CD44 variant 9 expression as a predictor for gastric cancer recurrence: immunohistochemical and metabolomic analysis of surgically resected tissues

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ABSTRACT

CD44 variant 9 (CD44v9) and the heavy chain of 4F2 cell-surface antigen (CD98hc) appear important for regulation of reactive oxygen species defence and tumor growth in gastric cancer. This study examined the roles of CD44v9 and CD98hc as markers of gastric cancer recurrence, and investigated associations with energy metabolism. We applied capillary electrophoresis time-of-flight mass spectrometry to metabolome profiling of gastric cancer specimens from 103 patients who underwent resection with no residual tumor or microscopic residual tumor, and compared metabolite levels to immunohistochemical staining for CD44v9 and CD98hc. Positive expression rates were 40.7% for CD44v9 and 42.7% for CD98hc. Various tumor characteristics were significantly associated with CD44v9 expression. Five-year recurrence-free survival rate was significantly lower for CD44v9-positive tumors (39.1%) than for CD44v9-negative tumors (73.5%; P < 0.0001), but no significant differences in recurrence-free survival were seen according to CD98hc expression. Univariate and multivariate analyses identified positive CD44v9 expression as an independent predictor of poorer recurrence-free survival. Metabolome analysis of 110 metabolites found that levels of glutathione disulfide were significantly lower and reduced glutathione (GSH)/glutathione disulfide (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.

Gastric cancer is the fifth most common malignancy (952,000 cases in 2012) and the third leading cause of cancer-related deaths (723,000 deaths in 2012) worldwide (1). Despite the decline in global patient numbers, gastric cancer remains one of the most prevalent cancers in Japan. Although progress in early diagnosis and adjuvant therapy has improved outcomes for gastric cancer patients, radical surgery remains the primary approach for gastric cancer treatment. Outcomes are still unsatisfactory, because gastric cancers relapse with local or distant metastasis even after radical gastrectomy. More specific diagnostic and therapeutic targets are urgently needed to achieve better clinical applications in this field.

CD44, a major adhesion molecule for the extracellular matrix, is a single-pass type I transmembrane protein that serves as a cell surface receptor for hyaluronic acid, and has been implicated in vari-
ous biological processes, such as cell adhesion, cell migration, and cancer metastasis (3, 12, 18, 38, 45). This molecule exists in numerous isoforms containing at least 20 exons in different combinations generated through alternative mRNA splicing (47). However, the functional relevance of CD44 variants in tumor cells remains unclear. Splice variants play important roles within the cell in both increasing proteome diversity and in cellular function. Splice variants are also associated with disease states and may play etiological roles (16).

CD44 has recently been identified as one of the cell surface markers associated with cancer stem cells in several types of tumor (2, 11). Among CD44 variants, CD44 variant 9 (CD44v9) is highly expressed by cancer stem cells in mouse gastric cancers (26). Cancer stem cells that exhibit stem cell-like characteristics such as multilineage potential and self-renewal potential have been identified in cancer tissues (41). Cancer stem cells are resistant to therapy because they show enhanced protection against reactive oxygen species (ROS) (13). In addition, CD44v9 may contribute to cancer survival under hostile conditions, such as with the condition of high ROS generation from chemoradiotherapy, by increasing intracellular levels of reduced glutathione (GSH) through an activating xCT, a glutamate-cysteine exchange transporter (25). Such findings suggest that CD44v9-positive cells have a specific function in protecting against ROS, and contribute to features of cancer stem cells. In addition, expression of CD44v9 in tumor tissues has also been associated with tumor recurrence and prognosis in patients with several types of cancer (4, 19, 20, 31, 33).

Recently, expression levels of CD44v9 in early gastric cancer have also been shown to correlate positively with the risk of recurrence in patients who underwent endoscopic submucosal dissection (21). Furthermore, expression levels of CD44v9 in head and neck cancer cell lines were associated with increased levels of intracellular GSH and resistance to cisplatin (52). Sulfasalazine, a drug used in the treatment of inflammatory bowel disease, is a specific inhibitor of xCT-mediated cysteine transporters and was recently shown to selectively damage CD44v9 expressing cells, suppress CD44v9-dependent tumor growth, and enhance sensitivity to cisplatin (25, 46). These findings suggest that CD44v9 might represent a good target for cancer treatment. However, whether CD44v9-positive cells demonstrate aspects of cancer stem cells and whether CD44v9 can be a biomarker for gastric cancer recurrence remain elusive.

On the other hand, xCT and L-type amino-acid transporter 1 (LAT1) transport large neutral amino acids, and require covalent association with the heavy chain of 4F2 cell-surface antigen (CD98hc) for functional expression in the plasma membrane (32, 48). CD98hc has a main biochemical function of amino acid transport, and appears important in tumor growth and metastasis (9). LAT1, the most well-studied CD98 light chain, is classified as an bi-directional transporter of large neutral amino acids. Recent studies have shown that high LAT1 expression is significantly associated with poor outcomes for various human cancers, such as gastric cancer, colorectal cancer, lung cancer, prostate cancer, breast cancer, and renal cell carcinoma (5, 17, 24, 28, 49, 50). However, no studies have clarified the relationship between CD98hc expression and gastric cancer recurrence. Metabolome analysis has recently been applied to the characterization of cancer-cell-specific metabolism. Levels of most amino acids and their primary derivatives have been found to be significantly higher in tumors than in normal tissue by quantitative metabolome profiling (22).

The development of metabolome analysis tools is allowing the application of comprehensive analyses of energy metabolism pathways such as glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, urea cycle, amino acid and nucleotide metabolisms, and GSH metabolism in cells and tissues. Several articles have been published on metabolomic applications in gastric cancer (27, 34, 35). However, none have focused on associations between energy metabolism and CD44v9 expression. The first aim of this study was thus to determine the role of CD44v9 and CD98hc as a marker of gastric cancer recurrence. The second was to identify associations of energy metabolism, especially GSH metabolism, with CD44v9 and CD98hc; to this end, we applied capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) to the metabolome profiling of human gastric cancer, and compared metabolite levels to CD44v9 and CD98hc expression in tumor tissues obtained by surgery.

MATERIALS AND METHODS

Patients. Participants in this study comprised 103 patients with gastric cancer who underwent resection with no residual tumor or microscopic residual tumor between February 2011 and May 2012 at Shizuoka Cancer Center. In general, patients received adjuvant chemotherapy with tegafur/gimeracil/oteracil known as S-1 for 1 year if the pathological stage was II or III. In addition, some stage IV patients also received
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chemotherapy with S-1 for 1 or 2 years, as decided by the physician. Patients were routinely followed-up every 3 to 6 months for up to 5 years. Median duration of follow-up was 61.0 months. Recurrence was diagnosed by image findings using computed tomography (CT), ultrasonography and magnetic resonance imaging (MRI). This study was approved by the Human Ethics Review Committee of Shizuoka Cancer Center, and written informed consent was obtained from each patient prior to enrolment.

**Tissue samples.** For metabolite extraction, tumor and surrounding non-tumor tissues were collected from surgically resected samples immediately after gastrectomy. The resected tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until metabolite extraction. The rest of all surgical specimens were fixed in 10% formalin neutral buffer solution and embedded in paraffin. All slides were reviewed by more than two pathologists and histologically confirmed to be gastric carcinoma. Tumors were staged according to the 14th Japanese Gastric Cancer Association staging system.

**Histology and immunohistochemistry.** We performed immunohistochemical staining for CD44v9 and CD98hc. Tissue was fixed in neutral-buffered 10% formalin solution, embedded in paraffin, and sectioned at a thickness of 4 μm. Sections were depleted of paraffin, then rehydrated in a graded series of ethanol solutions. For histology, sections were stained with hematoxylin and eosin.

Staining for CD44v9 was carried out using the avidin-biotin complex peroxidase method. For immunohistochemistry, following deparaffinization and rehydration, antigen for anti-CD44v9 (RV3; 1 : 5000 dilution; Cosmo Bio, Tokyo, Japan) was retrieved by heating samples in buffered Tris-EDTA (pH 9.0) for 40 min at 98°C. The sections were then incubated with the rat antibody for overnight at room temperature. After washing with phosphate-buffered solution, they were incubated with secondary antibody (BA-4000, biotinylated rabbit anti-rat IgG antibody; Vector Laboratories, Burlingame, CA) for 30 min. Immune complexes were detected using a Liquid DAB+ Substrate Chromagen System (K3468; Dako, Carpinteria, CA), and the sections were counterstained with hematoxylin.

Staining for CD98hc was carried out using the high-molecular polymer method. The Bond Polymer Refine Detection Kit (DS9800; Vision BioSystems, Melbourne, Australia) was applied using anti-CD98hc (sc-9160, 1 : 100 dilution; Santa Cruz Biotechnolo-

**Metabolite extraction.** Approximately 50 mg of frozen tissue was plunged into 1,500 μL of 50% acetonitrile/Milli-Q water containing internal standards (H3304-1002; Human Metabolome Technologies, Tsuruoka, Japan) at 0°C in order to inactivate enzymes. Tissue was homogenized three times at 1,500 rpm for 120 s using a tissue homogenizer (Microsmash MS100R; Tomy Digital Biology, Tokyo, Japan), then the homogenate was centrifuged at 2,300 × g and 4°C for 5 min. Subsequently, 800 μL of the upper aqueous layer was centrifugally filtered through a Millipore 5,000-Da cutoff filter at 9,100 × g and 4°C for 120 min to remove proteins. The filtrate was centrifugally concentrated and re-suspended in 50 μL of Milli-Q water for CE-TOFMS analysis.

**Metabolome analysis.** Metabolome analysis was conducted using the Basic Scan package of HMT using CE-TOFMS based on previously described methods (39, 40). Briefly, CE-TOFMS analysis was carried out using an Agilent CE capillary electrophoresis system equipped with an Agilent 6210 time-of-flight mass spectrometer (Agilent Technologies, Waldbronn, Germany). Systems were controlled using Agilent G2201AA ChemStation for CE version B.03.01 software (Agilent Technologies). The spectrometer was scanned from m/z 50 to 1,000 and peaks were extracted using MasterHands automatic integration software (Keio University, Tsuruoka, Yamagata, Japan) to obtain peak information including m/z, peak area, and migration time (43). Signal peaks corre-
Patient characteristics are shown in Table 1. Since participants comprised those whose tumors were large enough to allow tumor tissue collection for metabolite extraction, most patients (90.3%) showed advanced cancer. All patients who underwent resection with microscopic residual tumor showed positive peritoneal cytology. Patients who underwent resection with microscopic residual tumor included one patient with hepatic metastases and one patient with minimal peritoneal metastases that were completely resected simultaneously. Postoperative adjuvant tegafur/gimeracil/oteracil known as S-1 chemotherapy performed for stage II/III was administered to 61.2% of patients (41 patients).

Expression of CD44v9 and CD98hc in gastric cancer tumor tissue

All tissue slides were re-reviewed by two pathologists. Positive expression rates were 40.7% for CD44v9 (CD44v9-positive) and 42.7% for CD98hc (CD98hc-positive) (Supplementary Fig. 1). Representative images of immunohistochemical staining for CD44v9 and CD98hc are shown in Supplementary Fig. 2.
Clinically relevant background factors in the CD44v9-positive and CD98hc-positive groups are shown in Table 2. Tumor size, pathological T stage, pathological N stage, pathological TNM stage, and residual tumor were all found to be significantly related to CD44v9 expression. On the other hand, no significant differences in any characteristics were observed between CD98hc-positive and -negative groups. During the median follow-up of 61.0 months, tumor recurrence (including local and distant metastasis) was observed in 37 patients (35.9%). The most frequent recurrence pattern was peritoneal metastasis, which was observed in 24 patients (64.9%), followed by distant lymphatic metastasis in 14 patients (37.8%). Hepatic metastasis was observed in six patients (16.2%). Pulmonary metastasis was observed in one patient (2.7%). Multiple metastases were observed in seven (18.9%) of the 37 patients at initial recurrence.

During the study period, 35 patients (34.0%) died. Of these, 31 (88.6%) died of recurrent gastric cancer and four (11.4%) died of another disease without recurrence. No patients died of other malignancies.

**Prognostic role of CD44v9 and CD98hc in gastric cancer patients**

In this study, disease recurrence was identified in 26 patients (26/42, 61.9%) in the CD44v9-positive group and 9 patients (9/61, 14.8%) in the CD44v9-negative group. Fig. 1 shows the Kaplan-Meier curves for recurrence-free survival (RFS) in these two groups of gastric cancer patients. Five-year RFS rates were 39.1% for CD44v9-positive tumors and 73.5% for CD44v9-negative tumors, representing a significant difference between groups ($P < 0.0001$). Five-year RFS rate according to CD98hc expression was 60.7% for CD98hc-positive and 58.7% for CD98hc-negative. No significant difference was observed between the CD98hc-positive and -negative groups ($P = 0.690$) (Fig. 2). When we analyzed patients with gastric cancers of pathologic stages I through IV separately, CD44v9-positive patients showed worse
In CD98hc expression, GSH and cysteine levels and GSH/GSSG ratio were significantly higher in the CD98hc-positive group than in the CD98hc-negative group, although GSSG levels did not differ significantly between CD98hc-positive and -negative groups.

Concentrations of metabolites involved in glycolysis and PPP are illustrated on the metabolic pathway map in Fig. 4. Lactate levels were higher in the CD44v9-positive group than in the CD44v9-negative group. In addition, regarding glycolysis, 3-phosphoglyceric acid levels were significantly higher in CD44v9-positive patients than in the CD44v9-negative patients. Levels of ribulose 5-phosphate in PPP were significantly higher in the CD44v9-positive group than in the CD44v9-negative group, whereas metabolic intermediates in glycolysis and PPP showed no significant differences between CD98hc-positive and -negative groups.

Levels of amino acids such as Leu, Ile, Ser, Cys, Met, Asn, Try, Phe, Lys, Thr, Val, Pro, hydroxyproline, and His, with the exceptions of glutamine and glutamate, were significantly higher in the CD98hc-positive group than in the CD98hc-negative group. Levels of glutathione disulfide (GSSG) and GSH/GSSG ratio were significantly lower and higher, respectively, in gastric cancer tissue from the CD44v9-positive group than from the CD44v9-negative group.

DISCUSSION

Prior works have documented that expression of CD44v9, a marker of cancer stem-like cells, contrib-
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Our results revealed CD44v9 expression as an independent predictor of tumor recurrence in gastric cancer. Histological evidence of CD44v9 expression has also recently been reported for several types of cancer, such as gastric cancer, hepatocellular carcinoma, ovarian cancer, colorectal cancer, and urothelial cancer (19–21, 31, 33, 42). Hirata et al. reported that CD44v9 may serve as a novel indicator of stage I gastric cancer after endoscopic submucosal dissection (21). Specifically, they showed that among 65 patients who underwent endoscopic submucosal dissection for stage I gastric cancer, 13 developed multiple recurrences. Furthermore, Go et al. suggested that positive immunoexpression of CD44v9 serves as an independent predictor of tumor recurrence in gastric cancer.

CD44v9 expression was identified as an independent predictor of tumor recurrence in gastric cancer. Analysis of the metabolome found that metabolic intermediates in glycolysis and PPP were significantly higher, levels of GSSG were significantly lower, and GSH/GSSG ratio was significantly higher for CD44v9-positive tumors than for CD44v9-negative tumors, suggesting that CD44v9 may enhance PPP flux and maintain GSH levels in cancer cells.

**Table 3** Uni- and multivariate analyses for recurrence-free survival of the patients with gastric cancer by surgery

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Recurrence-free survival</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tr>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>P value</td>
<td>Hazard ratio (95% CI)</td>
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<tr>
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<td>Age (years)</td>
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<td>≥ 70</td>
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<td>≥ 5.0</td>
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<td>Venous invasion</td>
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<tr>
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<td>Positive</td>
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CD44v9 expression as an independent predictor of tumor recurrence in gastric cancer

Our results revealed CD44v9 expression as an independent predictor of tumor recurrence in gastric cancer. Histological evidence of CD44v9 expression has also recently been reported for several types of cancer, such as gastric cancer, hepatocellular carcinoma, ovarian cancer, colorectal cancer, and urothelial cancer (19–21, 31, 33, 42). Hirata et al. reported that CD44v9 may serve as a novel indicator of stage I gastric cancer after endoscopic submucosal dissection (21). Specifically, they showed that among 65 patients who underwent endoscopic submucosal dissection for stage I gastric cancer, 13 developed multiple recurrences. Furthermore, Go et al. suggested that positive immunoexpression of CD44v9 serves
as a predictor of poor prognosis in stage I, but not in stage II or III (19). They reported 5-year overall survival rates of 81.7% in the CD44v9-positive group and 95.2% in the CD44v9-negative group, showing a significant difference between groups. Consequently, the rate of stage I gastric cancer recurrence and poor prognosis was significantly higher in the CD44v9-positive group than in the CD44v9-negative group. In the present study, however, positive immunoexpression of CD44v9 served as an indicator of recurrence in all patients who underwent gastrectomy for gastric cancer. Then, in terms of TNM stage, we showed that the rate of recurrence was significantly higher in the CD44v9-positive group than in the CD44v9-negative group in stage III. That is, we showed that CD44v9 serves as a marker of recurrence for advanced gastric cancer after gastrectomy. In a previous study, CD44v9 was associated with lymph node metastasis, depth of invasion, and pathological TNM stage in gastric cancer (51). Those results support the present findings in which multivariate analysis identified undifferentiated histological type, positive lymph node metastasis and positive CD44v9 expression as independent prognostic factors for RFS.

**Metabolomic analyses of gastric cancer tissues**

To the best of our knowledge, this represents the first study to focus on energy metabolism in association with CD44v9 expression in surgically resected gastric cancer tissues. In metabolome analyses, levels of GSSG were significantly lower and GSH/GSSG ratio was significantly higher in the CD44v9-positive group than in the CD44v9-negative group for gastric cancer. On the other hand, GSH levels did not differ significantly between groups. In addition, levels of lactate, 3-phosphoglyceric acid and ribulose 5-phosphate were higher in the CD44v9-
CD44v and pyruvate kinase M2 suppress enzymatic activity via tyrosine phosphorylation and promote glycolysis, and increase flux to PPP that produces NADPH, contributing to increased GSH production from GSSG in glycolytic cancer cells. Our results support the notion that CD44v9 enhances PPP flux that produces NADPH, contributing to changes from GSSG to GSH and maintaining GSH levels in cancer cells.

Prognostic role of CD98hc and CD98 amino acid transport in gastric cancer

In the present study, expression of CD98hc was not identified as an independent prognostic factor for RFS in gastric cancer patients. A recent study reported that CD98 expression represents a significant predictor of poor outcome in squamous cell carcinoma (6). Furthermore, Martinez et al. reported that acidic pH, representing increased lactate, enhances the invasive behavior of human melanoma cells (36). However, no studies have reported on lactate associated with gastric cancer recurrence. High lactate levels clearly indicate enhanced lactate fermentation, which reaffirmed the Warburg effect in the CD44v9-positive group. Recent studies have shown that pyruvate kinase M2 is a key glycolytic enzyme that regulates the Warburg effect and is necessary for tumor growth (10). Tamada et al. reported that CD44 maintains GSH levels in cancer cells because CD44v and pyruvate kinase M2 interactions regulate GSH production (44). Interactions between CD44v and pyruvate kinase M2 suppress enzymatic activity via tyrosine phosphorylation and promote glycolysis, and increase flux to PPP that produces NADPH, contributing to increased GSH production from GSSG in glycolytic cancer cells. Our results support the notion that CD44v9 enhances PPP flux that produces NADPH, contributing to changes from GSSG to GSH and maintaining GSH levels in cancer cells.

Fig. 4  Quantified levels of metabolites involved in central carbon metabolism. Metabolite concentrations of CD44v9 expression and CD98hc expression groups superimposed on a metabolic pathway map that includes glycolysis and the pentose phosphate pathway. Columns, median concentration (nmol/g). All P-values were evaluated using the Wilcoxon matched pair test. *P < 0.05 G6P: glucose-6-phosphate; F6P: fructose 6-phosphate; 3PG: glycerate 3-phosphate; PEP: phosphoenolpyruvic acid; 6PG: 6-phosphogluconate; Ru5P: ribulose 5-phosphate; R5P: ribose 5-phosphate; X5P: D-xylulose 5-phosphate; NADPH: reduced nicotinamide adenine dinucleotide phosphate; S7P: sedoheptulose 7-phosphate; PRPP: 5-phosphoribosyl 1-diphosphate; 2OG: 2-oxoglutaric acid; Suc-CoA: succinyl-CoA; Suc: succinic acid; Fum: fumaric acid; Mal: malic acid.
Fig. 5 Metabolome data map of metabolites including amino acids and nucleotides (shown in the box) in CD98hc-negative and CD98hc-positive groups. Columns show median concentration (nmol/g) in CD98hc-negative and CD98hc-positive groups. All P values were evaluated using the Wilcoxon matched pair test. *P < 0.05 G6P: glucose-6-phosphate; 3PG: glyc-erate 3-phosphate; 2OG: 2-oxoglutaric acid; Suc-CoA: succinyl-CoA; Suc: succinic acid; Fum: fumaric acid; Mal: malic acid; AMP: adenosine monophosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; GMP: guanosine monophosphate; GDP: guanosine diphosphate; GTP: guanosine triphosphate

In the present study, metabolomic profiles suggested that intracellular amino acid concentrations and GSH were higher in CD98hc-expressing tissues, suggesting that CD98hc is associated with amino acid transport in gastric cancer tissues. However, CD98hc is a multifunctional molecule and interacts with many transporters, which is why expression of CD98hc may not have been identified as an independent prognostic factor for gastric cancer in the present study.

Limitations
Some limitations to this study must be considered when interpreting the present findings. First, selection bias was present in this study, with more than 90% of patients showing advanced cancer. Because specific amounts of tumor tissue were collected from surgically resected samples for metabolite extraction, patients with small tumors were excluded...
from the study. Improvements in the devices used for metabolomic analysis to allow measurements from smaller tumors are required.

Second, this study attempted immunohistochemical staining for xCT in surgically resected tissue samples according to the instructions from the manufacturer, but no tissue slides examined for xCT showed any staining. To the best of our knowledge, no reports have described staining of anti-xCT antibodies in patients with gastric cancer. This result remains unexplained. Further investigation is required with regard to xCT in gastric cancer.

Conclusions
In conclusion, our study confirmed that the recurrence rate of gastric cancer following surgery is higher in the CD44v9-positive tumors than in the CD44v9-negative tumors. The data presented in our study suggest that expression of CD44v9 may offer a new biomarker of recurrence in gastric cancer. To the best of our knowledge, this is the first study to focus on energy metabolism in association with CD44v9 expression in gastric cancer. Levels of GSSG were significantly lower and the GSH/GSSG ratio was significantly higher in the CD44v9-positive group than in the CD44v9-negative group in gastric cancer. These findings suggest that CD44v9 may enhance PPP flux and maintain GSH levels in cancer cells.

Acknowledgements
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REFERENCES


Supplementary Fig. 1  Histogram of CD44v9 and CD98hc immunoreactive scores for all 103 patients. The x axis shows the immunoreactive scores and the y axis shows the number of patients. CD44v9: CD44 variant 9

CD44v9 IHC score

CD44v9-positive = 42 / 103 (40.7%)

CD98hc IHC score

CD98hc positive = 44 / 103 (42.7%)
Supplementary Fig. 2A  Representative figures of immunohistochemical staining for CD44v9 expression on the basis of positive criteria. Membranous reactions were scored in accordance with the intensity of cellular staining and the proportion of stained tumor cells as follows: (A) 0, no staining; (B) 1+, weak staining; (C) 2+, moderate staining; (D) 3+, strong staining. The bar indicates 50 μm.

Supplementary Fig. 2B  Representative figures of immunohistochemical staining for CD98hc expression on the basis of positive criteria. Membranous reactions were scored in accordance to the intensity of cellular staining and the proportion of stained tumor cells as follows: (A) 0, no staining, (B) 1+, weak staining, (C) 2+, moderate staining, (D) 3+, strong staining. The bar indicates 50 μm.
Supplementary Fig. 3 Kaplan-Meier curves for RFS according to CD44v9 status in patients with TNM stage: stage I (A), stage II (B), stage III (C), and stage IV (D).