Table 1. Association between CD44v9 expression in resected specimens and clinicopathological factors in GC patients who underwent surgery without preoperative therapy

Factors		**CD44v9-negative n=142 (%)**	**CD44v9-positive n=51 (%)**	***P*-value**
Age (average ± SD)		64.2 ± 12.1	66 ± 11.5	0.3659
Sex	Male	87 (61.3)	41 (80.4)	**0.0154**
	Female	55 (38.7)	10 (19.6)	
Differentiation	Well/Moderately	67 (47.2)	27 (52.9)	0.5163
	Poorly/Signet-ring cells	75 (52.8)	24 (47.1)	
Depth of tumor invasion	T1/2	103 (72.5)	26 (51.0)	**0.0088**
	T3/4	39 (27.5)	25 (49.0)	
Lymphatic permeation	Absent	99 (69.7)	22 (43.1)	**0.0012**
	Present	43 (30.3)	29 (56.9)	
Vascular invasion	Absent	117 (82.4)	35 (68.6)	**0.0470**
	Present	25 (17.6)	16 (31.4)	
Lymph node metastasis	Absent	93 (65.5)	27 (53.0)	0.1309
	Present	49 (34.5)	24 (47.0)	
Distant metastasis	Absent	139 (97.9)	45 (88.2)	**0.0114**
	Present	3 (2.1)	6 (11.8)	
pStage	I/II	113 (79.6)	34 (66.7)	0.0839
	III/IV	29 (20.4)	17 (33.3)	
GPx2 expression	Negative	85 (59.9)	12 (23.5)	**<0.0001**
	Positive	57 (40.1)	39 (76.5)	

Abbreviations: CD44v9 = CD44 variant 9; GC = gastric cancer; pStage = pathological stage; GPx2 = glutathione peroxidase 2. Bold value indicates a significant difference.

Table 2. Univariate and multivariate analyses for OS and RFS in GC patients who underwent surgery without preoperative therapy (pStage I–IV)

Factors	Object	Control	OS						RFS					
			Univariate			Multivariate			Univariate			Multivariate		
			HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	≥65	<65	0.680	0.318–1.455	0.3208				0.642	0.328–1.257	0.1960			
Sex	Male	Female	1.043	0.471–2.308	0.9175				1.004	0.501–2.010	0.9921			
Differentiation	Poorly/ Sig	Well/ Moderately	1.886	0.850–4.183	0.1184				1.824	0.911–3.652	0.0897			
Lymphatic permeation	Present	Absent	3.769	1.704–8.334	**0.0011**	0.887	0.375–2.101	0.7855	3.728	1.862–7.464	**0.0002**	0.973	0.450–2.104	0.9442
Vascular invasion	Present	Absent	3.004	1.420–6.353	**0.0040**	1.338	0.600–2.983	0.4769	3.669	1.897–7.098	**0.0001**	1.602	0.799–3.211	0.1842
pStage	III/IV	I/II	19.970	7.553–52.799	**<0.0001**	18.898	6.441–55.447	**<0.0001**	15.955	7.240–35.163	**<0.0001**	13.830	5.731–35.375	**<0.0001**
CD44v9 expression	Positive	Negative	2.904	1.377–6.126	**0.0051**	2.393	1.110–5.159	**0.0259**	2.644	1.366–5.118	**0.0039**	2.395	1.216–4.714	**0.0115**
GPx2 expression	Positive	Negative	1.337	0.632–2.830	0.4474				2.060	1.029–4.123	**0.0412**	1.138	0.547–2.366	0.7298

Abbreviations: OS = overall survival; RFS = recurrence-free survival; GC = gastric cancer; Sig = Signet-ring cells; pStage = pathological stage; HR = hazard ratio; CI = confidence interval; CD44v9 = CD44 variant 9; GPx2 = glutathione peroxidase 2. Bold value indicates a significant difference.

Table 3. Association between CD44v9 expression in pretreatment biopsy specimens and clinicopathological factors in GC patients who underwent surgery with preoperative therapy

Factors		**CD44v9-negative n=15 (%)**	**CD44v9-positive n=14 (%)**	***P*-value**
Age (average ± SD)		63.1 ± 6.15	60.4 ± 12.6	0.4629
Sex	Male	10 (66.7)	12 (85.7)	0.3898
	Female	5 (33.3)	2 (14.3)	
Differentiation	Well/Moderately	2 (13.3)	8 (57.1)	**0.0209**
	Poorly/Signet-ring cells	13 (86.7)	6 (42.9)	
Depth of tumor invasion	T1/2	2 (13.3)	0 (0)	0.4828
	T3/4	13 (86.7)	14 (100.0)	
Lymph node metastasis	Absent	7 (46.7)	1 (7.1)	**0.0352**
	Present	8 (53.3)	13 (92.9)	
Distant metastasis	Absent	7 (46.7)	7 (50.0)	1.0000
	Present	8 (53.3)	7 (50.0)	
cStage	I/II	2 (13.3)	1 (7.1)	1.0000
	III/IV	13 (86.7)	13 (92.9)	
Grade	1a	5 (33.3)	11 (78.6)	**0.0253**
	1b/2	10 (66.7)	3 (21.4)	
GPx2 expression	Negative	10 (66.7)	2 (14.3)	**0.0078**
	Positive	5 (33.3)	12 (85.7)	

Abbreviations: CD44v9 = CD44 variant 9; GC = gastric cancer; cStage = clinical stage; GPx2 = glutathione peroxidase 2. Bold value indicates a significant difference.

Supplementary Table 1. Association between CD44v9 expression in resected specimens and Lauren classification in GC patients who underwent surgery without preoperative therapy

Factors		**CD44v9-negative n=134 (%)**	**CD44v9-positive n=43 (%)**	***P*-value**
Lauren classification	Intestinal type	67 (50)	27 (62.8)	0.1626
	Diffuse type	67 (50)	16 (37.2)	

Abbreviations: CD44v9 = CD44 variant 9; GC = gastric cancer.