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

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1 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.

5 Methods: CD44v9 and GPx2 expression were investigated by immunohistochemistry 6 in resected specimens from 193 gastric cancer (GC) patients without preoperative 7 chemotherapy and in pretreatment biopsy specimens from 29 GC patients with 8 preoperative chemotherapy. We analyzed the relationship between CD44v9 expression 9 and clinicopathological factors, prognosis, and pathological response to chemotherapy. 10 In GC cell lines, we examined the relationship between CD44v9 expression and 11 chemotherapeutic sensitivity.

Results: In patients without preoperative chemotherapy, CD44v9 expression was 12 significantly associated with depth of invasion, lymphatic permeation, vascular invasion, 13 14 distant metastasis and GPx2 expression. In multivariate analysis, CD44v9 expression was an independent poor prognosis factor for overall survival and recurrence-free 15 survival. In patients with preoperative chemotherapy, CD44v9 expression was 16 significantly associated with worse pathological response and GPx2 expression. In GC 17 cell lines, downregulation of CD44v9 expression enhanced chemotherapeutic sensitivity 18 19 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.

23

24 Keywords:

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

1 Introduction

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause 2 of cancer-related death in the world.¹ GC shows the highest estimated mortality rates in 3 Eastern Asia and is one of the most common neoplasms in Japan. Early detection and 4 resection of GC with gastrointestinal endoscopy and the development of various 5 anti-cancer drugs have improved the survival rates of GC. However, the treatment 6 outcome of advanced GC is still unsatisfactory. The reason is because some early and 7 8 advanced GC patients show recurrence and chemotherapy resistance, leading to poor prognosis. Therefore, investigation of poor prognostic biomarkers and predictive 9 biomarkers for the response to chemotherapy in GC is crucial. 10

CD44 is a cell surface marker that is associated with cancer stem cells (CSC) in 11 various solid tumors.²⁻⁵ CD44 variant 9 (CD44v9), a splicing variant of CD44, has been 12 reported to stabilize a glutamate-cystine transporter (xCT) at the cell surface and 13 promote the uptake of cystine required for intracellular glutathione (GSH) synthesis.⁶ 14 Glutathione peroxidase 2 (GPx2), the gastrointestinal form of glutathione peroxidases, 15 is an antioxidant enzyme that catalyzes the reduction of intracellular reactive oxygen 16 spices (ROS) using GSH as a reductant.^{7, 8} These mechanisms suggest that CD44v9 has 17 a specific function in the regulation of intracellular accumulated ROS. The regulation of 18 redox balance in cancer cells is reported to be an important factor in tumor development 19 and the response to anticancer therapies.^{8,9} 20

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 recurrence¹¹ 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.

1 Methods

2 Patients and specimens

3 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 4 and Science, Graduate School of Medical Sciences, Kyushu University. From this 5 6 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) 7 8 margins without preoperative chemotherapy between January 2008 and December 2012. We obtained pretreatment biopsy specimens from the remaining 29 primary GC patients 9 who underwent surgery after chemotherapy between January 2006 and December 2016 10 11 as the second sample set. TNM staging and pathological classification were defined 12 according to the Japanese Gastric Cancer Association (JGCA) staging system (14th edition).¹³ In the 193 primary GC patients who underwent surgery without preoperative 13 chemotherapy, postoperative adjuvant chemotherapy was completely performed to 36 14 patients with pathological stage II-III. Among these 36 patients, 32 were treated with 15 S-1 alone, one was treated with tegafur and uracil alone, and three were treated with 16 capecitabine plus oxaliplatin. Among the 29 patients who underwent surgery after 17 chemotherapy, six were treated with S-1 alone, nine were treated with S-1 plus cisplatin, 18 19 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 20 resected specimens of these patients, histological evaluation criteria of tumor response 21 22 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 23 (considerable effect); and Grade 3 (complete response).¹³ 24

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

3

4 Immunohistochemistry

5 CD44v9 and GPx2 immunohistochemistry were performed using a rat monoclonal 6 anti-CD44v9 antibody (LKG-M001, COSMO BIO CO LTD, Tokyo, Japan) at 1:5000 7 dilution and a rabbit polyclonal anti-GPx2 antibody (ab137431, Abcam, Cambridge, 8 UK) at 1:1000 dilution, respectively.

CD44v9 expression is mainly localized in the cell membrane, and GPx2 is mainly 9 localized in the cytoplasm. CD44v9 staining was scored as described previously.¹⁴ The 10 proportion of stained carcinoma cells was semi-quantitatively analyzed in whole-tumor 11 tissue in low-power fields (×40). The proportion scores were defined as follows: 0, 0% 12 (no positive cells); 1, 1% - 25%; 2, 26% - 75%; and 3, 76% - 100%. The intensity 13 scores were defined as follows: -1, no or weak staining homogeneously; 0, intermediate 14 or strong staining heterogeneously; and 1, strong staining homogeneously. The total 15 score was calculated as the sum of the proportion and intensity score of positively 16 stained carcinoma cells. Samples with scores from -1 - 1 were categorized as 17 CD44v9-negative and samples with scores from 2 - 4 were categorized as 18 CD44v9-positive. The GPx2 staining was scored as described previously.¹⁵ The 19 expression rate was quantified from 0% to 100%. The intensity score of positively 20 stained carcinoma cells was scored as follows: 0, no staining; 1, weak staining; 2, 21 intermediate staining; and 3, strong staining. Total scores were determined by 22 multiplying the expression rate and intensity scores. Samples with scores less than 0.5 23

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

3

4 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.

10

11 Quantitative RT- PCR

Total RNA was separated from cells using Maxwell RSC simplyRNA Tissue 12 KitRNeasy (Promega, Madison, WI, USA) and reverse transcribed into cDNA using 13 SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA, USA). 14 Real-time PCR was performed using StepOnePlus (Applied Biosystems, Foster City, 15 CA, USA). We determined mRNA expression with TaqMan qPCR using TaqMan probe 16 Hs01081475 m1 (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA 17 expression levels were measured in triplicate for each sample. B-actin mRNA level was 18 19 used as an internal control to normalize the mRNA levels.

20

21 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).

4

5 siRNA transfection

CD44v9 and negative control were obtained from Thermo Fisher Scientific, Inc. 6 siRNA sequences were as follows: CD44v9 siRNA sense, 5'-CUA CUU UAC UGG 7 AAG GUU Att-3' and antisense, 5'-UAA CCU UCC AGU AAA GUA Gtt-3'.17 Silencer 8 Select Negative Control siRNA was used as a non-targeting siRNA. Cells seeded in a 9 6-well plate (1×10⁵ cells per well) were reverse-transfected with 10 nmol CD44v9 10 11 siRNA with Lipofectamine RNAimax reagent (Thermo Fisher Scientific). mRNA knockdown and downregulated protein expression were verified by qRT-PCR and 12 western blotting, respectively, at three time points, 48 h, 72 h, and 96 h. 13

14

15 Cell viability assays

Cells transfected with CD44v9 or negative control siRNA were seeded into a 96-well plate $(2 \times 10^3 \text{ cells per well})$ 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). IC₅₀ values were calculated using XLfit (ID Business Solutions Ltd.).

23

24 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 (2×10³ cells per well), and GSH measurement was performed 48 h later
 using Cytation 5 Cell Imaging Multi-Mode Reader (BioTek).

5

6 Measurement of ROS levels

The intracellular ROS levels under normal and stress conditions were detected using 7 DCFDA/H2DCFDA-Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, 8 Cambridge, UK). Cells transfected with CD44v9 or negative control siRNA were 9 seeded into a 96-well plate $(2 \times 10^3$ cells per well) and incubated for 24 h. Various 10 concentrations of 5-FU were added, and cells were incubated for 72 h. Next, 20 µM 11 DCFDA was added and cells incubated for 30-45 min at 37°C in the dark. Fluorescence 12 intensity was immediately measured using Cytation 5 Cell Imaging Multi-Mode Reader 13 (BioTek). 14

15

16 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.

1 Results

2 CD44v9 expression in the resected specimens and clinicopathological factors in the 3 patients who underwent surgery without preoperative chemotherapy

Representative CD44v9 and GPx2 immunohistochemical staining patterns are shown 4 in Figure 2. Some cases showed heterogeneous expression of CD44v9 and GPx2 5 regardless of the infiltration of cancer cells. Positive CD44v9 staining in resected 6 specimens was observed in 51 (26.4 %) of the 193 cases who underwent surgery 7 8 without preoperative chemotherapy. Association between CD44v9 expression and clinicopathological factors in these GC patients is shown in Table 1. In patients without 9 preoperative chemotherapy, CD44v9 expression was significantly associated with sex (p 10 11 = 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 12 was also strongly correlated with GPx2 expression (p < 0.0001). In addition, the 13 association between CD44v9 expression and Lauren classification in 177 GC patients 14 pathologically solid-type poorly with diagnoses other than differentiated 15 adenocarcinoma (por1) and mucinous adenocarcinoma is shown in Supplementary 16 Table 1. 17

18

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 1 RFS among four groups of patients: patients with CD44v9-negative and GPx2-negative 2 3 expression (n = 85), patients with CD44v9-negative and GPx2-positive expression (n = 85) 57), patients with CD44v9-positive and GPx2-negative expression (n = 12), and patients 4 with CD44v9-positive and GPx2-positive expression (n = 39) (OS: p = 0.0350, RFS: p 5 = 0.0139). The double-positive group in CD44v9 and GPx2 expression showed 6 7 relatively poor outcomes in RFS (Supplementary Figure 1a, 1b). In multivariate analysis, 8 in all patients who underwent surgery without preoperative chemotherapy, pStage III/IV and CD44v9-positive expression were independent poor prognosis factors for OS (HR = 9 18.898; 95% confidence interval (CI), 6.441–55.447; p < 0.0001, HR = 2.393; 95% CI, 10 1.110-5.159; p = 0.0259, respectively), and pStage III/IV and CD44v9-positive 11 expression were also independent poor prognosis factors for RFS (HR = 13.830; 95% 12 CI, 5.731-35.375; p < 0.0001, HR = 2.395; 95% CI, 1.216-4.714; p = 0.0115, 13 respectively) (Table 2). 14

In addition, to evaluate the correlation of expression of CD44v9 and chemotherapeutic 15 effect, we analyzed the prognosis of the patients with postoperative adjuvant 16 chemotherapy. CD44v9-positive patients treated with completed postoperative adjuvant 17 chemotherapy (pStage II-III) showed significantly poorer prognosis (OS; p = 0.0297, 18 RFS; p = 0.0012) than those with negative expression (Supplementary Figure 2), 19 whereas CD44v9 expression did not have any prognostic impact for the patients without 20 postoperative adjuvant chemotherapy (OS; p = 0.8080, RFS; p = 0.5726) 21 22 (Supplementary Figure 3).

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

3 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). 4 In patients with preoperative chemotherapy, CD44v9 expression in biopsy specimens 5 was significantly associated with differentiation (p = 0.0209), lymph node metastasis (p 6 = 0.0352), and tumor response grade after preoperative chemotherapy (p = 0.0253) 7 (Table 3). In addition, CD44v9 expression in the biopsy specimens was also strongly 8 correlated with GPx2 expression (p = 0.0078). Supplementary Figure 4 shows a 9 representative immunohistochemical image from a patient in CD44v9- and GPx2-10 positive expression cases in the same biopsy specimens. 11

12

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

We found that CD44v9-positive expression was associated with resistance to 15 chemotherapy in GC in clinical specimens. We speculated that the acquisition of 16 antioxidant capacity through CD44v9 was related to chemotherapeutic sensitivity. We 17 therefore explored this possibility in GC cell lines. We first evaluated CD44v9 18 expression in MKN45, MKN74, NUGC4, KATOIII, and SNU-1, by qRT-PCR and used 19 HCT116 as a positive control.¹⁶ MKN45 and NUGC4 cells showed high CD44v9 20 mRNA expression (Supplementary Figure 5a). Western blot analysis of CD44v9 21 expression in MKN45 and NUGC4 cell lines corroborated the qRT-PCR results 22 (Supplementary Figure 5b). Supplementary Figure 5c and 5d confirms the efficacy of 23

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

3 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 4 (control) and CD44v9 knockdown cells (CD44v9 siRNA). Although no significant 5 difference was observed, CD44v9 siRNA cells showed a tendency to increase 6 chemotherapeutic sensitivity to 5-FU compared with controls in MKN45 and NUGC4 7 8 cells. The IC50 values of MKN45 control and CD44v9 siRNA cells were 8.02 and 3.83 μ g/ml, respectively (p = 0.4329). The IC50 values of NUGC4 control and CD44v9 9 siRNA cells were 8.91 and 4.50 μ g/ml, respectively (p = 0.1362) (Figure 4). 10

11

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).

1 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.

8 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 9 solid tumors.²⁻⁵ CD44, a major adhesion molecule for the extracellular matrix, is a cell 10 11 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 12 gene transcripts undergo complex alternative splicing, which results in many 13 functionally distinct isoforms, such as CD44 standard isoform (CD44s) and CD44 14 variant isoform (CD44v).²² CD44v is highly expressed in a number of carcinoma cells 15 and related to tumor progression and metastatic potential.^{19, 22-26} 16

Among the various CD44 isoforms, we focused on CD44v9 in this study because 17 CD44v9-positive expression was recently reported to be significantly associated with 18 poor clinicopathological findings and prognosis in GC patients.^{10, 11} CD44v9 stabilizes 19 xCT and promotes the uptake of cystine required for intracellular GSH synthesis.⁶ GSH 20 is the most abundant non-enzymatic antioxidant molecule in cells and acts directly on 21 eliminating intracellular ROS. GPx2, the gastrointestinal form of glutathione 22 peroxidases, is an antioxidant enzyme that catalyzes the reduction of intracellular ROS 23 such as H₂O₂ or hydroperoxide to water or the corresponding alcohols using GSH as 24

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 5 with not only poor clinicopathological factors and prognosis, but also poor response to 6 chemotherapy such as worse treatment response after preoperative chemotherapy and 7 8 poor prognosis after postoperative adjuvant chemotherapy in GC patients. These results indicated that CD44v9-positive GC patients showed chemotherapeutic resistance. We 9 performed an analysis comparing pretreatment biopsy specimens and resected 10 11 specimens in patients who received preoperative chemotherapy. If CD44v9-positive cells are resistant to chemotherapy, then CD44v9-positive cells are expected to increase 12 in the resected specimens after preoperative chemotherapy. However, CD44v9-positive 13 cells were not increased in the resected specimens. It may have been difficult to 14 evaluate tumor cells because the resected specimens after chemotherapy were highly 15 fibrotic and had undergone therapy-induced changes. 16

In this study, CD44v9 expression and GPx2 expression were strongly correlated in 17 clinical specimens, and GC patients with high expression of both indicated the relatively 18 19 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 20 analysis, which revealed that glutathione disulfide (GSSG) levels were significantly 21 lower and reduced GSH/ GSSG ratio was significantly higher in CD44v9-positive 22 tumors than in CD44v9-negative tumors, suggesting that CD44v9 may enhance pentose 23 phosphate pathway flux and maintain GSH levels in cancer cells.²⁷ Other studies 24

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.

8 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 9 NUGC4 cells, CD44v9 siRNA-transfected cells showed significantly reduced 10 11 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 12 incorporates into RNA and DNA, resulting in an intracellular increase in ROS levels.³⁰ 13 14 Similarly, in our study, both MKN45 and NUGC4 cells showed elevated intracellular ROS levels after exposure to 5-FU. Furthermore, CD44v9 siRNA-transfected MKN45 15 and NUGC4 cells showed elevated intracellular ROS levels compared with control cells. 16 Interestingly, in these cell lines, an increase in ROS was observed only by adding 17 CD44v9 siRNA with DMSO, respectively. Thus, we speculated that the reason for these 18 19 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.

8 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 9 cannot be ruled out. In particular, the sub-analyses were conducted in small populations. 10 11 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 12 adjuvant chemotherapy. Therefore, we think that postoperative adjuvant chemotherapy 13 was not the only cause of poor outcomes of CD44v9-positive patients. Second, we 14 evaluated CD44v9 and GPx2 immunohistochemical staining in whole-tumor tissue. 15 Some cases showed heterogeneous expression of CD44v9 and GPx2 regardless of the 16 infiltration of cancer cells. However, several recent studies showed that 17 CD44v9-positive cells located at the tumor invasive front (TIF) were important because 18 of the association between CD44v9 and the epithelial-mesenchymal transition (EMT).^{26,} 19 ³⁵ Thus, we think that it is necessary to evaluate CD44v9 expression at the TIF, focusing 20 on the intratumoral heterogeneity and the relationship between intracellular 21 accumulated ROS and EMT. Furthermore, in a future study, we will investigate a second 22 cohort to validate the findings of the current study. 23

1 In conclusion, we demonstrated CD44v9 expression was associated with 2 chemoresistance in GC by the regulation of intracellularly accumulated ROS. These 3 findings suggest that CD44v9 may be a not only a prognostic but also predictive 4 biomarker for the response to chemotherapy in GC patients.

5

6 Additional Information

7 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.

14

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18

19 Data availability:

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

22

23 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.

6

7 **Conflict of interest:**

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

9

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1 Figure legends

Figure 1. Flowchart depicting the patient selection process. This study included 596 2 3 primary GC patients who underwent surgery between January 2006 and December 2016. Among these patients, 193 GC patients were treated without preoperative chemotherapy 4 from 2008 to 2012 and 29 GC patients received preoperative chemotherapy from 2006 5 to 2016. Among the 69 GC patients with pathological stage II-III without preoperative 6 7 chemotherapy, 36 patients completed postoperative adjuvant chemotherapy, 12 patients 8 were treated with non-completed postoperative adjuvant chemotherapy, and 21 patients did not receive postoperative adjuvant chemotherapy. Abbreviations: GC = gastric 9 cancer; CD44v9 = CD44 variant 9. 10

11

Figure 2. CD44v9 and GPx2 expression in the resected specimens in GC patients. 12 Representative immunohistochemical staining of CD44v9 and GPx2 in resected 13 14 specimens of primary GC. CD44v9 intensity score (a) -1, no staining; (b) -1, weak staining homogeneously; (c) 0, intermediate staining heterogeneously; (d) 1, strong 15 staining homogeneously. Gpx2 intensity score (e) 0, no staining; (f) 1, weak staining; 16 (g) 2, intermediate staining; (h) 3, strong staining. (a–h, high-power view of square, $\times 20$ 17 objective lens, scar bar 100 μ m). Abbreviations: CD44v9 = CD44 variant 9; GPx2 = 18 19 glutathione peroxidase 2; GC = gastric cancer.

20

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.

3

Figure 4. The relationship between CD44v9 expression and chemotherapeutic 4 sensitivity to 5-FU in MKN45 and NUGC4 cells. Cell viability was measured after 5 treatment with different concentrations of 5-FU or DMSO (refer to Materials and 6 Methods for details) for 72 h in MKN45 and NUGC4 cells transfected with CD44v9 or 7 8 control siRNA. CD44v9 siRNA cells exhibited higher chemotherapeutic sensitivity to 5-FU than control cells in MKN45 and NUGC4 lines. Data are means ± standard 9 deviation from three independent experiments. Abbreviations: CD44v9 = CD44 variant 10 9; 5-FU = 5-fluorouracil. 11

12

13 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.

20

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.

5

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.

13

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 secure adjuvant chemotherapy in (a) OS = overall survival, RFS = recurrence-free survival.

20

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.

4

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.

596 GC patients who underwent surgery from 2006 to 2016

















Supplementary Figure 4



Supplementary Figure 5

а



b



С

MKN45





d



Factors		CD44v9-	-negative	CD44v9	CD44v9-positive		
		n=14	2 (%)	n=51			
Age (average \pm 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)		

 Table 1. Association between CD44v9 expression in resected specimens and

 clinicopathological factors in GC patients who underwent surgery without preoperative therapy

Abbreviations: CD44v9 = CD44 variant 9; GC = gastric cancer; pStage = pathological stage; GPx2 =

glutathione peroxidase 2. Bold value indicates a significant difference.

Factors	Object	Control			0	S					RI	7 S		
		_		Univariate			Multivariate			Univariate			Multivariate	
		_	HR	95% CI	<i>P</i> -value	HR	95% CI	<i>P</i> -value	HR	95% CI	<i>P</i> -value	HR	95% CI	<i>P</i> -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/	Well/	1.886	0.850-4.183	0.1184				1.824	0.911-3.652	0.0897			
	Sig	Moderately												
Lymphatic	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
permeation														
Vascular	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
invasion														
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	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
expression														
GPx2	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
expression														

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

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.

Factors		CD44v9	-negative	CD44v9	<i>P</i> -value	
		n=15	5 (%)	n=14		
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)	

Table 3. Association between CD44v9 expression in pretreatment biopsy specimens and

 clinicopathological factors in GC patients who underwent surgery with preoperative therapy

Abbreviations: CD44v9 = CD44 variant 9; GC = gastric cancer; cStage = clinical stage; GPx2 = clinical stage; GP

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	CD44v9-positive	<i>P</i> -value
		n=134 (%)	n=43 (%)	
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.